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The Effect of Simulated Flooding Post-anthesis on Pre-harvest Sprouting and Subsequent Seed Longevity in Contrasting Rice Cultivars

Sujittra Tejakhod

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School of Agriculture, Policy and Development

University of Reading, United Kingdom

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‘Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.’

Miss Sujittra Tejakhod

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Dedication

This thesis is dedicated to my Father and Mother, Assoc. Prof Adisorn Kraseachai, and all my great teachers for providing me knowledge of life.

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Acknowledgement

First and foremost, I would like to express my most sincere gratitude to my advisor, Professor Richard Ellis for his invaluable time, advice, insightful comments, feedback, and constant encouragement throughout my PhD work. I also would like to pass my thanks to Dr John Hammond, whose extremely useful suggestions, knowledge and skillful guidance added greatly to my PhD studies.

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Abstract

Unpredictable flooding is a major constraint to rice production. It can occur at any growth stage. The effect of simulated flooding post-anthesis on yield and subsequent seed quality of pot-grown rice (*Oryza sativa* L.) plants was investigated in glasshouses and controlled-environment growth cabinets. Submergence post-anthesis (9-40 DAA) for 3 or 5 days reduced seed weight of *japonica* rice cv. Gleva, with considerable pre-harvest sprouting (up to 53%). The latter was greater the later in seed development and maturation that flooding occurred. Sprouted seed had poor ability to survive desiccation or germinate normally upon rehydration, whereas the effects of flooding on the subsequent air-dry seed storage longevity (p_{50}) of the non-sprouted seed fraction was negligible.

The *indica* rice cvs IR64 and IR64Sub1 (introgression of submergence tolerance gene *Submergence1A-1*) were both far more tolerant to flooding post-anthesis than cv. Gleva: four days' submergence of these two near-isogenic cultivars at 10-40 DAA resulted less than 1% sprouted seeds. The presence of the *Sub1A-1* allele in cv. IR64Sub1 was verified by gel electrophoresis and DNA sequencing. It had no harmful effect on loss in seed viability during storage compared with IR64 in both control and flooded environments. Moreover, the germinability and changes in dormancy during seed development and maturation were very similar to IR64.

The efficiency of using chemical spray to increase seed dormancy was investigated in the pre-harvest sprouting susceptible rice cv. Gleva. Foliar application of molybdenum at 100 mg L^{-1} reduced sprouted seeds by 15-21% following 4 days' submergence at 20-30 DAA. Analyses confirmed that the treatment did result in molybdenum uptake by the plants, and also tended to increase seed abscisic acid concentration. The latter was reduced by submergence and declined exponentially during grain ripening.

The selection of submergence-tolerant varieties was more successful than application of molybdenum in reducing pre-harvest sprouting.

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Abbreviations and Symbols

σ	Sigma, standard deviation of seed death in time (days)
°C	Degree Celsius
°N	North (degree)
°S	South (degree)
ABA	Abscisic acid
ANOVA	Analysis of variance
AOSA	Association of Official Seed Analysts
cv., cvs	Cultivar, cultivars
cm	Centimetre
d	Day(s)
DAA	Day after anthesis
DAS	Day after sowing
DAT	Day after transplanting
d.f.	Degrees of freedom
eRH	Equilibrium relative humidity
FAO	Food and Agriculture Organization (United Nations)
FAOSTAT	Food and Agriculture Organization's statistics
g	Gram
g	Times gravity (xg), unit for relative centrifugal force
GA	Gibberellic acid
GBP	Great Britain Pound
Hz	Hertz
IART	Institute of Agrifood Research and Technology, Spain
Inc.	Incorporation

IPCC	Intergovernmental Panel on Climate Change
IRRI	International Rice Research Institute
ISTA	International Seed Testing Association
LSD	Least significant difference
Ltd	Limited
MC	Moisture content
M	Molar
mg	Milligram
mm	Millimetre
Mo	Molybdenum
mol	Mole
m/z	Mass/charge number of ion, Mass-to charge ratio
n	Number of observation
NED	Normal equivalent deviate
ng	Nanogram
NS	Not significant
P	Probability
p_{50}	Half viability period
PEL	Plant Environment Laboratory
ppm	Parts per million
RCBD	Randomized complete block design
REML	Restricted maximum likelihood analysis
s.e.	Standard error
sec	Second
s.e.m.	Standard error of means
UoR	University of Reading

USD	United States Dollar
USDA	United States Department of Agriculture
µ	Micro, $1x10^{-6}$
V	Volt
v/v	Volume by volume
W	Watt

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Chapter 1

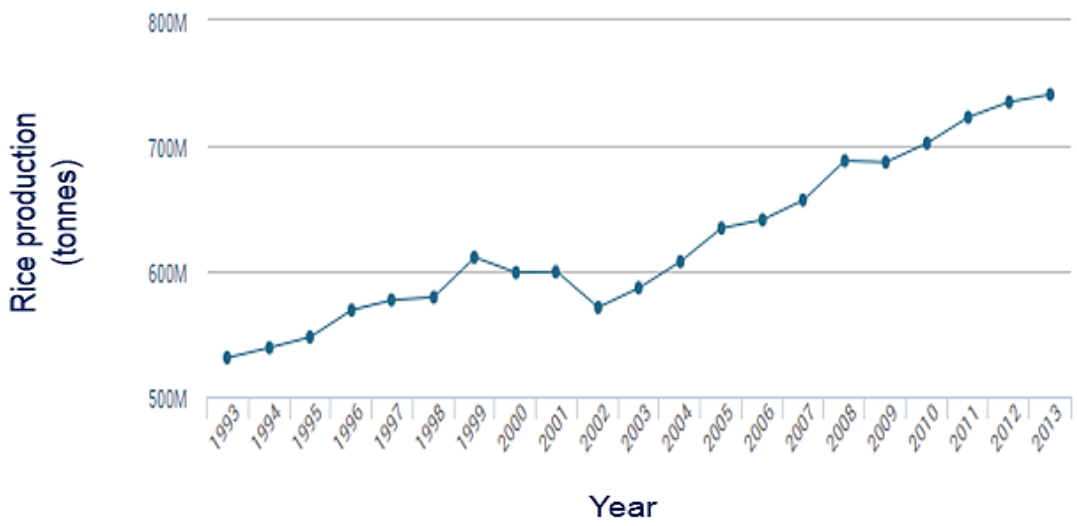
General Introduction

1.1 Rice in the global economy and constraints of rice production

Rice (*Oryza sativa L.*) is the most important staple food: it is consumed by approximately half of the world's population, i.e. 3.6 billion people (Tripathi *et al.*, 2011; Global Rice Science Partnership, 2013; FAOSTAT, 2015a). Global rice consumption has increased in parallel with the rapid growth of world population. Since the last decade, for example, world rice production has risen steadily from 586 million tonnes in 2003 to 740 million tonnes in 2013 (Fig. 1a). In accordance with the driving force of population growth, IRRI (1993) predicted that global rice demand would increase further, by at least 2.1% each year. For food security, more than 800 million tonnes of rice is forecast to be required to meet global rice demand in 2025 (IRRI, 2000; Kubo and Purevdorj, 2004; Smil, 2005). About 90 % of the major rice-producing and -consuming countries are in Asia (Fig. 1b) (Mohanty, 2013; FAOSTAT, 2015b). For these countries, rice is a vital source of food energy providing about 50 % of the calorie supply as well as 40% of protein intake (Redfern *et al.*, 2012; Mohanty *et al.*, 2013). China and India are the largest consumers globally and together account for half of global rice production (Mohanty *et al.*, 2013).

Rice productivity has improved greatly as a result of the green revolution in the second half of the twentieth century (Cantrell and Hettel, 2004; Tran and Kajisa, 2006; Estudillo and Otsuka, 2013). New technology and plant breeding has played an important role in rice production to produce sufficient rice yield for worldwide consumption. However, there are several factors that constrain rice yield; for example, the replacement of growing areas by human settlements, accelerating erosion of soil, salinization, high temperature, rise in atmospheric ozone concentration, and inappropriate water management (Nguyen, 2005; Ainsworth, 2008; Wassmann *et al.*, 2009; Adhya, 2011).

a.



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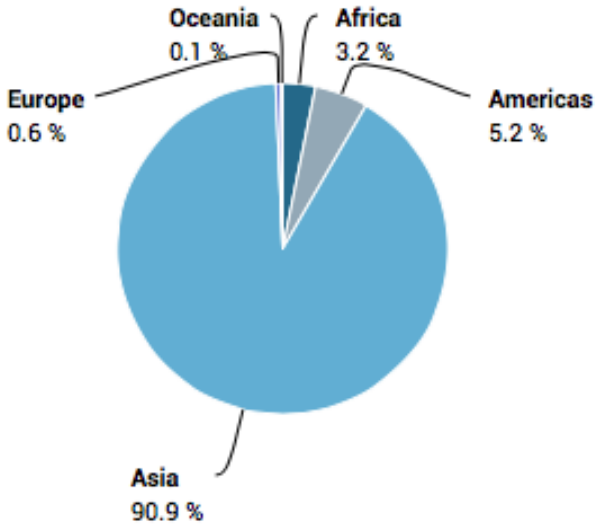

Figure 1.1 Annual world rice production (a, M = million) and world production by region (b) in 2003-2013 (Modified from FOODSTAT, 2015b).

Rice shows the ability to grow under varying environmental conditions where other crops might be less effective. According to IRRI (1985), rice is cultivated from 53 °N (Russia-China border) to 40 °S (Central Argentina). However, rice has more specific requirements, particularly in water management, than other crops because of its hydrological characteristics. It prefers to grow under shallow flooded conditions during the early stages of plant development, whereas irrigation is moderate or even negligible during flowering and subsequent grain filling and ripening stages (Mikkelsen and De Datta, 1980). Excessive water in the field may limit yield. Although some rice cultivars may withstand submergence during the vegetative stage for seven days, crop failure may approach 100% if the whole plants are flooded longer than 14 days (Mackill, *et al.,* 1993; Nguyen, 2005; Wassmann *et al.*, 2009). Undesirable rainfall patterns may damage grain production. Nguyen (2005) reported that high precipitation of more than 200 mm per day may lead to soil moisture stress due to oxygen depletion. This results in poor root respiration, and thus contributes to yield loss. Moreover, rainy weather coinciding with cool temperatures during flowering may lead to poor fertilization and grain sterility, and thus reduce filled grain at harvest (Mikkelsen and De Datta, 1980). Given that approximately three quarters of the world's rice production is cultivated in 93 million ha of irrigated lowland areas (Global Rice Partnership, 2013), flooding is likely to be the most important stress that may constrain rice production as well as increase the risks of rice farming (Mackill *et al*., 2010).

Flooding in rice fields varies greatly between locations and years. Singh *et al.* (2014) reported that rivers often overflow during seedling or early vegetative rice growth. In China, the most frequent flooding occurred from June to August when rice was tillering (Zhang *et al.*, 2015). Puckridge *et al.* (2000) reported that floods in Bangladesh frequently occur 20-60 DAS. On the other hand, greater variation in excessive water in paddy in Thailand was observed, where the onset of flooding may occur 50-120 DAS. The water that remains in the field could vary from 50 cm up to 4 m. In Thailand, the temporary flood surges, which are less than 1 m in height commonly occur during the wet season, with greater flood heights about 3 out of every 10 years (Puckridge *et al.,* 2000). Multi-cropping and continuous rice cropping in sub-tropical regions mean that rice plants may be subject to submergence during maturity – since this can occur at any time of the year.

In many previous studies, drought and heat stress effects during the reproductive stage of rice and other crops development have been well documented, whereas those of flooding have been fewer particularly for seed quality (Table 1.1). Besides the direct effect on agronomic performance, flooding that occurs during or after the end of grain filling stage may affect seed filling and/or subsequent seed quality development. Given the limited number of reports to date on the effect of submergence on rice seed quality, e.g. dormancy, germinability [the ability of

seed to germinate and produce normal seedling (Bewley and Black, 1978)], and storability [the ability of seed to survive and preserve its' viability post-storage (Rajjou and Debeaujon, 2008)], this thesis investigates the effect of simulated flooding at different seed developmental stages on subsequent seed quality (i.e. seed weight, pre-harvest sprouting, germinability, and storability). The impact of submergence duration, developmental stage susceptible to submergence, and varietal differences on sensitivity to damage are considered.

1.2 Taxonomy and geographical distribution of rice

Rice (*Oryza sativa* L.) is in *Gramineae*, genus *Oryza* (Chang and Bardenas, 1965; Grist, 1981). Cultivated rice was domesticated from the wild perennial grass *O. rufipogon* (formerly named *O. perennis*) and the wild annual weed *O. nivara* (Grist, 1891; Chang, 1976; Takahashi, 1984; Watanabe, 1993). *O. sativa* is the most common rice species which is cultivated widely in tropical and temperate zones. Another cultivated species, *O. glaberrima*, is cultivated less widely and is found especially in West Africa (Morishima, 1984; Sato, 1993).

The cultivated rice *O. sativa* is divided into three ecotypes; *japonica, indica*, and *javanica.* The criteria for this designation are based on plant characteristic differences (e.g. grain shape, the presence of awns, degree of grain shedding, and plant status) and geographical distribution. The latter being related to adaptation or sensitivity to different growing environments (Chang and Bardenas, 1965; Takahashi, 1984; Katayama, 1993; Rice Knowledge Bank, 2007; Tripathi *et al.*, 2011; Ricepedia, 2013). The general differences amongst the three ecotypes are as follows;

1) *Indica* rices commonly have high stature with broad to narrow light green leaves. Grains vary from long to medium with high amylose content. The spikelets are awnless and thresh easily. *Indica* rice cultivation occurs across the tropics and subtropics, for instance Philippines, India, Pakistan, Java, Sri Lanka, Indonesia, central and southern China, and some African countries.

2) *Japonica* rices show a medium-high tillering with narrow dark green leaves. The spikelets are awnless or long awned, with low shattering at harvest. The grains are short and round with low amylose content. *Japonica* varieties are generally less sensitive to cool temperature than *indica*, and therefore they are commonly grown in cooler zones of the subtropics and in the temperate zones of Indo-China.

Table 1.1 The number of rice studies documented by the two academic online databases on rice production and the effect of environment on rice seed quality

	ScienceDirect http://www.sciencedirect.com/ Search results			Google scholar https://scholar.google.co.uk/ Search results		
	Rice	Environmental factors		**Rice**	Environmental factors	
		Temperature	Drought	Submergence		Temperature	Drought	Submergence
Rice	**277,332**				**2,210,000**			
Production	**122,184**				**2,460,000**			
Effect on rice		115,457	13,649	1,316		2,070,000	327,000	31,100
Effect on rice seed quality		16,156	4,942	402		288,000	82,300	19,600
Effect at grain filling maturation on rice dormancy germination		135	89	11		7,140	5,210	1,160
Effect at grain filling maturation on rice dormancy germination viability longevity		37	24	**5**		2,770	2,320	**252**

Search date 10 October 2015

3) *Javanica* varieties originated from indica and japonica types, but are more closely related to the latter (Weising *et al*., 1994). The plants are tall with broad stiff light green leaves. The spikelets can be awned or awnless. The grains are larger and bolded than the other two ecotypes with little shattering. The plants are tolerant to cooler temperatures, and are commonly grown in the upland high-elevation rice terraces of Philippines and Indonesia.

1.3 Morphophysiology of rice

The crop duration from germination to maturation of cultivated rice varies from three to six months depending upon variety and growing environment (Yoshida, 1981a; Vergara, 1991). Development may be divided into three main growth phases; vegetative, reproductive, and ripening (Vergara, 1971; De Datta; 1981; Vergara, 1991).

The vegetative phase is from the start of seedling growth to panicle initiation. After sowing, seed generally germinate and seedlings emerge within three days. Thereafter changes in leaf and tiller production are the most visible signs of development. A new leaf is produced every 3-5 days during the early vegetative stage, whereas the rate becomes slower (8-9 days) later in development. Tillers may begin to develop from the main culm when the 5^{th} or 6^{th} leaf is produced (tillering). Maximum tillering (the number of tillers per unit area) is generally observed about 30 days after sowing (DAS). Tillers may bare panicles or die later on.

Stem elongation is the subsequent event after tillering, in which the last leaf of main culm, called the flag leaf, develops. It represents the boundary between vegetative and reproductive phases. At the cellular level, the later stage begins with the differentiation and development of panicle primodia. Panicle initiation may become visible via a light microscope following dissection of the apex when the size of panicle reaches 1-2 mm 10-14 days later. During panicle development, the flag leaf sheath swells with the developing panicle, and this is called booting. Meiosis is also occurring at this stage. The emergence of the panicle from the flag leaf sheath, panicle exsertion or heading, normally occurs about 30 days after panicle initiation. The flowering of the panicle (anthesis) begins from the top, followed by the middle, and then the bottom part of the panicle. It may take seven days for complete flowering of the whole panicle. Pollination and fertilization occur in the morning and may last until mid-afternoon (9.00 – 15.00) depending on cultivar and weather during anthesis.

After fertilization, grain development then begins. The seed of rice is technically termed a caryosis, because the pericarp layer is fused with the testa. Thus this organ is actually a fruit enclosed by the covering structure of bracts; lemma and palea (Morris and Bryce, 2000). The differences in the starchy portion of the developing seed divides seed development into three stages; milk grain in which the endosperm is first watery then turns to milky, dough grain stage, where it changes to soft and later hard dough, and finally mature stage in which it becomes hard and the seed coat turns to yellow from green. Although caryopsis development depends on genotype and environment during maturation, the milk, dough, and fully ripe stages are normally detected about 7, 14, and 30 days after anthesis (DAA), respectively (Fig. 1.2).

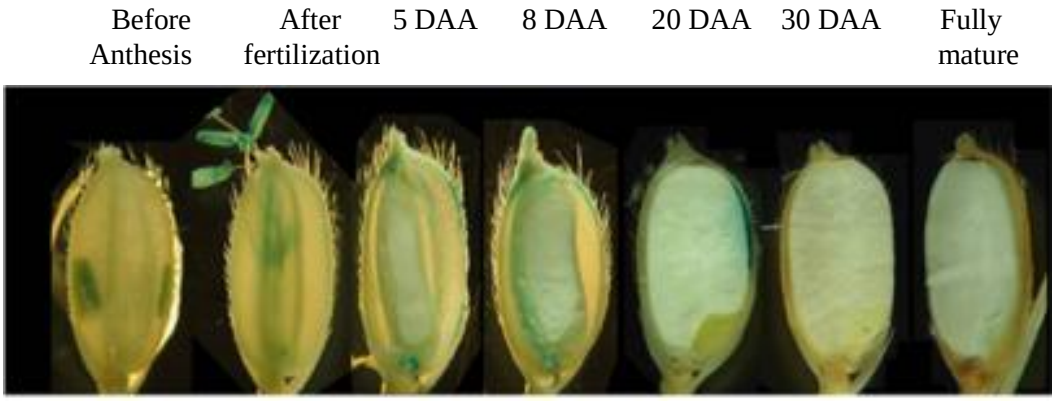

Figure 1.2 Caryopsis development of *japonica* rice cv. Nipponbare shows grain formation at different days after flowering (anthesis; DAA) (From Nozoye *et al.*, 2011).

1.4 Seed quality

1.4.1 Seed quality development

High quality seed is one of the most important basic inputs in food crop production. Differences in seed quality can result in 5-20% higher cereal crop yields (Ousmane and Ajeigbe, 2008; IRRI, 2009; Agrawal and Jacob, 2010). From an agriculturalist's point of view, good quality seed emerges promptly and uniformly after sowing and establishes vigorous, uniform seedlings (i.e. seed vigour, defined by AOSA, 2002). These healthy plants will subsequently contribute uniformity in plant growth that provide high productivity and manageable, timely harvests (Ellis, 2009).

For seed scientists and technologists, seed quality is assessed more precisely. Almekinders and Louwaars (2009) suggest that seed quality can be classified into four aspects; first, seed physiological quality [germinability, vigour, storability, dormancy; physiological stage that viable seed temporarlily are unable to germinate even though favourable conditions are provided (Takahashi, 1984)], and physical criterion, e.g. seed size and seed weight], second seed sanitation quality (absence of seed-borne diseases), third seed analytical quality (percentage of full seed without inert matter or weed seed), and finally, genetic seed quality (varietal uniformity and purity).

Seed physical and physiological qualities develop during seed development. Maximum seed quality has been discussed in many studies. To describe the end of seed filling in agronomical research, i.e. where the seed first achieved maximum seed dry weight, Shaw and Loomis (1950) defined this stage as physiological maturity. Harrington (1972) supported this statement rather more broadly and proposed that aspects of seed performance such as seed vigour and germination capability also reach maxima at this point, and thereafter deterioration commences.

There are, however, many contradictory findings to Harrington's statement. Seeds of barley, wheat, pearl millet, lentil, common bean, faba bean, soya bean, tomato, pepper, aubergine, marrow, and the wild species foxglove obtain maximum seed dry weight at the end of seed filling (by definition), whereas several parameters of seed quality are still improving thereafter for a considerable period (Ellis *et al.*, 1987; Pieta Filho and Ellis, 1991; Rao *et al*., 1991; Ellis and Pieta Filho, 1992; Demir and Ellis, 1992a, b, 1993; Hay and Probert, 1995; Sanhewe and Ellis, 1996; Demir *et al.*, 2002; Ghassemi-Golezani and Mazloomi-Oskooyi, 2008). In rice (*Oryza sativa* L.), ability to germinate of freshly-harvested and dried seeds, storability, desiccation tolerance (i.e. ability of seed to survive without damage after drying to low moisture content), and potential air-dry longevity improve continuously for a further 2-4 weeks after the end of seed filling before deterioration then begins (Ellis *et al*., 1993; Ellis and Hong, 1994; Rao and Jackson, 1996; Ellis, 2011). These observations of rice seed quality development are similar to those with the others crops above, and are clearly contrary to Harrington's hypothesis. Thus, Ellis and Pieta Filho (1992) suggested that the achievement of maximum dry weight at the end of seed filling be termed "mass maturity", and that this is preferable to the term "physiological maturity" because the latter term might be appropriate for agronomists but is misleading for seed scientists.

1.4.2 Seed deterioration

After seeds attain maximum quality, seed deterioration in mature dry seed is a natural consequence, which is observed ultimately by loss of seed viability. Germination percentage may remain high at harvest and the beginning of storage. After a certain storage period, seed may take longer to germinate and establish a normal seedling (Hong and Ellis, 1990; Gurusamy and Thiagarajan, 1998; Bewley *et al*., 2013). Low germination percentage, high number of abnormal seedlings, low vigour seedlings, and finally zero germination are subsequent consequences of seed deterioration (ageing) (Roberts, 1972; Ellis *et al.*, 1987; Copeland and McDonald, 1999; Rao *et al.*, 2006; Khaldun and Haque, 2009; Nik *et al.*, 2011; Bewley *et al*., 2013; Veselova *et al.*, 2015), which normally show as the pattern of seed loss in viability in storage of a seed lot. The seed lot is a population of seeds harvested from the same area and treated identically through drying and storage (ISTA, 2013a).

It is suggested that seed deterioration is caused by three processes: destabilization of cell membranes during the drying process, degradation of seed storage reserves, and inability to control leakage of cellular contents during rehydration (imbibition) (Bewley and Black, 1994; Bewley *et al.*, 2013). Moreover, the production of abnormal seedlings from aged seeds may be due to over accumulation of cytotoxic components (e.g. olefins, alcohol, alkanes, and carbonyl compounds) during seed storage which lead to DNA damage and result in chromosome abnormality (Bewley *et al.*, 2013).

The ability to predict seed viability during storage is valuable. Not only for crop production so that farmers and seed producers can estimate the value of their seed stocks and seed storage potential and avoid substaintial loss in viability, and so manage the risk of potential crop failure, but also for the benefit of long-term seed storage for genetic resources conservation to maintain germplasm and plant diversity. To assess loss of seed viability, repeated germination tests on samples removed serially during the storage period is acknowledged as a useful index in order to determine the proportion of seeds in the population able to germinate and produce a normal seedling. The germination test is also one practical method to evaluate seed vigour indirectly where germination rate can also be considered during the period of the test (Copeland and McDonald, 1999). Moreover, such results can be used to quantify seed life-span during storage in a constant, known environment (Ellis and Roberts, 1980a).

1.4.3 Seed viability equation

Roberts (1960) is one of the pioneers who attempted to analyze the loss of seed viability in cereals. He proposed that the pattern of survival of seed in storage follows a particular negative sigmoidal shape, a negative cumulative normal frequency distribution, when germination percentage is plotted against storage period (the seed survival curve). According to this, therefore the negative cumulative frequency normal distribution pattern represents the many viability periods (death points) of the individual seeds in the population. Consequentially, the curve will become a straight line if the percentage values of germination are transformed to probit values. From this and other relationships noted by Roberts (1960), he developed three basic equations to quantify loss of seed viability under constant storage conditions:

$$\log_{10} \bar{p} = K_v - C_1 m - C_2 t \quad (1.1)$$

$$\sigma = K_\sigma \bar{p} \quad (1.2)$$

$$y = \frac{1}{\sigma\sqrt{2\pi}\, e} - \frac{(p-\bar{p})^2}{2\sigma^2} \quad (1.3)$$

where in equations (1.1) and (1.2), $\bar{p}$ is the period (days) that reduces seed lot viability to half (50%), *m* is seed moisture content (%, fresh weight basis), *t* is storage temperature (°C), σ is the standard deviation of the frequency distribution of seed deaths in time (days), and K_σ, K_v, C_1, and C_2 are constant values. The relative frequency of seed deaths (*y*) after *p* days in storage is described by equation (1.3), based on the normal frequency distribution.

The three basic viability equations above were applied successfully to quantify seed longevity of single seed lots of wheat, barley, rice, broad beans, and peas over certain ranges of the storage environment (Roberts, 1960; Roberts, 1961; Roberts and Abdalla, 1968; Roberts, 1972). However, seed longevity varies amongst seed lots even in identical storage conditions (for example, due to different production environments), and also equation (1.1) was known to be unsuitable for application to a wider range of storage environments (Roberts, 1972). Thus, Ellis and Roberts (1980a) developed a modified seed viability equation with two components. The first component (1.4) described the seed survival curve;

$$v = K_i - p/\sigma \quad (1.4)$$

where v is probit percentage viability after p days in storage under a constant environment. The reduction in seed viability overtime during storage depends on the slope ($1/\sigma$) of the seed survival curve (when percentages are transformed to probits) which relates to the particular storage conditions (σ is the standard deviation of the frequency distribution of seed deaths in time (days) as above). K_i is the value of seed lot constant (intercept at the beginning of storage on Y-axis of the probit-transformed seed survival curve), reflecting the difference in the initial quality of different seed lots.

The second component describes the effect across wide ranges of the storage environment on the estimate of σ:

$$\log_{10} \sigma = K_E - C_W \log_{10} m - C_H t - C_Q t^2 \quad (1.5)$$

where m and t are as above, and K_E, C_W, C_H, and C_Q are constant values applicable to a species. Equations (1.4) and (1.5) can be combined to provide the modified viability equation (Ellis and Roberts, 1980a):

$$v = K_i - p/10^{K_E - C_W \log_{10} m - C_H t - C_Q t^2} \quad (1.6)$$

Subsequently, Ellis and Roberts (1981) showed how the pattern of seed deterioration quantified by equation (1.4) could be related to many different aspects of seed physiological quality, such as time to germinate and ability to germinate in poor environments (Fig. 1.3).

1.4.4 Analytical method development for seed quality assessment

Accelerated-ageing is widely acknowledged as a potential technique for seed quality assessment. The test was first introduced by Grabe (1965) who attempted to determine methods for ageing assessment in stored maize seed. Delouche and Baskin (1973) developed the accelerated-ageing test further to estimate storability of onion seed. The technique has been well accepted and recommended officially by the Association of Official Seed Analysts (AOSA, 1983) and International Seed Testing Association (Hampton and Tekrony, 1995), respectively. The principle of accelerated ageing is that seed lots are subjected to rapid-ageing at 40-45 °C at more than 95% relative humidity. After a single standard period, seeds are withdrawn for a germination test which evaluates the percentage of seeds that remain capable of producing normal seedlings. The results are used to rank the seed lots in terms of likely field emergence potential.

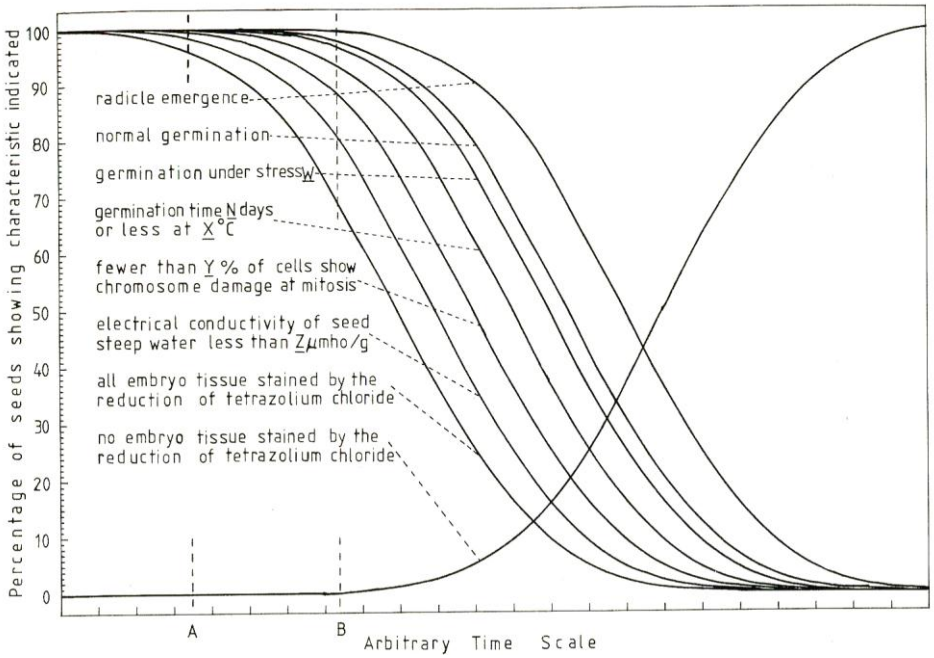

Figure 1.3 The pattern of deterioration amongst individual seeds within a seed lot during storage, in which each ageing symptom can be quantified in a form where the variation can be described by equation (1.4) (From Ellis and Roberts, 1981)

Accelerated ageing is reported to be a practical and efficient method to estimate seedling emergence potential under field conditions of seed lots of onion (Torres and Marcos Filho, 2003; Madruga de Tunes *et al.*, 2011), carrot (Rodo *et al.*, 2000), broccoli (Martínez *et al.*, 2014), tomato (da Silva Almeida *et al.*, 2014), melon (Mavi and Demir, 2007), sorghum (Ibrahim *et al.*, 1993), and corn (Bennett *et al.*, 2004). There can, however, be substantial variations in results especially with small seeds. Matthews (1980) comments that differences in water uptake rates of seeds during the first part of the rapid-ageing test is the main cause of variable results. Therefore, he proposed that initial seed moisture content must be adjusted to similar values before beginning the rapid-ageing test. This amended method is known as the 'controlled deterioration test'. The conditions of the controlled deterioration test and its success may vary amongst species and genotype (Powell and Mathews, 1981; Burgass and Powell, 1984; Osman and George, 1988; Fujikura and Karssen, 1992; Tesnier *et al.*, 2002; Demir *et al.*, 2005; Kavak *et al.*, 2008). Nevertheless, an initial seed moisture content of 20%, then exposure of those seeds to 45°C over a water bath for 24 h are the conditions for the controlled deterioration test recommended by Powell and Matthews (2005).

The fact that either accelerated ageing or controlled deterioration test may help to determine the subsequent field emergence ability of stored seeds, means that these methods are well-adopted by commercial seed companies and are suggested by seed industry organizations. However, Ellis and Roberts (1980b) argued that there are four major concerns of rapid-ageing techniques that may lead to misleading conclusions; first, the difficulty to maintain a high relative humidity accurately and consistently; second, unavoidable changes in seed moisture content during the accelerated ageing test; third, it is difficult to provide identical conditions for each of the different samples and within them to the whole seed population if moisture content is not controlled precisely; and finally, there are sampling errors because only a single germination test is conducted at the end of the ageing treatment.

For these reasons, Ellis and Roberts (1980b) recommended that seed survival curves be determined under constant, hermetic storage conditions, and hence with several samples tested for ability to germinate after different storage periods. Therefore, to assess seed quality by determining estimates of seed longevity throughout this thesis, constant hermetic storage conditions were applied, and the results were analyzed using equation (1.4). This provides a direct estimate of seed storage life, a concern to farmers and seedmen, and an indirect indicator of a seed sample's field emergence ability, a close concern of farmers.

1.5 Flooding- Abiotic induced stress

Armstrong and Drew (2002) found that oxygen diffusivity in flooded water is 10,000 times slower than in air. Furthermore, they reported that oxygen flux in water-logged soil was 320,000 times less than when soil pores are filled with gas rather than water. Therefore, submergence can stress plants and affect respiration as well as photosynthesis because of significant lower oxygen concentration, poor aeration, limited soil nutrition diffusion, and reduced light intensity (Ram *et al.,* 1999), and this may constrain aerobic respiration (Pucciariello and Perata, 2012).

If oxygen supply is limited, the plant will respond to this suboptimal condition by switching its carbohydrate catabolism through anaerobic respiration (Dennis *et al.,* 1992; Perata and Alpi, 1993; Ricard *et al.,* 1994; Quimio *et al.,* 2000; Kato-Noguchi and Morokuma, 2007; Miro and Ismail, 2013). To provide sufficient energy for living cells under oxygen deficiency, sucrose is first broken-down into the three-carbon pyruvate molecules. Then pyruvate is converted to acetaldehyde by the activity of the enzyme pyruvate decarboxylase (PDC), which reaction carbon dioxide. The acetaldehyde is then

metabolized to produce the end product of alcohol fermentation, ethanol, by alcohol dehydrogenase (ADH). In this final step, the essential oxidizing agent NAD^+, which plays an important role to maintain glycolysis and substrate level phosphorylation, is also regenerated releasing ATP from the anaerobic metabolism (Conn *et al.,* 1987).

1.6 Adaptability of rice to flooding conditions

Excessive water in lowland rice fields can generally be classified into two types: deep-water flooding and flash-flooding (Jackson and Ram, 2003; Bailey-Serres *et al.,* 2010; Hattori *et al.,* 2011; Mickelbart *et al.,* 2015). The former refers to the annual occurrence of complete submergence where water from 0.5-4 m deep remains in the paddy fields for several weeks or months during the crop season (Jackson and Ram 2003; Bailey-Serres *et al.* 2010; Nishiuchi *et al.,* 2012). The landraces of rice adapted to deepwater ecosystems avoid the conditions of oxygen depletion by promoting internode elongation, and thus the upper foliage is kept above the water surface for respiration and photosynthesis (Hattori *et al.,* 2011; Mickelbart *et al.,* 2015). The molecular mechanism of this escape strategy is controlled by *SNORKEL1* (*SK1*) and *SNORKEL2* (*SK2*) on Chromosome 12 (Hattori *et al.,* 2009): the high accumulated ethylene during submergence can induce *SK1* and *SK2* expression, which promote gibberellin synthesis and accumulation inside the stem, and hence elongation of internodes aids survival in flood conditions. Kende *et al.* (1998) and Hattori *et al.* (2011) found that the internodes of deepwater cultivars could elongate up to 25 cm per day under complete submergence. The number of nodes and elongated internodes encouraged by flooding depend upon depth of flood water and variety. For instance, rice cv. Pin Gaew produced 12 elongated internodes with 137 cm plant height under normal shallow flooded water of 25 cm (Kupkanchanakul *et al.,* 1988 as cited in Catling, 1992). On the other hand, under 3 m flooded conditions of the same cultivar, 18 elongated internodes were observed with total 453 cm stem length. In some ecosystems with flooded water over 5 m, the Bangladesh rice cvs Chota Bawalia and Raza Mondal could produce up to 21-23 elongated internodes under such environments (Catling, 1992). As a result, such rice cultivars are sometimes called floating rice.

On the other hand, some rice varieties follow a quiescence strategy by minimizing biological processes and avoid internode elongation under unexpected temporary inundation from flood or heavy rainfall in flash-floods (i.e. flood of shallower water about 50 cm deep for 1-2 weeks) (Bailey-Serres *et al.,* 2010; Hattori *et al.,* 2011). The stunted growth is controlled by *Submergence1* gene of Chromosome 9 resulting in reduced energy

consumption for shoot elongation, and thus rice could survive as well as renew growth when the water recedes (Xu *et al.,* 2006).

Despite rice having two adapting strategies to cope with submergence, rice breeding lines with the quiescence mechanism more favoured in plant breeding programmes and by farmers (Bailey-Serres *et al*., 2010; Hattori *et al.,* 2009). Deepwater rice cultivars provide comparatively low yield (about 1 tonne of grain per hectare), and the elongated long stem rice is also susceptible to lodging with difficulty for harvest after the flood water subsides (Bailey-Serres *et al.,* 2010). Furthermore, rapid elongation of deepwater rice may lead to over consumption of carbohydrate for growth, therefore causing more recovery requirement for submergence injury when the flood recedes, or even death if the plant runs out of energy reserves (Jackson and Ram, 2003; Bailey-Serres *et al.*, 2010). Hence, rice landraces for shallow paddies have become widespread, whereas floating cultivars are preserved only in negligible areas of deepwater ecosystems: there are approximately 7% of deepwater rice cultivation in Africa and Asia of the total 150 million hectares of rice grown globally (Ferrero and Tinarelli, 2008; Mickelbart *et al.,* 2015).

1.7 Flooding damage in rice production

Several reports show dramatic loss in rice yield due to severe flooding during the growing season. In 2006, floods in the Philippines destroyed rice production in up to 50 provinces, which cost the industry USD 65 million (IRRI, 2011). In 2011, about 13% of the rice crop area was destroyed by floods and strong winds in Thailand, Cambodia, Philippines, Lao (PDR) and Vietnam (Redfern *et al.,* 2012). These events in Southeast Asia in 2011/2012 led to an estimated loss of 112.5 million tonnes of total rice production because of heavy monsoon, rainfall, and multiple typhoons (USDA, 2011). Redfern *et al.* (2012) and Global Rice Science Partnership (2013) estimated that there are 16 million hectares of irrigated rice fields in South and Southeast Asia that are subjected to flooding, incurring a loss of up to USD 1 billion every year. In Bangladesh and India, more than 20% of the rice-cultivated area is flooded annually, contributing to a loss of at least 4 million tons of rice per annum (Barclay, 2009).

Sharma and Ghosh (1999), Facon (2000) and Singh *et al.* (2009, 2011) suggested that improving drainage management, changing crop practices and using submergence-tolerant rice varieties could alleviate the losses of yield and subsequent damage in the above environments. However, according to Tuong and Bouman (2003), 75% of the world's rice is

produced in irrigated lowland fields and deltaic regions in which flooding is likely to occur often. Moreover, the change in global temperature due to increasing emission of anthropogenic carbon dioxide is tending to alter weather seasons that lead to more frequent intense heavy storms combined with sea level rise (IPCC, 2007, 2013). Thus, flooding is unpredictable varying in depth, duration and can occur at any rice growth stage, and is likely to increase in the future.

1.8. Pre-harvest sprouting

In cereal production, pre-harvest sprouting is a serious problem because pre-germinating grain lead to a decrease in seed quantity and quality (Derera, 1989; Moot and Every, 1990; Every and Ross, 1996; Lorenz and Valvano, 2006). Premature sprouting has been found often in cereals especially when maturing seeds are exposed to rain (Gelin *et al.,* 2006; Lin *et al.,* 2008; Gualana and Benech-Arnold, 2009). Suitable field conditions such as high humidity as well as warm temperature close to harvest time enhance pre-harvest sprouting (Modi and Cairns, 1995; Gualana and Benech-Arnold, 2009; Mahbub *et al.,* 2005). Seed dormancy during late maturity, which varies amongst genotypes, make some genotypes more susceptible to sprouting damage (Gelin *et al.,* 2006, Gualana and Benech-Arnold, 2009). As well as the dormancy level, dependent upon species and cultivars, the environment experienced by the mother plant also affects the crop's susceptibility to damage by wet weather (Hilhorst, 1995; Gualano and Benech-Arnold, 2009; Sugimoto *et al.*, 2010; Bewley *et al.,* 2013).

Dormant seed provides both advantages and disadvantages for grain or seed users. For utilization, deep dormancy may cause a problem in beverage manufacture, i.e. the malting process, in which prompt germination of, for example, barley is required to avoid the cost of medium-term storage until dormancy is released (Benech-Arnold, 2001; Durantini *et al.,* 2008; Sugimoto *et al.,* 2010). Nevertheless, in terms of cultivation and crop management practices, weak dormancy is detrimental especially in tropical and sub-tropical areas where plants may be subject to untimely rainfall or unpredictable floods during late maturity (Tung and Serrano, 2011).

Pre-harvest sprouting leads to substantial damage to crop yield, processing quality, and seed viability in storage (Derera, 1989). High humidity in the field triggers the germination process of mature non-dormant seed, and the hydrolysis of starch in the endosperm resulting in a reduction in grain quantity and weight (Derera, 1989; Morris and

Rose, 1996). Furthermore, premature sprouting enhances grain shattering, and thus increases yield loss during harvest (Mahbub *et al.*, 2005). In the industrial production of bread, pasta, and spaghetti, sprouted grain contributes substantially to processing losses as the flour from sprouted grain yields low quality in terms of firmness, stickiness, short shelf-life, and discoloration of these products (Derera, 1989; Grant *et al.*, 1993; Troccoli *et al.*, 2000; Gelin *et al.*, 2006). In addition to the reduction in seed quality, subsequent post-harvest storage of sprouted seed is problematic in terms of poor ability to germinate due to loss in viability and mould growth in the sprouted seed (Castor and Frederiken, 1977; Moor, 1987; Bason *et al.*, 1991; Sugimoto *et al*., 2010).

1.9 Controlling mechanisms of seed dormancy and germination

There are two main mechanisms controlling seed dormancy; coat-imposed and embryo dormancy (Bewley *et al.*, 2013). The physical barrier (e.g. from hull, kernel, or pericarp) in the case of the former reduces water absorption and gaseous exchange rate, increasing the accumulation of toxic compounds (e.g. phenolic acids, flavonoids, coumarins, tannins, alkaloids, terpenoids, and quinones), and also provides a physical obstacle to the growth and extension of the radicle during seedling emergence (Roberts, 1961; Seshu and Sorrells, 1986; Rao, 2000; Sugimoto *et al*., 2010; Bewley *et al.*, 2013). In embryo dormancy, the germination process is affected by undifferentiated or immature embryos (Bewley *et al.*, 2013). The phytohormone inhibitor abscisic acid (ABA) is one of the most essential factors that plays a pivotal role in regulation of maturation and in embryonic dormancy (Leung and Giraudat, 1998; Finkelstein *et al.*, 2002; Hilhorst, 2007; Bewley *et al.*, 2013).

Synthesis and accumulation of ABA increases during early seed development and declines during the maturation phase (Karssen *et al.*, 1983; Koornneef *et al.*, 1989; Fang and Chu, 2008; Nambara *et al*., 2010). The highest accumulation period was found during the middle of seed development, and in some plants two peak accumulation periods were found. In Arabidopsis, the maximum amounts of ABA were detected at 10 DAA, with a second minor peak at 16 DAA (Karssen *et al.*, 1983). Finkelstein *et al.* (2002) and Kanno *et al.* (2010) proposed that the ABA content in early seed development is from the passing down of maternal ABA to the immature seed, whereas the second peak was the result of ABA synthesized in the developing embryo (*de novo* ABA biosynthesis). The onset of seed dormancy during seed development was found to parallel in time the increase of ABA content (Hilhorst, 1995).

ABA content in seed declines during the maturity stage, synchronously with desiccation, and hence dormancy release, allows germination in favourable conditions (Bewley *et al.,* 2013). Besides natural degradation of ABA, gibberellic acid (GA), phytohormone, functions antagonistically with ABA. GA leads to the onset of dormancy break and germination begins. Fincher (1989) and Gubler *et al.* (1995) described the pathway by which GA promotes germination after imbibition as follows. First, inactive GA derived from maternal tissue is converted into the active form (Liu *et al.,* 2014), alternatively *de novo* GA biosynthesis occurs. Second, GA moves to the aleurone layer where it stimulates the secretion of the starch hydrolysis enzyme, α-amylase. Third, α-amylase metabolizes the carbohydrate in the endosperm into sugar, providing energy for seedling growth (i.e. radicle, shoot, and root) and establishment of the seedlings in the field (Akazawa and Hara-Mishimura, 1985; Beck and Ziegler, 1989). The balance of ABA/GA is crucial to the control of seed dormancy. For instance, a high ABA to GA ratio is found throughout seed development in deep dormant rice seed (cv. N22), whereas a lower proportion may be detected at mid or late-grain filling of medium (cv. ZH11) and non-dormant (cv. G46B) rice, respectively (Liu *et al.,* 2014).

1.10 Pre-harvest sprouting and seed dormancy of rice

In rice, there are several reports indicating genetic and environmental influences on the severity of seed dormancy. In general, wild species (*O. rufipogon*) and African rice (*O. glaberrima*) show greater dormancy than domesticated varieties (*O. sativa* L.) (Misro and Misra, 1969; Sugimoto *et al.,* 2010; Tung and Serrano, 2011). Amongst the latter, the strongest dormancy was found in *indica*, followed by *javanica* and *japonica* sub- species, respectively (Beachell 1943; Tang and Chiang, 1955). Moreover, the lack of dormancy is more severe in modern rice cultivars resulting in up to half of the grain sprouting when developing in very humid weather (Sarma and Parnaik, 1980; Seshu and Sorrells, 1986; Guo *et al.,* 2004; Wan *et al.,* 2006; Fan *et al.,* 2007; Tao *et al.,* 2007).

The relevant genes that control seed dormancy and premature germination resistance of rice have been mapped on Chromosomes 1, 2, 3, 4, 5, 6, 7, 8, and 12 accounting for 10-45% the total phenotypic variation (Lin *et al.,* 1998; Dong *et al.,* 2003; Gu *et al.,* 2004; Gao *et al.,* 2008; Hori *et al.,* 2010; Sasaki *et al.,* 2013). Sugimoto *et al.* (2010) studied this further and found that the substantial sensitivity to pre-harvest sprouting of high-yielding inbred rice varieties has been due to the mutation of an allele during domestication. To date, although the major gene loci controlling seed dormancy and resistance to pre-harvest sprouting in rice

have not been identified precisely, the results of Sugimoto *et al.* (2010) confirmed that there are three alleles responsible for seed maturation and dormancy of rice (*Seed dormancy 4, Sdr4*) on chromosome 7: *Sdr4-k* was present in wild rice, *Sdr4-k and Sdr4-n* were distributed throughout the *indica* sub-species, whereas *japonica* group inherited only *Sdr4-n*.

1.11 Effect of growing environment on seed quality and subsequent longevity

Although seed quality improves during grain-filling and maturation, unfavourable growing conditions (i.e. high temperature, drought, salinity, as well as improper crop practices) may limit the maximum quality attained and shorten seed storability after harvest (Green *et al.*, 1965; Austin, 1972; Grass and Burris, 1995; Sanhewe *et al.*, 1996; Spears *et al.*, 1997; Taub *et al.*, 2008; Kochanek *et al.*, 2011; Hampton *et al.*, 2013). There is much evidence to confirm that the growing environment of mother plants impacts on subsequent seed performance. High-growing temperature before and during seed development causes shorter grain-filling durations, poor yield components (e.g. seed setting, seed number, seed weight, and seed size), low germination, and decrease in seedling potential [e.g. seedling biomass, number of shoots and roots, root length, and seed steep water conductivity (the electrical conductivity of the water in which seeds have been steeped, which indicates the degree of damage to seed membrane integrity)] (Green *et al.*, 1965; Grass and Burris, 1995; Sanhewe *et al.*, 1996; Spears *et al.*, 1997; Kochanek *et al.*, 2011). Detrimental effects of high temperature on seed quality can be more severe if soil moisture content is low or carbon dioxide is elevated (700±50 μmol mol^{-1}), although impacts depend upon plant species (Muasya *et al.*, 2008; Hikosaka *et al.*, 2011; Hampton *et al.*, 2013). For example, Taub *et al.* (2008) reported a reduction of protein content in wheat, barley, and rice seeds grown in elevated carbon dioxide. Shading crops after flowering time delays the end of dry matter accumulation and reduces the rate of seed filling in barley (Pieta Filho and Ellis, 1991). Limiting irrigation improved grain-filling rate of rapid-cycling brassica (Sinniah *et al.*, 1998) and common bean (Ghassemi-Golezani and Mazloomi-Oskooyi, 2008), also maximum seed viability achieved sooner though seed filling ended earlier. On the other hand, Olivares *et al.* (2009) reported that a high rainfall regime (671mm) late in the growing season of wild oat (*Avena barbata*) gave higher mass production and improved seed viability. Furthermore, improper crop practices, for example delayed harvest, can encourage deterioration by increasing seed steep water electrical conductivity in common bean (Ghassemi-Golezani and Mazloomi-Oskooyi, 2008). In *japonic*a rice, seed quality was reduced slightly when plants were exposed to high temperature after the end of grain filling (Ellis, 2011), whereas

exposure during both seed filling and maturation drying provided dramatically poorer ability to germinate, potential longevity and desiccation tolerance (Ellis *et al*., 1993; Ellis and Hong 1994).

1.12 Effect of submergence on rice seed quality and storability post-harvest

Plants generally produce reactive oxygen species (ROS) such as OH^-, O_2^- and H_2O_2 when they experience harsh growing conditions, i.e. drought, osmotic stress and salinity (Apel and Hirt, 2004). High concentration of ROS in seed may lead to damage and malfunction of cell structure, nucleic acid, proteins, enzymes, and ion exchange, which perturb cells and increase seed ageing (McDonald, 1999; Bailly, 2004). Plants respond to abiotic stress by generating antioxidants together with enzymatic (catalase, superoxidase) and non-enzymatic compounds (glutathione, ascorbic acids) to scavenge these free radical ROS (Bailly *et al.*, 2000; Chen and Arora, 2011). Over accumulation of ROS products in developing seed may restrict seed to obtain maximum seed quality. Furthermore, seed that has experienced stress, e.g. water submersion during development, may potentially show accelerated seed deterioration post-harvest despite no visible submergence damage (e.g. pre-harvest germination, discolouring of seed coat, or fungus infection) being detected.

1.13 Similarity of submergence during seed development to 'priming'

In the circumstance that complete submergence during grain filling or maturation results in some seed on mother plants germinating (i.e. pre-harvest sprouting) and some not, the non-sprouted seed may nevertheless experience unintended priming. The initial definition of priming by Heydecker *et al.* (1973) was the pre-treatment of seed by controlling their hydration just underneath the point of full imbibition for a set period of time. This triggered the early stages of germination, but radicle protrusion was prevented due to insufficient moisture availability. After a set period of rehydration, seed was dried and stored for subsequent sowing. Submergence during seed development has some differences from priming: water availability is unlimited and dependent on flooding duration, with rehydration occurring in the remaining pre-harvest period.

Advantages of priming have been well documented and included advanced germination, i.e. improved germination rate, ability to germinate, and enhanced root growth rate of various crops, such as lettuce, spinach, tomato, pepper, okra, bitter gourd, beetroot,

sugar beet, and wheat (Heydecker *et al.*, 1973; Heydecker and Gibbins, 1978; Georghiou *et al.*, 1987; Argerich and Bradford, 1989; Argerich *et al.*, 1989; Demir and Ellis, 1992a and 1992b; Demir and Ellis, 1993; Tarquis and Bradford, 1992; Lanteri *et al.*, 1993; Sarocco *et al.*, 1995; Gurusinghe *et al.*, 2001; Powell *et al.*, 2000; Demir, 2003; Cortez-Baheza, 2007; Catusse *et al.*, 2011; Chen and Arora, 2011). Furthermore, primed seedlings are better able to tolerate unsuitable growing conditions for instance drought, chilling, and saline soil (Farooq *et al.*, 2009; Sun *et al.*, 2010; Farooq *et al.*, 2011, Sarkar, 2012). Primed seed, including those of rice, require less time to germinate (Lee *et al*., 1998a, b). Vigorous seedlings with enhanced seedling establishment, and so greater plant population density, tiller number, earlier maturity, and high yield even under unfavourable growing conditions have also been reported from primed seeds (Lee *et al.*, 1998a, b; Du and Tuong, 2002; Harris *et al.*, 2002; Basra *et al.*, 2003; Farooq *et al.*, 2005; Farooq *et al.*, 2006; Sarkar, 2012).

Enhancement of germination potential due to priming involves several cellular and molecular mechanisms. Immediately after seed imbibes water from its surroundings (Phase I), up-regulation of germination-related genes and encoding of various enzymes are detected in primed seed, which are nearly absent in non-primed seed (Bewley *et al.*, 2013). Essential proteins involved in DNA synthesis, cell cycle (Sarocco *et al.*, 1995), enzyme activity (Gao *et al.*, 1999; Basra *et al.*, 2003; Willigen *et al.*, 2006; De Lespinay *et al.*, 2010), energy metabolism (Bray, 1995; Benamar *et al.*, 2003; Li *et al.*, 2010; Sun *et al.*, 2011; Weitbrecht *et al.*, 2011), reserve mobilization (De Lespinay *et al.*, 2010), antioxidant system (Bailly *et al.*, 2000; Lin *et al.*, 2005; Chen and Arora, 2011), and protection from desiccation tolerance (Close, 1996; Gallardo *et al.*, 2001; Gurusinghe *et al.*, 2002; Rorat, 2006; Cortez-Baheza, 2007; Kosová *et al.*, 2007; Catusse *et al.*, 2011) are then produced and accumulated (Phase II) in primed seed. These advances in physical and biochemical development also allow primed seed to approach endosperm weakening earlier (Toorop *et al.*, 1998; Chen and Bradford, 2000; Anese *et al.*, 2011). Furthermore, primed seed that has undergone phase I and II might skip to Phase III (radicle emerge) to complete germination upon reimbibition in phase III (Bewley *et al.*, 2013). Therefore, rapid radicle emergence (i.e. reduced period to germinate), vigorous seedling growth, better crop establishment and uniformity are the main benefits of seed priming.

Although seed priming is useful in agricultural practices, the consequence of priming on seed storability can be either positive or negative, in some cases both, depending upon species, pre-treatment method and priming conditions. There were findings that pre-treatment of seeds via priming could extend seed longevity (Gegorhiou *et al.*, 1987; Probert *et al.*, 1991; Wechsberg, 1994; Kuppusamy and Ranganathan, 2014), however priming

causing poor longevity tended to be reported more often. Reduced longevity was found in primed seed of lettuce (Tarquis and Bradford, 1992; Schwember and Bradford, 2005; Hill *et al.*, 2007), pepper (Saracco *et al.*, 1995), tomato (Argerich and Bradford, 1989), cauliflower (Powell *et al.*, 2000), sugar beet (Śliwińska and Jendrzejczak, 2002), bitter gourd (Lin *et al.*, 2005) and okra (Kuppusamy and Ranganathan, 2014). Similarly in cereals, comparison of germination rate over storage time found that sprouted seed lost vigour faster than non-sprouted seed (Hofmann and Sterner, 1994; Stahl and Steiner, 1998).

1.14 Implications for yield stability under flood-threatened rice production

Given the conflict between food security to meet the future growing demand and the ongoing severe threat of climate change on crop production described above, mitigation of crop damage and loss due to submergence is necessary.

1.14.1 *Sub1* rice varieties

Selection of appropriate rice varieties for different production ecosystems is essential. Although the traditional-local-rice landraces have biomechanical adaptation to flash- or deepwater flood, these cultivars provide low grain yield (i.e. 1-2 tonnes) per hectare (Bailey-Serres *et al.*, 2010). In contrast, the semi-dwarf-high-yielding-lowland-rice varieties produce greater grain yield of 6-8 tonnes ha^{-1}. Nevertheless, the latter are vulnerable to flooding, and may not be able to survive if the paddy fields are flooded for longer than one week (Mackill *et al.*, 1993; Bailey-Serres *et al.*, 2010; Ismail *et al.*, 2013).

Development of submergence-tolerant rice varieties is a target developed intensively by IRRI since 1975 (Bailey-Serres *et al.*, 2010).The submergence-tolerant improved varieties designated Sub-1 mega-varieties are new rice cultivars in which the flood resistant *Sub1* gene has been introgressed into high-yielding parents (producing about 6 tonnes of grain ha^{-1}; such as Swarna, Samba Mahsuri, IR64, TDK1, BR11 and CR1009) by marker-assisted backcrossing (Septiningsih *et al.*, 2009). This gene in submergence-tolerant rice allows plants to survive 10–14 days of complete submergence and to renew growth when the water subsides without negative impact on yield (Xu *et al.*, 2006; Septiningsih *et al.*, 2009). *Sub1* varieties showed greater yield stability (i.e. reduction to 1-3.5 tonnes ha^{-1}, rather than zero in the conventional varieties) when subject to flood during the early vegetative stage up to 14 days (Das *et al.*, 2009). Furthermore, no detriment to the parental agronomic traits were found in *Sub1* introgressed rice under non-submergence growing conditions (Ram *et*

al., 2002; Das *et al.*, 2009; Sarkar *et al.*, 2009; Singh *et al.*, 2009). Given these advantages, the improvement of submergence tolerance by this route has now involved more local elite rice landraces, for example Thai KDML105 (Siangliw *et al.*, 2003), Vietnamese Bacthom 7 (Khanh *et al.*, 2013; Linh *et al.*, 2013), Indonesian Ciherang (Septiningsih *et al.*, 2015), and Philippino PSB Rc18 (Septiningsih *et al.*, 2015).

1.14.2 Application of molybdenum

Pre-harvest sprouting is highly likely in rice production if inundation occurs during late maturation. The foliar application of molybdenum (Mo) is a well-acknowledged method to prevent pre-harvest sprouting in cereal crops, i.e. maize, barley, and wheat (Tanner, 1978; Walker-Simmons *et al.*, 1989; Cairns and Kritzinger, 1992; Modi and Cairns, 1994; Modi and Cairns, 1995; Cairns *et al.*, 1997). Therefore, the application of Mo to rice plants may aid sprouting resistance, and Mo-treated plants may thus be more flood tolerant.

Molybdenum is a trace element in soil with atomic mass of 95.96 (Gupta, 1992). In general, it is found in agricultural land in the form of the transition metal Mo (VI) oxide (Kaiser *et al.*, 2005). Molybdate (MoO_4^{2-}) is the common anion form that plants utilize for growth and development (Lindsay, 1979; Mengel *et al.*, 2001; Kaiser *et al.*, 2005). The uptake of Mo by plants depends upon the pH of the growing media: the lower the soil pH, the greater the availability of MoO_4^{2-}, with pH 4 to 5 the most efficient range for Mo to become soluble and accessible to plants (Reddy *et al.*, 1997; Smith *et al.*, 1997; Kaiser *et al.*, 2005). Small amounts of Mo are sufficient for plants to retain normal growth and development. Availability of Mo at 50 g ha^{-1} (Weir, 2004) or 0.1-1.0 ppm molybdate in soil (International Molybdenum Association, 2014) were recommended amounts to meet the nutritional requirement of most plants. In young leaves of cereals, less than 0.075 mg Mo kg^{-1} tissue fresh weight is classified as Mo deficiency (Department of Agriculture and Food, Government of Western Australia, 2015).

In the living cell, Mo itself is biologically inactive, but it is essential for molybdenum-requiring enzymes (molybdoenzymes) in both prokaryotes and eukaryotes (Zimmer and Mendel, 1999; Mendel and Hänsch; 2002, and Williams and Frausto da Silva, 2002). There are two kinds of Mo coenzymes; FeMoco, an iron-molybdenum cluster of coenzymes of nitrogenase, and Moco, which consists of a pterin subunit (Zimmer and Mendel, 1999; Williams and Frausto da Silva, 2002). In plants, Hille (1996), Mendel and Schwarz (1999), and Mendel and Hänsch (2002) reported that there were four Moco

dependent enzymes, which could be classified into two subgroups based on the structural differences. The first category was dioxo Mo hydroxylases, including nitrate reductase (NR) and sulphite oxidase (SO), and the second, mono oxo-Mo hydroxylases, include aldehyde oxidase (AO) and xanthine dehydrogenase (XDH). The above molybdoenzymes are the key participants in plant inorganic nitrogen assimilation, detoxifying excess sulphite, carotenoids and phytohormone biosynthesis (i.e. IAA and ABA) and purine catabolism, respectively.

1.15 Research purpose, objectives, and general hypotheses

Despite the projected steadily increasing demand of global rice consumption, variable rainfall, heavy storms, and sea-level rise are likely to be the major threats that may constrain rice production as they are predicted to increase in frequency in the late 21st century (IRRI, 2000; Kubo and Purevdorj, 2004; Mackill *et al.,* 2010; Mirza, 2011; IPCC, 2013). According to previous reports, most climate models have projected that flood risk is becoming greater due to the variable pattern of monsoon (i.e. the onset and departure date), frequency of intense rainfall, and expansion of flood-prone areas. The occurrence of flood can be severe and can happen any time throughout the rice growing season, whilst submergence tolerance in rice will differ amongst genotypes. But even in submergence-tolerant varieties, the effects of submergence during grain ripening are not well-known.

In this thesis, I use the seed viability equation (Ellis and Roberts, 1980a) to study changes in seed quality during seed development and maturation in rice in different production environments (flooded or not). This approach has been used successfully before to study the effect of seed production temperature on rice seed quality (Ellis *et al.*, 1993; Rao and Jackson, 1996; Ellis, 2011).

Therefore, the fundamental aims of this thesis are;

- to study the effect of submergence (i.e. simulated flooding) at different seed developmental stages on seed quality and subsequent storability.

  The differences in vulnerability to flood damage between genotypes are determined using *indica* (cvs IR64 and IR64 Sub1) and *japonica* (cv. Gleva) rices, in which the most susceptible seed developmental stage to submergence will be investigated. In terms of subsequent impact of submergence on seed quality post-harvest, the germinability and longevity of non-sprouted seed samples are examined.

- to investigates the impact of introgression of the *Sub1* gene on subsequent seed

storability using a pair of near isogenic cultivars, IR64 and IR64 Sub1.

- finally, evaluated the practical implication of Mo foliar spray as a possible technique to alleviate damage to seed due to submergence.

1.16 Thesis outline

In this thesis, there are three main experimental chapters; Chapter 2 focuses on the impact of submergence on yield and seed quality; Chapter 3 investigates the effect on seed longevity of submergence-tolerant rice with the *Sub1* gene introgressed; and Chapter 4 studies the potential of Mo to reduce pre-harvest sprouting. The general discussion is provided in Chapter 6.

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Chapter 2

The Effect of Simulated Flooding on Yield, Seed Weight, Germinability, and Longevity

2.1 Introduction

In terms of the projections of future climate change, ICCP (2007, 2013) predicted that there is more than a 90 and 65% chance that heavy rainfall and tropical cyclones, respectively, will increase by the mid to late 21st century. These extreme events not only increase variation in the pattern of precipitation, they can result in sea-level rise and more frequent inundations in low-lying estuary delta regions.

The losses in rice yield from flooding have been reported to vary from 10-100% depending on variety, growth stage, plant nutrition, crop practices, duration of submergence, and flood water conditions (e.g. water depth, water temperature, concentration of dissolved oxygen and carbon dioxide, water pH, light intensity, turbidity, and siltation on leaves during submergence) (Yoshida, 1981a; Reddy *et al*., 1985; Ram *et al*., 1999; Sharma and Ghosh, 1999; Kotera *et al*., 2005; Kotara and Nawata, 2007; Das *et al*., 2009; Dar *et al*., 2013).

Submergence at the seedling and early vegetative stage (14-50 days old) can be deleterious to crop establishment and affect number of tillers, number of panicles, panicle size, and plant survival after the water level subsides (Devender-Reddy and Mittra, 1985; Reddy *et al.*, 1985; Shama and Ghosh, 1999; Das *et al.*, 2009; Singh *et al.*, 2009; Singh *et al.*, 2011). Severe loss of yield caused by flooding during the reproductive stage of development is also documented (Devender-Reddy and Mittra, 1985; Reddy *et al.*, 1985; Kotera *et al.*, 2005). Form former reports, panicle initiation and booting are claimed to be the most susceptible of all developmental stages to damage from floods; the impairment of the development of the pollen mother cell, panicle formation, panicles failing to emerge, and delay to flowering are the main factors reducing grain yield when flooding occurs during this stage (70-80 day after transplanting: DAT). Submergence at one week after heading can produce sterility with less ripened grain, ultimately due to poor or failed fertilization (Devender-Reddy and Mittra, 1985; Kotera and Nawata, 2007). Submergence at the initial ripening stage is also detrimental to grain development. For example, in the study of Kotera *et al.* (2005) 40, 55, and 60% yield reduction was found from five days of full submergence

at seven days after anthesis (DAA) of rice cvs Tap Giao 4, CR203, and Moc Tuyen, respectively.

Finally, towards the end of seed development and maturation, pre-harvest sprouting, or vivipary, is one of the limiting factors that cause worldwide yield and quality losses in cereal production (Gelin *et al.,* 2006). This character, defined as in-spike or on-panicle germination close to harvest time of mature seeds that lack dormancy, coincides with wet conditions and warm temperatures in rice fields. In rice, pre-germination is undesirable in terms of both grain quality and quantity because it leads to deterioration such as discolouring, cracking, reduced grain weight, and poor cooking quality (Derera, 1989; Mahbub *et al.,* 2005). Especially in subtropical climates, precocious germination is troublesome and an ongoing problem which constrains rice production. In southwest China, pre-harvest sprouting can be detected in more than 6% of rice farmlands when prolonged rainfall occurs during early summer and autumn (Guo *et al.,* 2004; Wan *et al.,* 2006). The problem can become more severe when hybrid rice is subjected to unusual patterns of rainfall before harvest, with 10-50% lower yield (Tao *et al.,* 2007).

Submergence tolerance of rice has been improved by introgression of *Submergence1* (*Sub1*) gene into high-yielding cultivars and these has been promoted to farmers in East and Southeast Asia since 2009 (Septiningsih *et al.,* 2009; Wassmann *et al.,* 2009). Septiningsih *et al.* (2009) reported that the introgression line IR64 Sub1 was effective in enduring submergence for up to 17 days. Furthermore, they found that the grain quality of the original recurrent parent (IR64) had been inherited by the introgressed-line IR64 Sub1 without severe yield penalty. Nevertheless, whilst disadvantages to crop performance from introgression of *Sub1* into high-yielding varieties (i.e. IR64, Swarna, BR11, TKD1, Samba Mahsuri, and CR1009) has not been reported (Septiningsih *et al.,* 2009; Wassmann *et al.,* 2009), it is notable that submergence tolerance was studied during the vegetative growth phase of rice only (Septiningsih *et al.,* 2009; Mackill *et al.,* 2010; Singh *et al.,* 2011). Hence, an examination of the effect of submergence during the reproductive stage would be of benefit to evaluate the efficiency of *Sub1* introgressed-varieties.

It has been well documented that seed quality (germinability, desiccation tolerance, and potential air-dry storability) improves considerably during grain filling and maturation (Ellis *et al*., 1993; Ellis and Hong, 1994; Rao and Jackson, 1996; Ellis, 2011). However unfavorable growing conditions, i.e. high temperature, drought, salinity, as well as improper crop practices could limit attainable maximum quality and shorten the storability after harvest (Green *et al.*, 1965; Austin, 1972; Grass and Burris, 1995; Sanhewe *et al.*, 1996;

Spears *et al.*, 1997; Tuab *et al.*, 2008; Kochanek *et al.*, 2011; Hampton *et al.*, 2013). Nevertheless, the subsequent seed quality due to unpredictable rainfall and flash-floods during seed development and maturation drying (as opposed to earlier in seed development) has been poorly addressed.

In rice, the *japonica* subspecies is more sensitive to seed production environment than *indica* rice (Chang, 1991; Ellis *et al.*, 1992). Thus, to better understand the effect of submergence, contrasting genotypes of rice (cultivars selected to be high-yielding or from delta regions) were chosen for this study. The effect of submergence at different times of grain filling and maturation on yield, weight and the occurrence of pre-harvest sprouting were examined in two experiments. In 2012, to assess the critical seed developmental stage and the effect of duration of submergence, mimicking of natural flooding was imposed at different seed developmental stages for different periods (0-5 days). *Japonica* rice cv. Gleva, the round grain rice developed by Arroz de Valencia, was selected for this experiment. Gleva is early-maturing (Martínez-Eixarch and Ellis, 2015) and mainly cultivated in the wetlands of the Mediterranean coast. This cultivar is well adopted by farmers in Spain because it is a shorter-stemmed cultivar with high resistance to cryptogamic diseases and striped rice stem borer (Anonymous, 2014). In 2013, *indica* rice cvs IR64 and the submergence-tolerant IR64 Sub1 were selected to compare the influence of genotypic differences on flood tolerance along with *japonica* rice cv. Gleva. The occurrence of pre-harvest sprouting after submergence at different seed developmental stages was recorded, together with subsequent damage to yield, grain weights, and longevity in these contrasting genotypes. Furthermore, the ability of sprouted seed to re-germinate was investigated.

Although it is well-known that seed deterioration during storage is inevitable, there are methods to improve seed quality before sowing. Priming is one of the important techniques that is frequently applied in the seed industry (particularly for high-value crops). The procedure involves incubating dry seed in conditions of controlled water availability (often with osmotica), which encourage the germination process to start, and then dehydrate the seed at the point before radicles emerge (Bewley and Black, 1994; Bewley, *et al.*, 2013). If successful, this enhances subsequent plant growth in vegetable and cereal crops (Heydecker *et al*., 1973; Heydecker and Gibbins, 1978; Georghiou *et al.*,1987; Argerich and Bradford, 1989; Argerich *et al.*, 1989; Demir and Ellis, 1992a and b; Tarquis and Bradford, 1992; Demir and Ellis, 1993; Lanteri *et al.*, 1993; Sarocco *et al.*, 1995; Gurusinghe and Bradford, 2001; Powell *et al.*, 2000; Demir, 2003; Cortez-Baheza, 2007; Catusse *et al.*, 2011; Chen and Arora, 2011). The storability of primed seed may, however, be shorter depending on plant species and the osmotic treatments (Argerich and Bradford, 1989;

Tarquis and Bradford, 1992; Saracco *et al.,* 1995; Powell *et al.,* 2000; Śliwińska and Jendrzejczak, 2002; Lin *et al.,* 2005; Schwember and Bradford, 2005; Hill *et al*, 2007; Kuppusamy and Ranganathan, 2014).

The effect of (simulated) flooding to developing seed in this study has some similarities with seed priming. Nevertheless, there were two differences in the present study; water was freely available and the imbibition period was during seed development instead of post-harvest. In this Chapter, I report the possible effects of simulated flooding during seed development on the subsequent seed storage longevity of non-sprouted rice seed.

2.2 Null hypotheses

The main objective of this study was to examine the effect of submergence at different seed developmental stages on seed physical (i.e. yield and seed weight), visible susceptibility to pre-harvest sprouting, and physiological quality (i.e. germinability, and potential longevity), and to determine the stage at which the developing seed was most vulnerable to damage from flooding. Thus, the null hypotheses were;

2.2.1 There is no effect of submergence on rice seed productivity

- 2.2.1.1 Submergence has no effect on seed yield, weight or the occurrence of pre-harvest sprouting.
- 2.2.1.2 The duration of submergence has no effect on seed yield, weight or the occurrence of pre-harvest sprouting.
- 2.2.1.3 Stage of seed development at the time of submergence has no effect on seed yield, weight or the occurrence of pre-harvest sprouting.
- 2.2.1.4 Contrasting genotypes show no difference in the response of seed yield, weight, or the occurrence of pre-harvest sprouting (i.e. as above) to submergence.

2.2.2 There is no effect of submergence tolerance on rice seed germinability and subsequent longevity

- 2.2.2.1 There is no difference of dormancy level and hence to vulnerability to pre-harvest sprouting between rice sub-species *japonica* and *indica.*

2.2.2.2 Submergence has no effect on subsequent germinability and storability of seeds not sprouted after flooding.

3.2.2.3 The duration of submergence has no effect on subsequent germinability and storability

2.2.2.3 Stage of seed development at the time of submergence has no effect on subsequent germinability and storability

2.2.2.4 Contrasting genotypes show no difference in seed longevity following submergence.

2.2.2.5 There is no difference in seed longevity between the submergence-tolerant introgression cultivar and the parental variety.

2.3 Materials and Methods

2.3.1 Plant culture

Two experiments were conducted, one in 2012 and one in 2013, with pot-grown plants under controlled environments provided by a heated and vented glasshouse (6.40 x 8.60 m, Appendix 2.1) with adjoining dark compartments (four, 3.50 x 2.07 x 2.10 m each) at the Plant Environment Laboratory (PEL) (51°27'N latitude and 00°56'W longitude), University of Reading. Three cultivars of rice were selected for study. Seeds of *japonica* rice cv. Gleva (2012 and 2013) were provided by the Institute of Agrifood Research and Technology (IART), Barcelona, Spain, and *indica* rice cvs IR64 and IR64 Sub1 (2013) by the International Rice Research Institute (IRRI), Los Baños, Philippines.

The growing media comprised steam-sterilized coarse sand, crushed gravel, peat compost, and vermiculite in the ratio of 2:4:1:4 (v/v), respectively. Slow release fertilizer (Osmocote Pro 3-4M, Everris International BV, The Netherlands) containing $N:P_2O_5:K_2O:MgO$ (17:11:10:2) was added at 3 kg m^{-3}. The 18 cm diameter (3 litre capacity) plastic pots were filled with the mixture, then pre-soaked with tap water and left to drain overnight before sowing. The positions of pots on four trolleys (2.84 x 1.05 m each) were recorded. Each trolley contained 75 pots, with seven rice seeds sown directly into each pot. After that, they were kept inside complete dark compartments where the temperature was maintained constantly at 25 °C until seedlings began to emerge (7-10 days).

A short-day environment was provided from seedling emergence onward. Photoperiod control was achieved by moving trolleys containing the plants from and into dark compartments at 8 a.m. and 7 p.m. (11 hours/day), respectively, every day. The temperature inside the glasshouse and dark compartments was maintained at 28/20 °C day/night (11 h/13 h). The thermoperiod and photoperiod were synchronous. This regime is known to be suitable for rice seed production in controlled environments (Ellis *et al.*, 1993). The number of plants per pot was reduced to four at the four-leaf stage (16-30 days after sowing, DAS, depending on cultivar).

During the beginning of experiment when seeds were germinating and seedlings emerging, plants were irrigated by hand with tap water (until 14 DAS). An automatic drip feed irrigation system (6 times/day) was then installed in each individual pot. Pots had drain holes and were irrigated to overflowing on each occasion. The top of pots was 2 cm above the top of the growing media. The nutrient solution was based on common practice following Yoshida *et al.* (1976). It contained 100 mg L^{-1} inorganic nitrogen and was

acidified to pH 5.0 ± 0.2. Savona (Koppert B.V., The Netherlands), an insecticidal soap containing 50% fatty acid from potassium salt, was used occasionally during plant development to control spider mites and aphids when detected. The criteria for the identification of anthesis were that at least three-quarters of the panicle had exserted and more than half of florets on the panicle were flowering, as determined by close examination of a sample. In 2012, 10% of 1,200 plants from different pots were examined to identify the timing of anthesis, meanwhile in 2013 the sample size per cultivar increased to 16% (50 from 320 plants in total).

2.3.2 Submergence treatments

Submergence ("simulated flooding") was carried out in the glasshouse under the controlled environments above (i.e. the same glasshouse that plants were grown in). Temperature was maintained at 28 °C in the glasshouse and combined with natural light (ambient photoperiod for the whole duration of the submergence treatments). To prevent lodging, all plants within a pot were supported by a wooden stick and bound together with wool, a wrap-around plastic (self-tie loop-lock label, 19.1 x 2.5 cm) tag, or a plastic cable tie (20.3 x 0.25 cm) (depending on available materials in each experiment). Steam-sterilized gravel was placed as the top layer of each pot to avoid the growing media being dislodged under simulated flooding. The details of treatment combinations for each experiment are provided in Table 2.1.

Table 2.1 Details of submergence experiments conducted under glasshouse conditions during 2012 and 2013

Experiment (Year)	Sowing date	Submergence treatment	Submergence duration	Cultivar	50% Anthesis date (DAS)[1]	Harvest
2012	25 May	1. Control (No submergence) 2. 9 DAA[2] 3. 30 DAA 4. 35 DAA 5. 40 DAA	1. 3 days 2. 5 days	1.Gleva	69	47 DAA (116 DAS)
2013	9 May	1. Control (No Submergence) 2. 10 DAA 3. 30 DAA 4. 40 DAA	1. 4 days	1. Gleva 2. IR64 3. IR64 Sub1	73 87 93	46 DAA (119 DAS) 46 DAA (133 DAS) 46 DAA (139 DAS)

[1] DAS = days after sowing

[2] DAA = days after 50% anthesis

Due to the work in 2012 was the first experiment (not just mine but covering a gap in previous research), intended to obtain basic data about the effect of submergence at different grain ripening stages of rice cv. Gleva. Those results were applied to fine-tune the experimental design in the following year (2013). This experiment was designed to investigate the potential effects of two factors;

Factor 1: Seed development stage: 9, 30, 35, 40 DAA or Non-submergence as Control. These different timings of submergence were designed to represent seed filling, or early-, mid-, or late-maturation drying.

Factor 2: Submergence duration: two periods of submergence (3 or 5 days) were designed to represent shorter or longer temporary (flash) flooding events.

As the experiment was carried out with pot-grown plants under glasshouse conditions with limitation of space to 300 pots (on 4 trolleys, 75 pots each), the non-submergence plant treatments for comparison with 3 days' or 5 days' submergence were merged into a single Control group. Although the latter led to non-orthogonality, i.e. nine treatment conditions from eight submergence treatment combinations with one non-submergence Control, it provided benefits to achieve maximum basic information for subsequent years with limited resource. The benefits obtained from reducing the Control groups to one included;

1) extra pots of plants available to determine through close destructive examination patterns of plant growth and seed development

2) more pots within treatments, up to ten, and thus larger seed samples (i.e. non-sprouted seed fraction after submergence treatment) for subsequent work on longevity

3) a sensible number of blocks, i.e. three blocks to aid statistical analyses of the results."

Flooding treatments were provided by submerging rice plants growing in their pots, gently in a wooden tank approximately 2.4x1.2 m (1.1 m in depth), with black polyvinyl as the waterproof membrane (Fig. 2.1). The experiment was laid out as a randomized complete block design (RCBD) with 10 pots per treatment combination. The space on four trolleys was divided into the three blocks (18 rows of 5 pots/block, 75 pots per trolley, four trolleys in total). In each block, there were 90 pots from treatment combinations plus 10 more pots that were used to allow very close examination of the growth and development of the plants.

Hence, the total number of pots in this experiment was 300. To match the blocking the space on trolleys, the area in the water tank for the flooding treatment was similarly blocked from east to west in three (0.8 x 1.2 m) (Appendix 2.2).

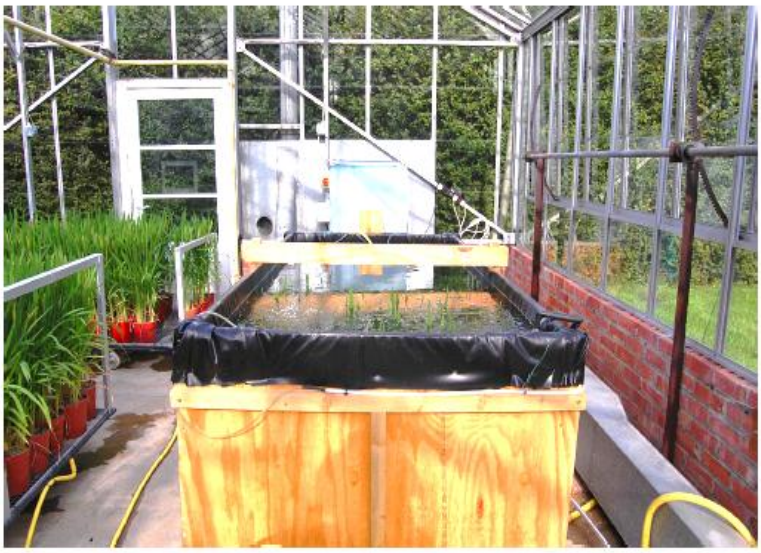

Figure 2.1 Rice plants in pots in water tank during submergence treatment in the glasshouse

For each separate submergence date, the tank was filled with new tap water before the date of application to eliminate contamination from previous floodwater - such as pH level, turbidity, accumulation of micro-organism, algae, as well as oxygen or carbon dioxide concentrations (Ram *et al*., 1999; Das *et al*., 2009; Singh *et al*., 2009). Water was circulated and aerated for the whole duration of treatment to avoid it becoming stagnant and anoxic (Appendix 2.2). A pump (RG25 Stuart-Turner, 240V, 180W, Stuart-Turner, UK) was used to circulate water around the container, with four air-stones (50 mm diameter) from Precision Air Pump (Aqua One 12000, 240V, 14W, Precision Manufactured, Australia) placed under water at each corner of the tank to provide better oxygen diffusion. The water temperature was associated with air temperature but water temperature was not controlled directly; it ranged from 20 to 25 °C. Hence, during the duration of submergence, treated plants differed slightly in temperature from those on trolleys. Nevertheless, the difference in mean temperature was small. When submerged, all panicles were completely covered by water up to 80-95 cm deep, the depth being controlled to be 2 cm above the highest panicle. After the treatment, the treated plants were returned carefully to their previous position on

the trolley and irrigation supply reconnected (and hence the nutrient solution was provided again).

The experiment in 2013 was conducted in a similar manner to that described above for 2012, but involved two geographical races, *japonica* rice cv. Gleva and *indica* rice cvs IR64 and IR64 Sub1. Thus two *indica* rices were included in the study, which enabled also comparison of IR64 with its submergence–tolerant relative, IR64 Sub1, as well as between *indica* and *japonica* rices. According to the results from 2012, precise durations and timing of submergence were adjusted in order to obtain sufficient non-sprouted seeds for longevity determination despite adding two cultivars to the investigation. Simulated flooding for four days (one duration only) was applied at 10, 30 and 40 DAA, in which non-submergence was represented as a Control. The experiment was designed as a RCBD with two blocks and 10 pots per treatment combination. Thus the total number of pots for the 12 treatment combinations including the control was 240. The level of dissolved oxygen and temperature of the water during submergence were recorded hourly by a Pro Optical Dissolved Oxygen instrument (YSI Incorporated, Ohio, USA). The submergence treatment was conducted on different dates amongst the three cultivars (Table 2.1, Fig. 2.2) because flowering date varied. Similarly, harvest date varied amongst cultivars.

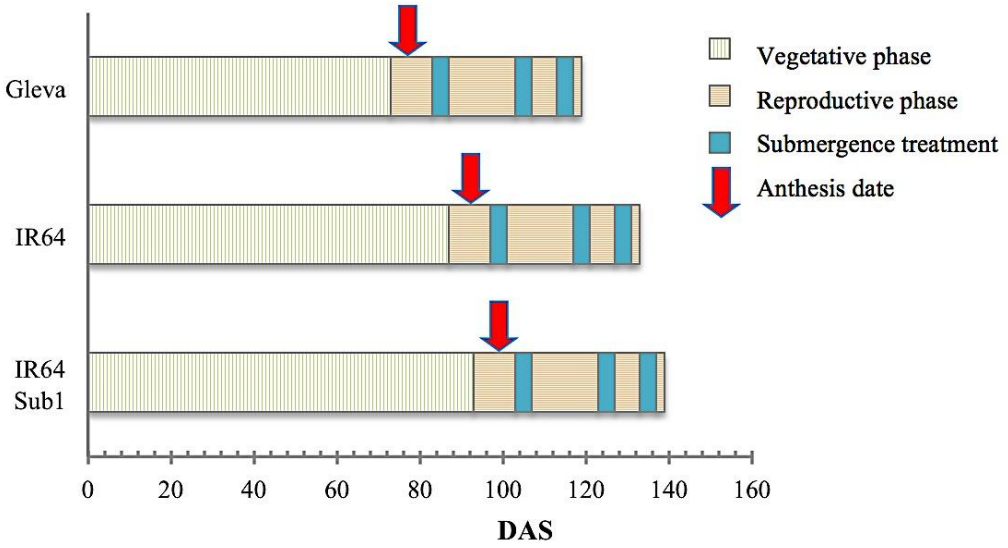

Figure 2.2 Timeline in 2013 to show timing of the three different four-days-duration simulated flooding treatments of rice cvs Gleva, IR64, and IR64 Sub1 in relation to plant development

2.3.3 Harvest

Irrigation stopped two days before harvest towards the end of each experiment. A single harvest of seeds was made at maturity when seeds had dried naturally ("harvest maturity"). This is when panicles became yellow and grains firm. All panicles in each treatment combination within a block were collected together. That is the seed samples from each block for each treatment were kept separate throughout harvest and subsequent laboratory investigations. The ripened seed was threshed from panicles by hand.

2.3.4 Assessment

2.3.4.1 Effect of submergence on crop performance

2.3.4.1.1 Crop yield and the occurrence of pre-harvest sprouting

Seed samples from each of the treatment combinations at harvest maturity were assessed for yield per pot. In some treatment conditions, sprouted seeds were observed at harvest maturity. These were separated by eye and counted to provide numbers of sprouted seeds (caryopses enclosed by paleas and lemmas: photographs of seeds defined as sprouted, i.e. radicle, shoot, or micropyle observed, is provided in Fig. 2.3) or non-sprouted seed. The yield per pot reported is the weight of all sprouted and non-sprouted seeds determined when seed were dried to 12-14% moisture content. Pre-harvest sprouting was calculated as the percentage of sprouted seed of the total number of seeds produced.

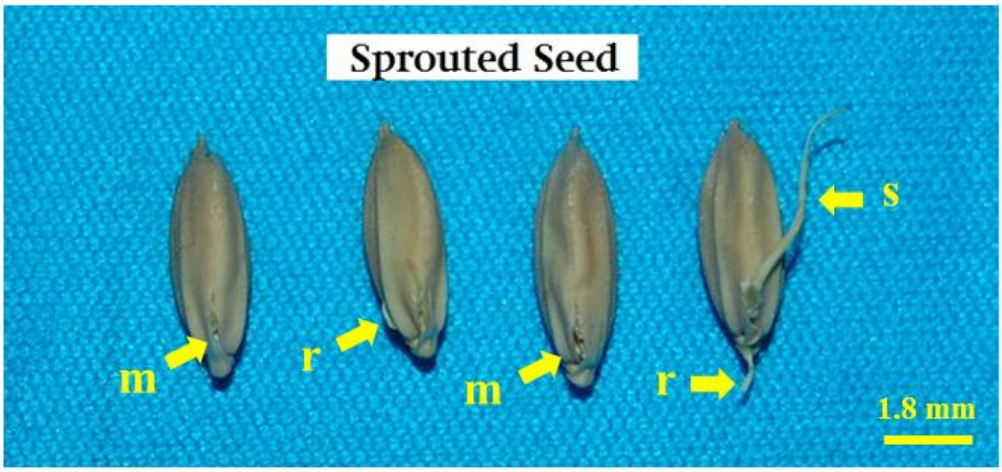

Figure 2.3 Sprouted seeds from submergence treatments (cv. Gleva, 2012) that showed visible signs of germination (shoot [s], radicle [r], or micropyle [m] are identified)

2.3.4.1.2 Seed moisture content determination

The moisture content of non-sprouted seeds was measured immediately after harvest. Two replicates of 2.5-3.0 g of seed were sampled from each seed lot to determine moisture content by the high-constant-temperature-oven method (International Seed Testing Association, 2013b). Each seed sample was ground in a mill (Laboratory Mill 3303, Perten Instruments Ab, Stockholm, Sweden). The fine powder after grinding was placed in a glass Petri-dish that had been weighed before (*M1*). The initial weight of sample and container together before (*M2*) and after drying at 130 ± 2 °C for 2h (*M3*) in a forced air oven (Thermocenter, Savis Lab, Rotkeuz, Switzerland) were recorded on a balance to 0. 1 mg (Mettler Toledo, AE160, Greifensee, Switzerland). Percentage moisture content was calculated on the fresh weight basis (International Seed Testing Association, 2013b) by the following formula;

$$\text{MC}\ (\%) = (M2 - M3) + \frac{100}{(M2 - M1)}$$

2.3.4.1.3 1000 seed weight

To determine the fresh weight of 1000 seeds, 800 non-sprouted seeds of known moisture content were randomly selected from the sample for each treatment combination using the hand sampling method to provide a working sample. These seeds were then weighed (W_{800}) using a balance with 0.1 mg accuracy (as above). Thousand seed fresh weight was calculated by the formula;

$$1000\ \text{Seed Weight}\ (\text{g}) = \frac{1000 \times W800}{800}$$

In the case of thousand seed dry weight, this former value was then converted to dry weight using the estimated moisture content;

$$\text{Dry weight}\ (\text{g}) = \text{Initial weight}\ (\text{g})\ \text{x}\ \frac{100 - \text{Initial moisture content}}{100 - \text{Final moisture content}}$$

2.3.4.2 Effect of submergence on seed quality and subsequent longevity

2.3.4.2.1 Germination test

2.3.4.2.1.1 Non-sprouted seed

To determine the ability of seeds to germinate, two replicates of 50-70 seeds each were placed between moist rolled paper towels [Kimberley Clark Professional, Hostess Natural Hand Towels - S Fold (Natural, 24 × 35 cm), Greenham Sales, UK], in which the lower and upper layer consisted of three and one sheets, respectively. The sample size for this germination test was limited in order to conserve seed for all of the assessments to be made. The towels were first soaked in deionized water and hand wrung almost dry. Up to four tests were wrapped together in a polyethylene bag, folded up at the top but left loose enough to allow sufficient air exchange. The bags were placed in an upright position in a germination cabinet (LMS Cooled Incubator, LMS Ltd., Kent, UK) maintained at the alternating temperature regime of 34/11°C (16 h/8 h) for 21 days, as recommended by Ellis *et al.* (1983) to break dormancy.

Throughout this thesis, viability is defined as the proportion of seeds that germinated normally in test after dormancy had been broken. The first count of seedlings was conducted after seven days. Normal seedlings with well-developed root (i.e. a long-slender-primary-root) and shoot axis (i.e. a straight-slender-elongated hypocotyl) were identified, removed, and recorded (ISTA, 2013a). In some germination tests (freshly-harvested seed), seed covering structures were removed from seeds that remained firm after 21 days in test to promote loss in dormancy. In this case, the total test period was extended to 28 instead of 21 days. Subsequent spraying of deionized-water during each counting was necessary to retain sufficient moisture of the paper towels. The paper substrate was changed at intermediate counts if the test became infected by fungi.

2.3.4.2.1.2 Sprouted seed

Eighteen samples of dry sprouted seeds (11-12% moisture content) obtained from imposing simulated-submergence for 3 or 5 days on 30, 35, and 40 DAA (three blocks each) in 2012 were used to assess the capability of such seeds to (continue to) germinate upon reimbibition. The germination test procedure was as described above, except that the number of replicates was increased to four. Thus the total number of sprouted seeds used in this test was 200-280 per sample.

2.3.4.2.2 Seed storage longevity

The remainder of non-sprouted seeds of each sample harvested at maturity was kept individually in muslin bags and air dried in the laboratory at 22 ± 2 °C for 14-20 days. After that, each seed sample was sealed in a laminated aluminium foil bag and stored at 2-4 °C until the determination of seed storage longevity commenced.

For experimental storage, the moisture content of samples was first determined and then adjusted to 15 % by humidification above water or by drying over silica gel (depending upon the extent of increase or reduce in moisture content required) at 20 °C. Seeds in sealed containers were then left to equilibrate at 2-3 °C for 2-3 weeks and moisture content re-determined indirectly at 20 °C by Humidat IC1 (Novasina, Switzerland) in 2012 or dew point hygrometer (Aqualab, 3TE, Decagon Devices, Inc. Pullman, USA) in 2013 to provide an estimate of the equilibrium relative humidity (eRH) (Probert *et al.*, 2003). The weight (and hence indirectly moisture content) of seed samples were monitored regularly until they were close to 15 ± 0.2 %, which moisture content was finally confirmed by the high-constant-temperature-oven method (Section 2.3.4.1.2).

Longevity was determined in hermetic storage, using laminated aluminium foil bags (Moore & Buckle Ltd. St Helens, UK), in a heated incubator at 40 ± 0.5 °C with about 15 % moisture content (Table 2.2). For each seed sample, 10-15 sub-samples of seed (about 150-200 seeds each) were stored. The sub-samples were withdrawn at different periods from the incubator at regular intervals during storage. The maximum storage period varied between 28 and 49 days depending on treatment, investigation, and variety. The 150-200 seeds in each sub-sample were tested for ability to germinate in three replicates of 50-70 seeds. These determinations were conducted as described above (Section 2.3.4.2.1.1). Seed survival curves (ability to produce normal seedlings was used as the criterion of viability) were fitted to the observations by probit analysis (GenStat 13th edition, released on July 2010, VSN International Ltd.) for each sample to provide the estimates of K_i and σ for the seed viability equation (1.4) as suggested by Ellis and Roberts (1980a). This required the fitting of 27 survival curves in 2012 (9 treatments x 3 blocks) and 24 in 2013 (3 cvs x 4 treatments x 2 blocks) (Table 2.2).

Table 2.2 Details of seed longevity determinations on seed samples produced in 2012 and 2013.

Experiment (Year)	Cultivar	Sowing date	Harvest date	Submergence duration	Submergence treatment	Number of blocks	Determination of longevity		
							Moisture content (%)	Start date	Maximum storage period (days)
2012	Gleva	25 May	17 Sep	1. 3 days 2. 5 days	1. Non-submergence 2. At 9 DAA[1] 3. At 30 DAA 4. At 35 DAA 5. At 40 DAA	3	15.1±0.2[2]	15 Jan 2013	32
2013	1. Gleva 2. IR64 3. IR64 Sub1	09 May 09 May 09 May	05 Sep 19 Sep 25 Sep	4 days	1. Non-submergence 2. At 10 DAA 3. At 30 DAA 4. At 40 DAA	2	14.7±0.2[3]	08 Feb 2014	49

[1] DAA = days after 50% anthesis

[2] Mean and standard error of 27 seed samples

[3] Mean and standard error of 24 seed samples

2.3.4.3 Seed development and maturation of rice

To determine if there were any differences in seed development and maturation amongst the *japonica* and *indica* rice cvs Gleva, IR64 and IR64 Sub1, serial harvests were conducted in 2013. In each cultivar, panicles with the same date of anthesis were tagged and sampled destructively every 3-5 days from 8 DAA until harvest maturity at 47 DAA. There were 10 sampling times, with six panicles harvested randomly from six pots in each destructive sampling. Therefore, the total number of panicles harvested from four treatments for assessment of seed development amongst cultivars was 60. Within a treatment of each cultivar, 15 panicles from two blocks (7 or 8 in each block depending on available panicles) were selected from different pots (i.e. 15 of 20 pots). Each selected panicle was threshed separately to extract the seed, and hence contributed six replicate samples. Empty (unfilled) seeds were discarded. To assess seed development and maturation of rice, all six replicates were weighed separately for mean seed weight. After that, two of those six replicates were used in subsequent determination of seed moisture content, and ability to germinate normally of freshly-harvested and dried seed, respectively.

2.3.4.3.1 Seed weight

Mean seed fresh weight was calculated from total seed weight harvested divided by the total number of seed from that selected panicle. Mean seed dry weight was calculated as described above in Section 2.3.4.1.3.

2.3.4.3.2 Moisture content

For fresh seeds harvested before harvest maturity during seed development, pre-drying was necessary to determine moisture content. At each sampling date, one panicle from each block was used in this determination. Seeds were weighed and placed in a pre-weighed Petri-dish and left overnight in an incubator (LEEC drying cabinet 226L Sliding door, LEEC Limited, Nottingham, UK) maintained at 30 ± 2 °C. The loss in moisture in this first stage was determined similarly to Section 2.3.4.1.2. Immediately thereafter the seed was ground, then subjected to the high-constant-temperature-oven method (above) to determine the moisture content loss in the second stage of drying. The final moisture content of the sample was then calculated by using this formula;

$$\text{MC}\ (\%) = (S1 + S2) - \frac{100(S1 \times S2)}{100}$$

where *S1*and *S2* were the moisture loss in the first and second stage, respectively (ISTA, 2013c).

2.3.4.3.3 Changes in ability to germinate during seed development and maturation

Following the observation of pre-harvest sprouting after submergence in 2012, changes in the ability to germinate during grain filling and maturation were investigated in 2013. The main purpose of this experiment was to indicate whether there were varietal differences in dormancy during seed development and whether or not these might be associated with pre-harvest sprouting.

Germination tests were carried out as above (Section 2.3.4.2.1.1). Because only filled-seeds were used in this test, therefore the number of seeds per replicate varied from 24 to 70 depending on seed developmental stage and variety. Due to workload pressures, the contrast of ability to germinate between *japonica* and *indica* rice was tested with cvs Gleva and only IR64.

2.3.5 Statistical analyses

One-way analysis of variance (ANOVA) with blocking was applied to the results of each of yield per pot, grain weight, and percentage pre-harvest sprouting (with arcsine transformation) to assess the effects of treatments. GenStat 13th edition (released on July 2010, VSN International, Ltd.) was performed to analyze these data. The multiple comparisons of means using least significant differences at $P = 0.05$ were performed using Tukey's test and represented with different lowercase or uppercase letters where statistical significance was detected. To test for interactions between treatments, two-way ANOVA where randomized block results were available, or restricted maximum likelihood analysis (REML) to analyze linear mixed models for unbalanced experimental designs were applied.

2.4 Results

2.4.1 Effect of submergence on seed production and quality of *japonica* rice cv. Gleva (2012)

2.4.1.1 Yield and the occurrence of pre-harvest sprouting

Effect of submergence on yield per pot was evaluated from each sample by using the dry matter of sprouted and non-sprouted seed. Grain yield of rice cv. Gleva submerged at 9 DAA was less than the Control (non-submergence) (32% less for 3 days' submergence and 33% for 5 days'). There was substantial variation in yield per pot, however, and hence no significant effects of the treatments (Table 2.3).

The submergence treatments affected pre-harvest sprouting (P<0.001, Appendix 2.4) considerably (Figure 2.4), with significant effects of the duration of submergence (P<0.001), plant developmental stage at submergence (P<0.001), and their interaction (P<0.05). The largest percentage (53%) of sprouted seeds was from 5 days' submergence at 40 DAA (i.e. the longest and last treatment). Figure 2.4 shows clearly that submergence at 9 DAA had no effect, but thereafter the later during development that the plants were submerged, and the longer they were submerged for, the greater the proportion of sprouted seed.

Table 2.3 Effect of submergence for 3 or 5 days at different stages of plant development on dy matter yield pot^{-1} of cv. Gleva (2012)

Treatments	**Yield of seed pot^{-1} (g)[1]**					
	0 days		**3 days**		**5 days**	
	Mean	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**
Control	11.8	1.39				
9 DAA			8.0	0.41	7.9	0.89
30 DAA			15.4	3.72	9.8	1.02
35 DAA			9.9	1.36	10.0	1.23
40 DAA			10.4	2.64	12.1	1.70
F Test[2]	0.235	NS				

[1] At 0% moisture content

[2] One-way ANOVA for 9 treatments (included Control), see Appendix 2.3a

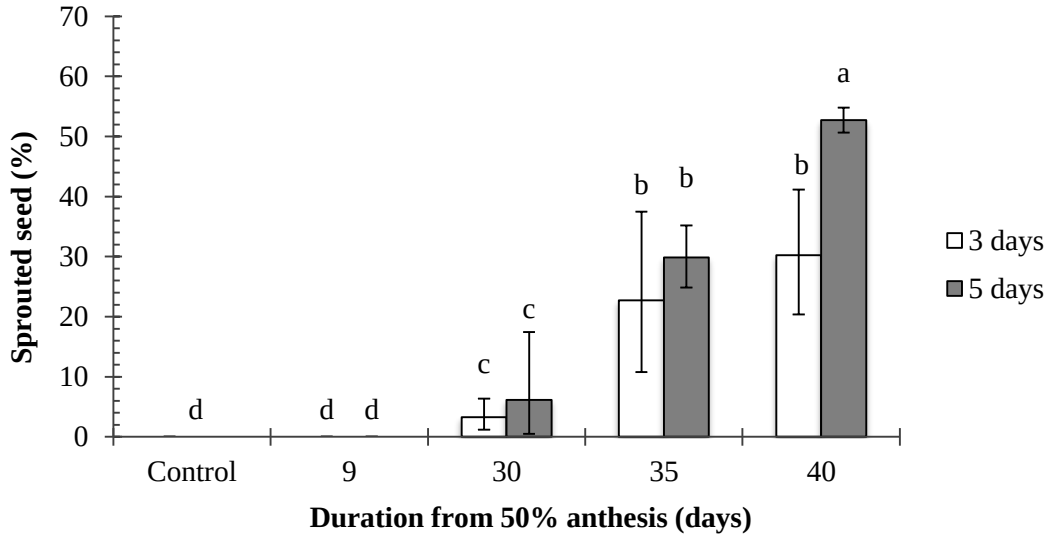

Figure 2.4 The impact of different durations of submergence, i.e. 3 (□) or 5 (■) days at various stages of development on pre-harvest sprouting assessed at harvest maturity of *japonica* rice cv. Gleva (2012). The percentage of sprouted seed was assessed after seed was dried to 11-12% moisture content. The vertical bars represent ± s.e. (n=3). Superscripts with a different letter indicate significant difference (P<0.05) using Tukey's Multiple Range Test (Appendix 2.4a).

2.4.1.2 Seed moisture content and 1000 seed weight at harvest maturity

To determine the effect of simulated flooding at different seed developmental stages on seed moisture content and dry seed weight at harvest maturity, sprouted seeds were separated from the remainder of the sample. Therefore, the values of those two parameters in this experiment were for the non-sprouted fraction only.

Moisture content of freshly-harvested seed of cv. Gleva in 2012 varied significantly around 18-22% depending on treatment (P = 0.019, Table 2.4). Seeds of the control had a higher moisture content (21.7%) than all submerged treatments. The lowest moisture content (17.9%) was found in freshly-harvested-non-sprouted seeds with 5 days' submergence at 40 DAA. There was a general trend amongst nine treatments that the more mature the developing seed when submerged or the longer the duration of flooding the lower the moisture content of non-sprouted seed at harvest: only the developmental stage at flooding had a significant effect (P < 0.001), that of flooding duration not being significant (Table 4.2, P = 0.150, Appendix 2.7a). Nevertheless, the former factors was less significant (P=0.058) if the interaction term between two effects of seed development stage and submergence duration was considered without Control's group (Table 4.2, Appendix 2.7b).

Table 2.4 Moisture content of freshly-harvested-non-sprouted rice cv. Gleva at harvest maturity (47 DAA) (2012) after submergence for 0, 3, or 5 days at different seed developmental stages. Testing of the interaction of submergence developmental time (DAA) with duration was performed by restricted maximum likelihood (REML) or 2-ways ANOVA.

Treatments	**Moisture content (% fresh weight)**		
	0 days	**3 days**	**5 days**
Control	21.7		
9 DAA		19.7	20.7
30 DAA		20.5	19.6
35 DAA		20.0	18.1
40 DAA		18.7	17.9
F Test [1] ***P***	0.019		
LSD = 0.05	2.048		
	REML[2]	**2-ways ANOVA**[3]	
Seed development stage (DAA)	<0.001	0.058	
Submergence duration (d)	0.150	0.233	
DAA . d	0.133	0.279	

[1] One-way ANOVA for 9 treatments (included Control), see Appendix 2.6

[2] Control was included in interection analysis using REML, see Appendix 2.7a

[3] Control was excluded in interection analysis using 2-ways ANOVA, see Appendix 2.7b

In the case of 1000 seed weight at harvest maturity, significant differences (*P*<0.001, Appendix 2.8) were found for 1000 seed dry weight (0% moisture content; Table 2.5). Dry seed of all submergence treatments were lighter than the control, significantly so for 9 DAA for both durations of submergence as well as 5 days' submergence at 30 DAA. Submergence for 5 days provided consistently lighter thousand seed dry weight than 3 days' submergence and significantly so for submergence at 9 DAA (Table 2.5). The greatest reduction from the control in seed dry weight (3.5%) was for 5 days' submergence at 9 DAA. In the facts that there was no significant interaction within submergence treatments (Control was excluded, Table 2.5, Appendix 2.9b), both seed development stage and submergence duration affected seed dry matter significantly: 35, 40 > 30, 9 DAA and 3 days' submergence > 5 days' submergence. Moreover, note that the mean seed dry weight from the treatments does not contradict the conclusions for the average yield per pot from Table 2.3: Control > 3 days' submergence > 5 days' submergence; and Control > 30, 35 or 40 DAA > 9 DAA. That is, early and longer submergence reduced yield and seed dry weight the most.

Table 2.5 1000 seed dry weight (0% moisture content) of non-sprouted rice cv. Gleva at harvest maturity (47 DAA) (2012) after submergence for 0, 3, or 5 days at different seed developmental stages. Testing of the interaction of submergence conducting time (DAA) with duration was performed by restricted maximum likelihood (REML) or two-ways ANOVA.

Treatments	**Weight of 1000 dry seed (g)[1]**					
Submergence:	**0 day**		**3 days**		**5 days**	
	Mean	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**
Control	29.5	0.22				
9 DAA			28.4	0.19	27.4	0.16
30 DAA			28.6	0.19	27.9	0.19
35 DAA			29.3	0.15	28.7	0.18
40 DAA			29.1	0.35	28.5	0.21
F Test[1] ***P***	<0.001					
LSD = 0.05	0.547					

	REML[2]	**Two-ways ANOVA[3]**
Seed development stage (DAA)	<0.001	<0.001
Submergence duration (d)	0.618	<0.001
DAA . d	1.000	0.701

[1] One-way ANOVA for 9 treatments (included Control), see Appendix 2.8

[2] Control was included in interection analysis using REML, see Appendix 2.9a

[3] Control was excluded in interection analysis using two-ways ANOVA, see Appendix 2.9b

2.4.1.3 Ability to germinate

2.4.1.3.1 Non-sprouted seed

Ability to germinate normally of mature freshly-harvested seed of rice cv. Gleva from the different treatments were all greater than 90%, and significant differences were nevertheless detected amongst treatments (Fig. 2.5, $P = 0.014$, Appendix 2.10). Estimates for seed first dried (to 15.1 ± 0.2% moisture content) varied between 89 and 97%, but these differences were not quite significant ($P = 0.073$, Appendix 2.11). The lowest germination was found from the 5 days' submergence at 40 DAA treatment (i.e. longest duration and latest in development), however low values also were detected at 9 DAA with 5 days' submergence. There were only slight differences with no significance ($P = 0.161$, Appendix 2.12) between fresh and dried seed in ability to germinate of fresh and dry seed at harvest maturity within each treatment. Figure 2.5 shows that freshly-harvested seeds resulted in slightly higher germination percentage than dried seeds in almost all treatments, except submergence at 30 and 40 DAA where the dry seed had equal or higher germinability than the fresh seeds, respectively.

Although only one significant difference in germination ability of fresh and dry rice cv. Gleva seeds was detected, there were differences in germination rate. The vigour of freshly-harvested-non-sprouted-submerged seeds was comparatively poor compared with the Control when the number of normal seedlings emerged after only seven days in the germination test was considered (Fig. 2.6). In the case of germination of mature freshly-non-sprouted seed, high ability to germinate at the first count (more than 97%) was found only in the Control (non-submergence) treatment or 3 days' submergence at 9 DAA. On the other hand, it was noted that delay in germination was considerably less with dried seed. Therefore, at the first count of freshly-harvested-non-sprouted seed, the longer submergence or the more mature the stages of development the slower the germination.

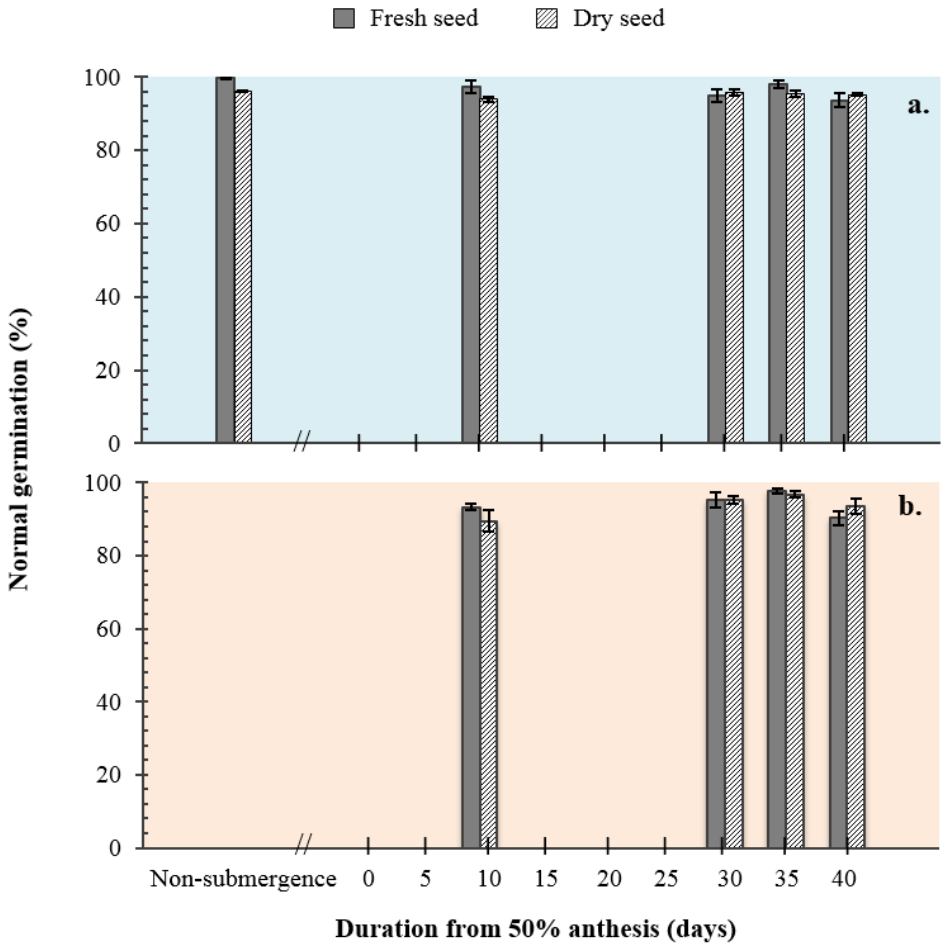

Figure 2.5 Ability to germinate normally of freshly-harvested (■) and dried (▨) non-sprouted-seed (15.1 ± 0.2% moisture content) of cv. Gleva after submergence for 3 (a) or 5 (b) days at different seed developmental stages (2012). The vertical bars represent ± s.e. (Appendices 2.10 and 2.11).

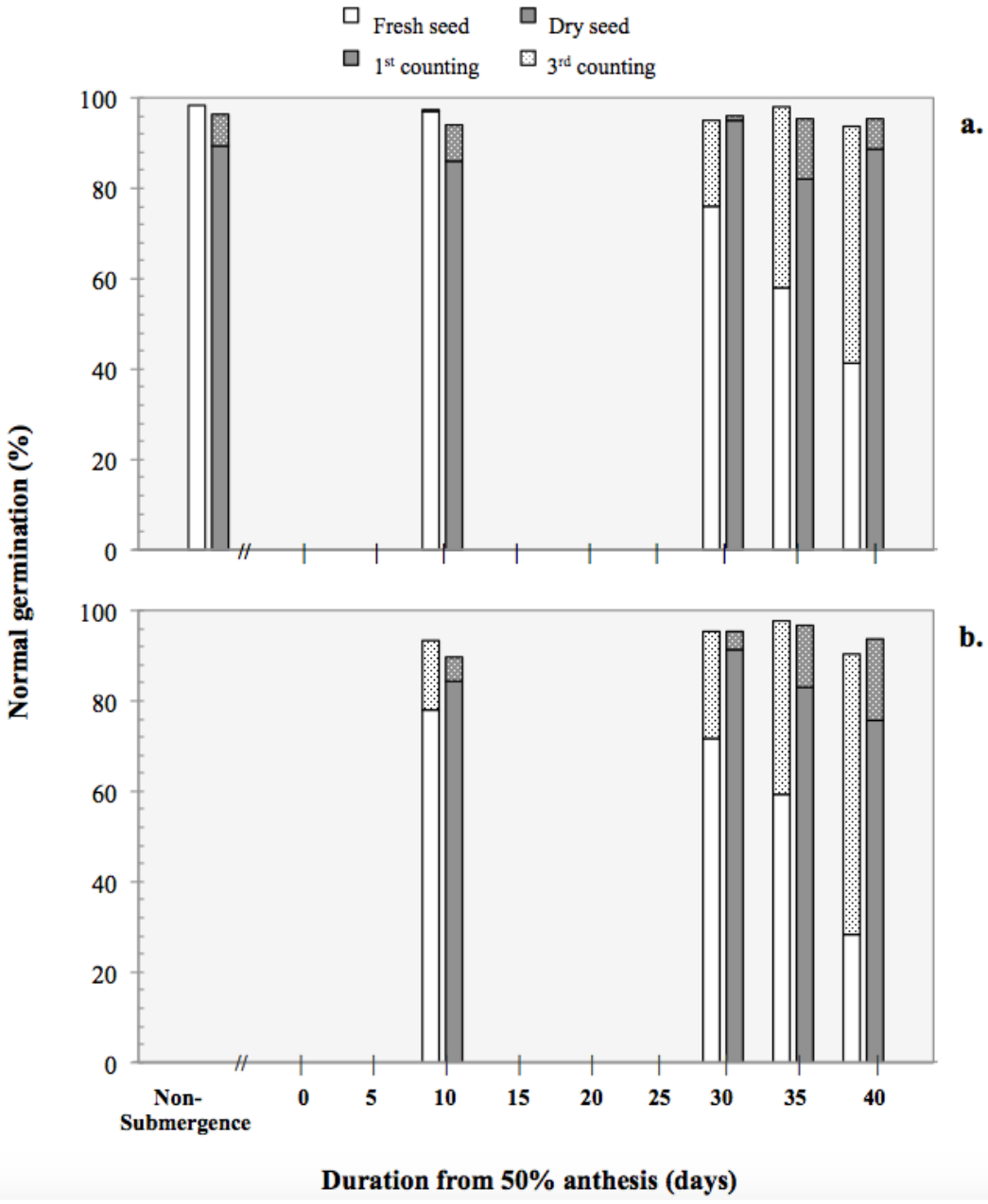

Figure 2.6 Comparison of germination ability of freshly (□,⬚) and dried (■,▩) (15.1 ± 0.2% moisture content) non-sprouted seeds of cv. Gleva submerged for 3 (a) or 5 (b) days at different seed developmental stages. The first (□,■) and third (⬚,▩) counts of normal seedlings at 7 and 21 days, respectively, in test are shown (2012).

2.4.1.3.2 Post-harvest germination of pre-harvest sprouted seed

Pre-harvest sprouting was one of the main negative impacts due to submergence during seed development and, especially, late in maturation (Fig. 2.4). Figure 2.7 shows the adverse effect of submergence on the ability of dried pre-harvest sprouted seeds to germinate following drying and reimbibition: the more mature seed or the longer duration that plants were submerged for, the lower germination ability of dried sprouted seeds. Following submergence for 3 days at 30, 35 or 40 DAA about half of the pre-harvest sprouted seeds were able to germinate normally following desiccation and rehydration (49-66%, Fig. 2.7a). Whereas, samples from those treatments with submergence for 5 days provided negligible normal germination (<5%, Fig. 2.7b). Dividing the pre-harvest sprouted seeds into three subsets before these germination tests showed that seed with micropyle open only at harvest showed subsequent normal germination after desiccation and rehydration (Fig. 2.8a). Whereas these with radicle first emerged (Fig. 2.8b) or shoot and root first emerged (Fig. 2.8c) were progressively less able to germinate normally, or indeed at all.

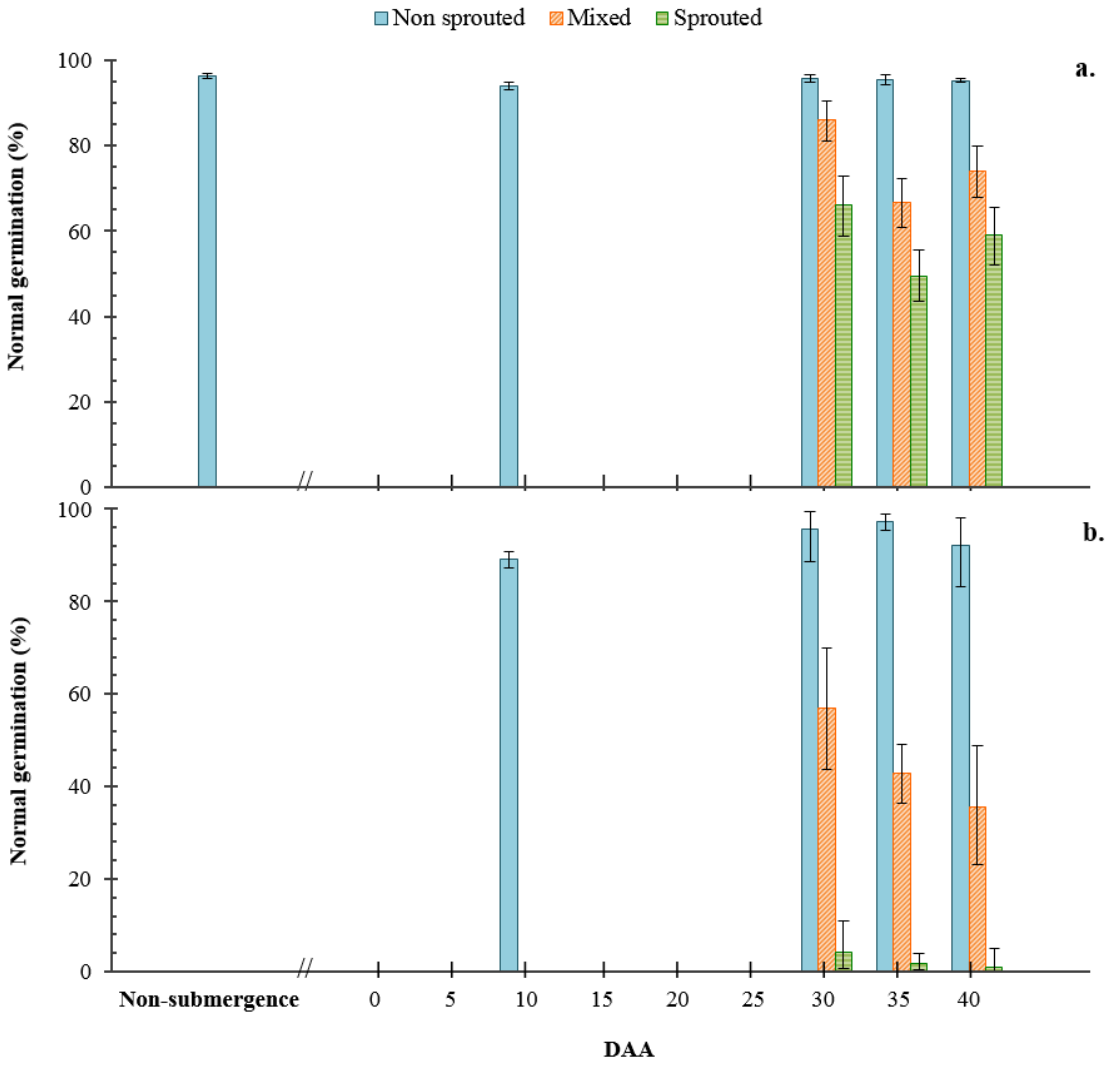

Figure 2.7 The effect of submergence on ability to germinate (non-sprouted seeds) or re-germinate (pre-harvest sprouted seeds) of dried seeds of cv. Gleva after submergence for 3 (a) or 5 (b) days at different seed developmental stages (2012). The vertical bars represent ± s.e. (n=3) of normal germination of non-sprouted ( ), pre-harvest sprouted ( ) or both cohorts combined ( ). The results for non-sprouted seeds are repeated from Fig. 2.5.

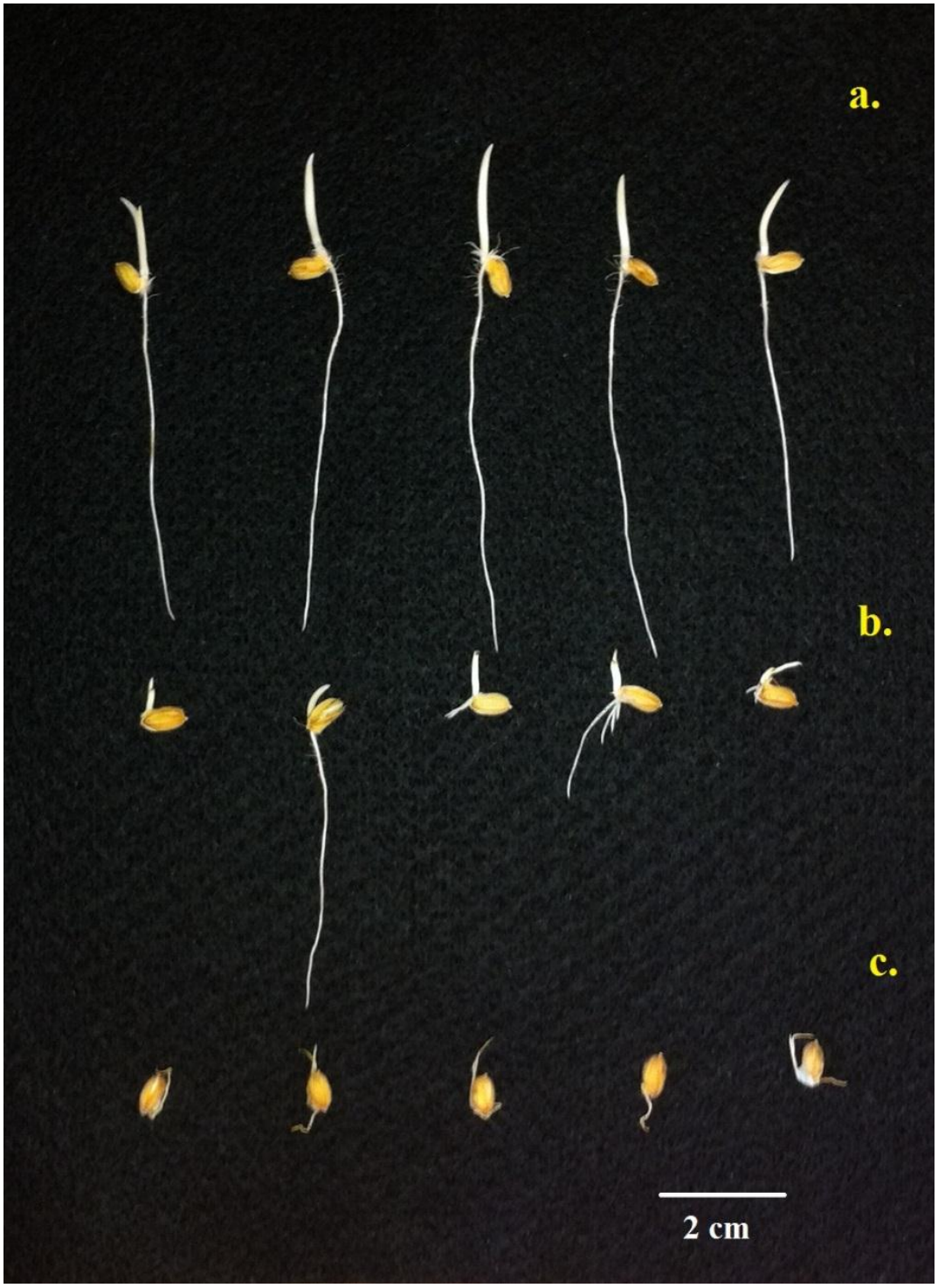

Figure 2.8 Germination after seven days of test following desiccation and rehydration for three types of pre-harvest sprouted seeds of cv. Gleva (2012); seeds with opened-micropyle only (a), seeds with radicles emerged (b), and seeds with well-developed shoot and root structures emerged (c) before harvest

2.4.1.4 Longevity

The effect of complete crop submergence for 3 or 5 days at different developmental stages on subsequent longevity of *japonica* cv. Gleva was examined. Normal germination of control and submerged non-sprouted seeds were plotted against time in experimental storage at 40 °C and seed survival curves were fitted by probit analysis (Fig. 2.9, Table 2.6). All seed lots lost viability in a negative sigmoidal pattern until none were able to germinate (after 32 days in storage). The differences amongst blocks within a treatment were generally small in terms of the seed survival pattern. Similarly, any difference amongst treatments was apparently small. In general, seed survival during storage corresponded to the expected sigmoidal pattern and the survival curves confirmed to the cumulative negative normal distribution of seed deaths in time and were subjected to probit analysis. The treatment for 5 days' submergence at 9 DAA (Fig. 2.9f) was the most different to the other survival curves, with less evidence of a "shoulder" before loss in viability was detected.

To identify acceptable models for fitting the seed survival curves, analysis of deviance was performed. The results revealed that individual (different) lines for each seed sample was the best model to fit since the 27 curves could not be constrained to neither a common line [F (16,725) = 1.657, P= 0.000] nor a common slope [F (8,733) = 1.951, P= 0.001] (Appendix 2.14). According to this analysis, Figure 2.9 shows the fitted survival curves for each treatment in each block separately. The small variation in the fitted curves amongst blocks within a treatment were least with 3 days submergence at 30 or 35 DAA (Figs 2.9c, d) and greatest after 5 days submergence at 9, 30 or 35 DAA (Figs 2.9f, g, h).

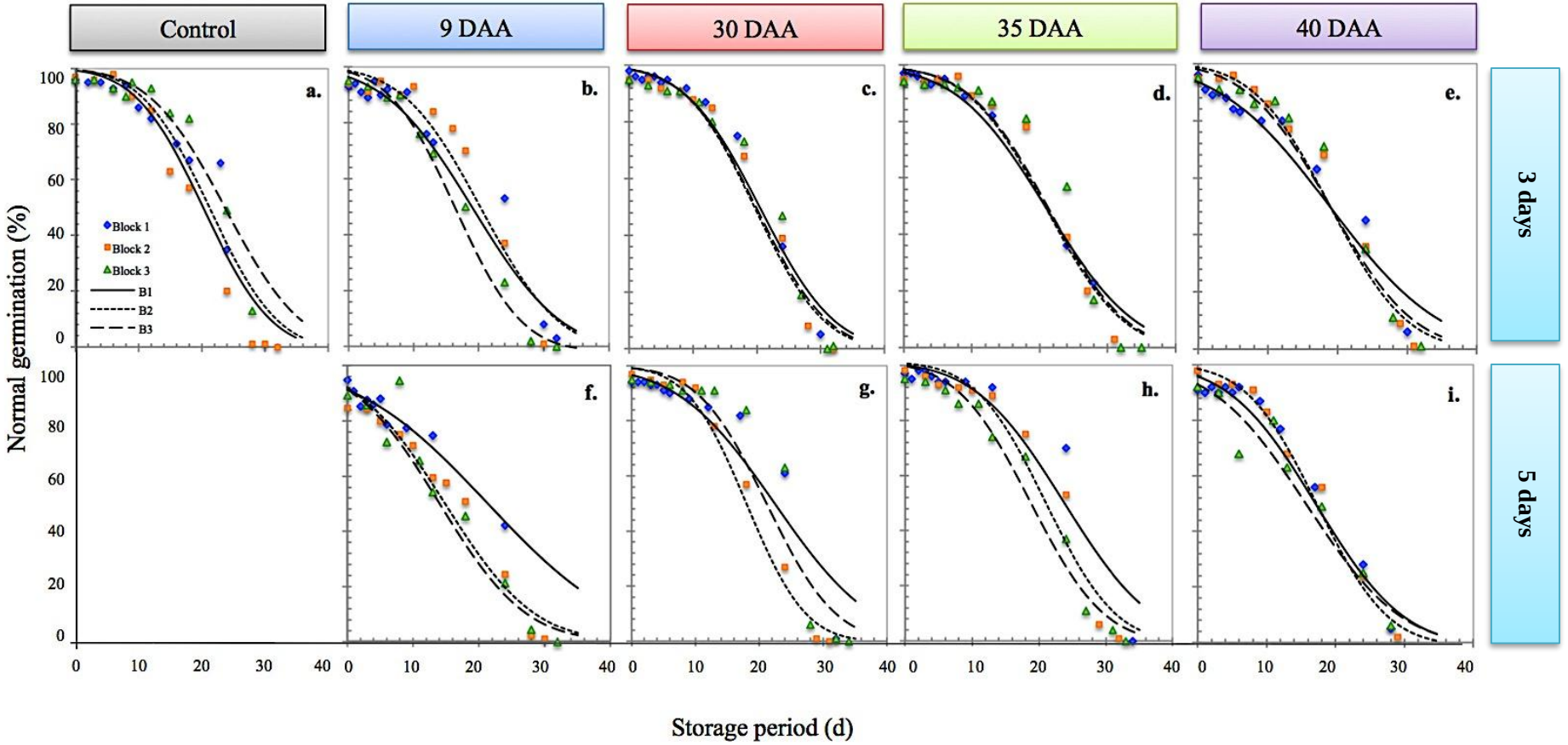
Control
9 DAA
30 DAA
35 DAA
40 DAA
3 days
5 days
a.
b.
c.
d.
e.
f.
g.
h.
i.
Block 1
Block 2
Block 3
B1
B2
B3
Normal germination (%)
Storage period (d)
0
10
20
30
40
0
20
40
60
80
100

Figure 2.9 Seed survival curves (% normal germination plotted against period in hermetic storage at 40 °C with 15.1 ± 0.2% moisture content) of *japonica* rice cv. Gleva for non-sprouted seeds harvested at harvest maturity after no submergence (Control, a), or submerged for 3 days at 9, 30, 35 or 40 DAA (b, c, d and e), or for 5 days at 9, 30, 35 or 40 DAA (f, g, h and i) (2012). The symbols; ♦, ■, and ▲ represent the values for Blocks 1, 2, and 3, respectively. The different lines represent the negative accumulative normal distributions for seeds from Blocks 1 (——), 2 (-----), or 3 (— — -). The parameters of these fitted curves provided by probit analysis are shown in Table 2.6.

Table 2.6 Longevity (parameters of seed viability equation fitted by probit analysis) of *japonica* rice cv. Gleva harvested at harvest maturity after submergence for 0 (Control), 3 or 5 days at 9, 30, 35 or 40 DAA in hermetic storage at 40° C with 15.1 ± 0.2% moisture content (2012). The 95% confidence intervals are shown for p_{50}

Developmental stage	Submergence duration	Block	Moisture content (%)	K_i		Slope (1/σ)		σ (days)	p_{50} (days)			
				Estimate	s.e.	Estimate	s.e.	Estimate	Estimate	s.e.	lower 95%	upper 95%
Control	None	1	15.1	1.92	0.103	-0.081	0.0064	12.3	23.7	0.95	22.05	25.77
		2	15.2	2.68	0.136	-0.152	0.0074	6.6	17.6	0.36	16.91	18.33
		3	14.9	2.37	0.125	-0.104	0.0068	9.6	22.8	0.65	21.58	24.13
9 DAA	3d	1	15.2	1.79	0.074	-0.093	0.0044	10.7	19.2	0.57	18.17	20.40
		2	15.0	2.17	0.113	-0.106	0.0064	9.4	20.5	0.56	19.45	21.62
		3	15.0	2.07	0.107	-0.125	0.0063	8.0	16.6	0.43	15.74	17.42
	5d	1	15.2	1.35	0.070	-0.063	0.0063	15.7	21.3	1.58	18.77	24.92
		2	15.0	1.40	0.083	-0.094	0.0051	10.6	14.9	0.47	13.95	15.78
		3	15.0	1.52	0.088	-0.103	0.0055	9.7	14.8	0.47	13.89	15.73
30 DAA	3d	1	15.1	2.19	0.094	-0.108	0.0056	9.3	20.3	0.59	19.17	21.5
		2	15.1	2.24	0.113	-0.115	0.0059	8.7	19.4	0.48	18.53	20.39
		3	15.0	2.22	0.109	-0.113	0.0053	8.9	19.7	0.44	18.84	20.58
	5d	1	15.3	1.86	0.080	-0.083	0.0048	12.0	22.4	0.80	20.97	24.08
		2	15.3	2.47	0.123	-0.137	0.0068	7.3	18.0	0.42	17.17	18.81
		3	14.9	2.49	0.121	-0.118	0.0056	8.5	21.1	0.45	20.26	22.01

Table 2.6 (continued)

Developmental stage	Submergence duration	Block	Moisture content (%)	K_i		Slope (1/σ)		σ (days)	p_{50} (days)			
				Estimate	s.e.	Estimate	s.e.	Estimates	Estimate	s.e.	lower 95%	upper 95%
35 DAA	3d	1	15.1	2.04	0.090	-0.098	0.0055	10.2	20.8	0.71	19.52	22.31
		2	15.3	2.33	0.119	-0.112	0.0060	8.9	20.8	0.50	19.86	21.79
		3	15.0	2.35	0.114	-0.111	0.0053	9.0	21.1	0.47	20.22	22.04
	5d	1	15.1	2.23	0.098	-0.095	0.0052	10.6	23.6	0.80	22.13	25.24
		2	15.2	2.51	0.126	-0.120	0.0062	8.3	20.8	0.48	19.92	21.78
		3	15.0	2.14	0.105	-0.115	0.0054	8.7	18.6	0.44	17.72	19.42
40 DAA	3d	1	15.0	1.54	0.070	-0.081	0.0047	12.4	19.1	0.72	17.80	20.60
		2	15.2	2.24	0.112	-0.117	0.0059	8.5	19.2	0.47	18.29	20.12
		3	14.9	2.06	0.105	-0.107	0.0056	9.3	19.2	0.49	18.25	20.18
	5d	1	14.9	1.73	0.076	-0.100	0.0052	10.0	17.4	0.56	16.35	18.55
		2	15.1	2.16	0.113	-0.123	0.0069	8.1	17.5	0.48	16.62	18.48
		3	15.1	1.48	0.098	-0.092	0.0060	10.8	16.0	0.58	14.90	17.15

From Table 2.6, analysis of variance of the estimates of potential longevity (K_i) revealed that the values between treatments differed significantly ($P<0.001$, Appendix 2.15). The highest value of K_i was obtained from non-submerged (Control) seed (2.33 ± 0.221) and the lowest for 5 days' submergence at 9 DAA (1.79 ± 0.198). Despite the significant differences in the estimated K_i values, neither σ (the period for viability to fall by 1 NED) nor p_{50} (period for viability to fall to 50%) differed significantly amongst treatment ($P > 0.05$, Appendices 2.16 and 2.17). The estimates of σ varied (insignificantly) from 6.6 to 15.7 days, whilst those for p_{50} varied from 14.8 to 23.7 days (Table 2.7). The Control provided the greatest longevity (p_{50}) of 21.4 days, whereas the shortest p_{50} of about 17.0 days was found following 5 days' submergence at 9 or 40 DAA (Fig. 2.10). Although no significant difference of p_{50} was detected at $P=0.05$, the value of this parameter for 5 days' submergence at 35 DAA (21.0 days) did differ significantly with 9 or 40 DAA (both 17.0 days) when the comparison of differences was tested at $P=0.20$ (Fig. 2.10, Appendix 2.17).

Paired F-Tests were used to clarify any differences in p_{50} resulting from the main treatment factors, i.e. developmental stage or submergence duration. Duration of simulated flooding had no effect on seed storage survival [$F(4,725) = 2.387$, $P = 0.432$, Appendix 2.18.1]. In contrast, developmental stage at submergence was significant [$F(6,725) = 2.110$, $P = 0.023$, Appendix 2.18.2]: the effect of submergence on subsequent longevity (p_{50}) following submergence was ranked 9 or 40> 30, 35 DAA, or non-submergence treatment (Appendix 2.19). This would suggest that early grain filling and late maturation stages are the most critical seed developmental stages for submergence in terms of detrimental effects on subsequent seed viability period in storage.

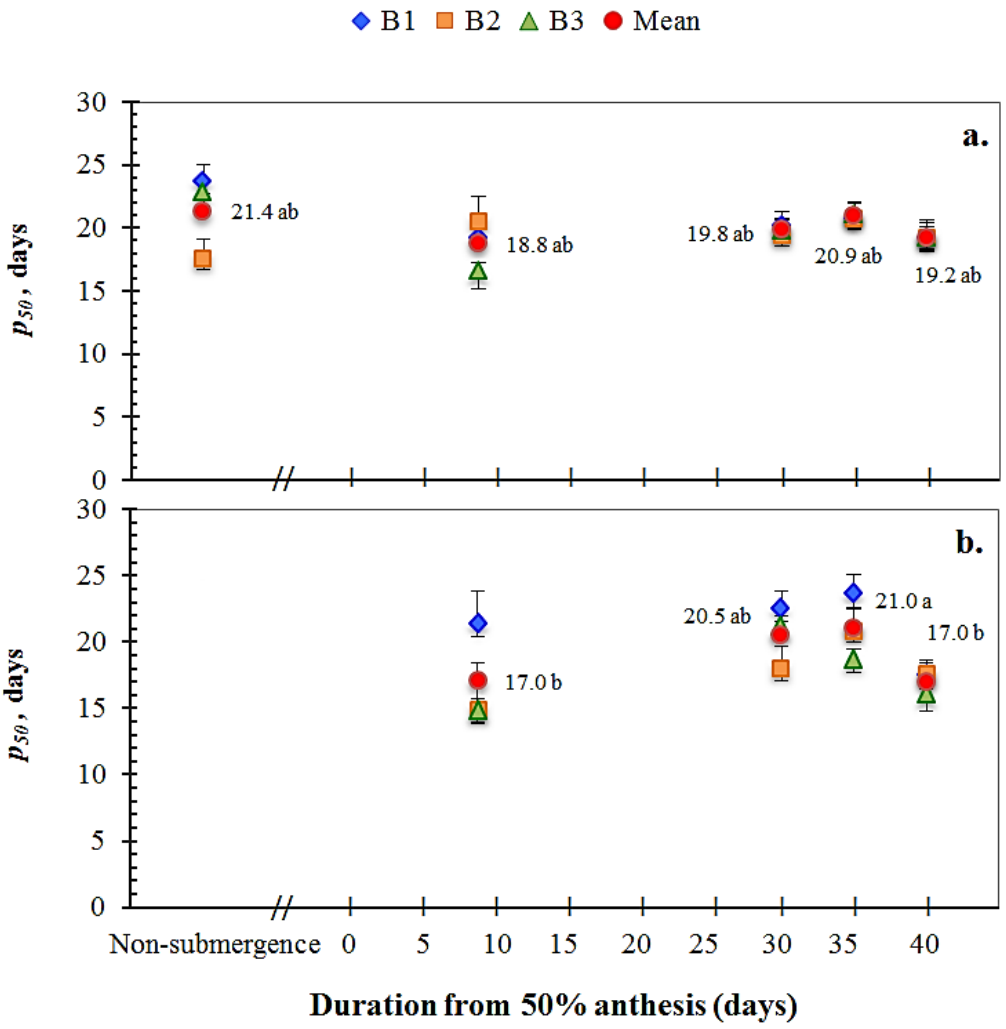

Figure 2.10 Effect of complete submergence for 3 (a) or 5 days (b) at different seed developmental stages on the period for viability to fall to 50% (days) of *japonica* rice cv. Gleva (2012). The symbols ♦, ■, ▲ and ● represent the values for Blocks 1, 2, 3 and their mean, respectively. The vertical bars represent ± estimates s.e. of each block, when larger than symbols size. Superscripts with a common letter indicate no significant difference at $P = 0.20$ using Tukey's Multiple Range Test. No significant differences were detected at $P = 0.05$ (Appendix 2.17).

2.4.2 Seed development and maturation of rice (2013)

2.4.2.1 Changes in seed weight and moisture content

The *japonica* rice cv. Gleva flowered earlier (73 DAS) than the *indica* rice cvs IR64 (87 DAS) and IR64 Sub1 (93 DAS), with IR64 Sub1 being 6 days later than IR64 (Table 2.1). The broad patterns of developmental changes in seed weight, maturation and moisture content from anthesis other than timing, however, were similar amongst cultivars (Fig. 2.11).

From 8-9 DAA, fresh and dry seed weight increased rapidly reaching maxima at about 31, 26, and 20 DAA in cvs Gleva (Fig. 2.11a), IR64 (Fig. 2.11b), and IR64 Sub1 (Fig. 2.11c), with values plateauing thereafter until harvest maturity (at 47 DAA). The end of grain filling (i.e. mass maturity) was estimated from the intercept of the two lines shown for each cultivar (a broken-stick model of two lines, the first of positive slope, the second a constant values) to be 27.6 (s.e. 1.17), 27.5 (s.e. 1.21) and 24.0 (s.e. 3.10) DAA, with mature mean seed dry weights of 29.5 (s.e. 0.34), 19.6 (s.e. 0.26), and 20.3 (s.e. 0.31) mg, for cvs Gleva, IR64, and IR64 Sub1, respectively. The broken-stick models fitted well with R^2 values of 97.2, 97.0, and 78.9%, which provided rates of seed filling of 1.37 (s.e. 0.118, from 6.1 to 27.6 DAA), 0.93 (s.e. 0.081, from 6.3 to 27.5 DAA), and 1.08 mg day^{-1} (s.e. 0.361, from 5.2 to 24.0 DAA), respectively (Appendix 2.20). Changes in seed size and colour of rice cv. Gleva during seed development and maturation are shown in Fig. 2.12.

Moisture content of cvs Gleva, IR64, and IR64 Sub1declined sharply from 66.7, 64.8, and 55.0 % at the earliest harvests (early grain filling) to 37.7, 35.1, and 30.3% at mass maturity, but then decreased far more gradually after seed had attained maximum dry weight. At harvest maturity, the seed moisture content of cvs Gleva, IR64, and IR64 Sub1 had reduced to 26.7, 28.2, and 27.6 %, respectively.

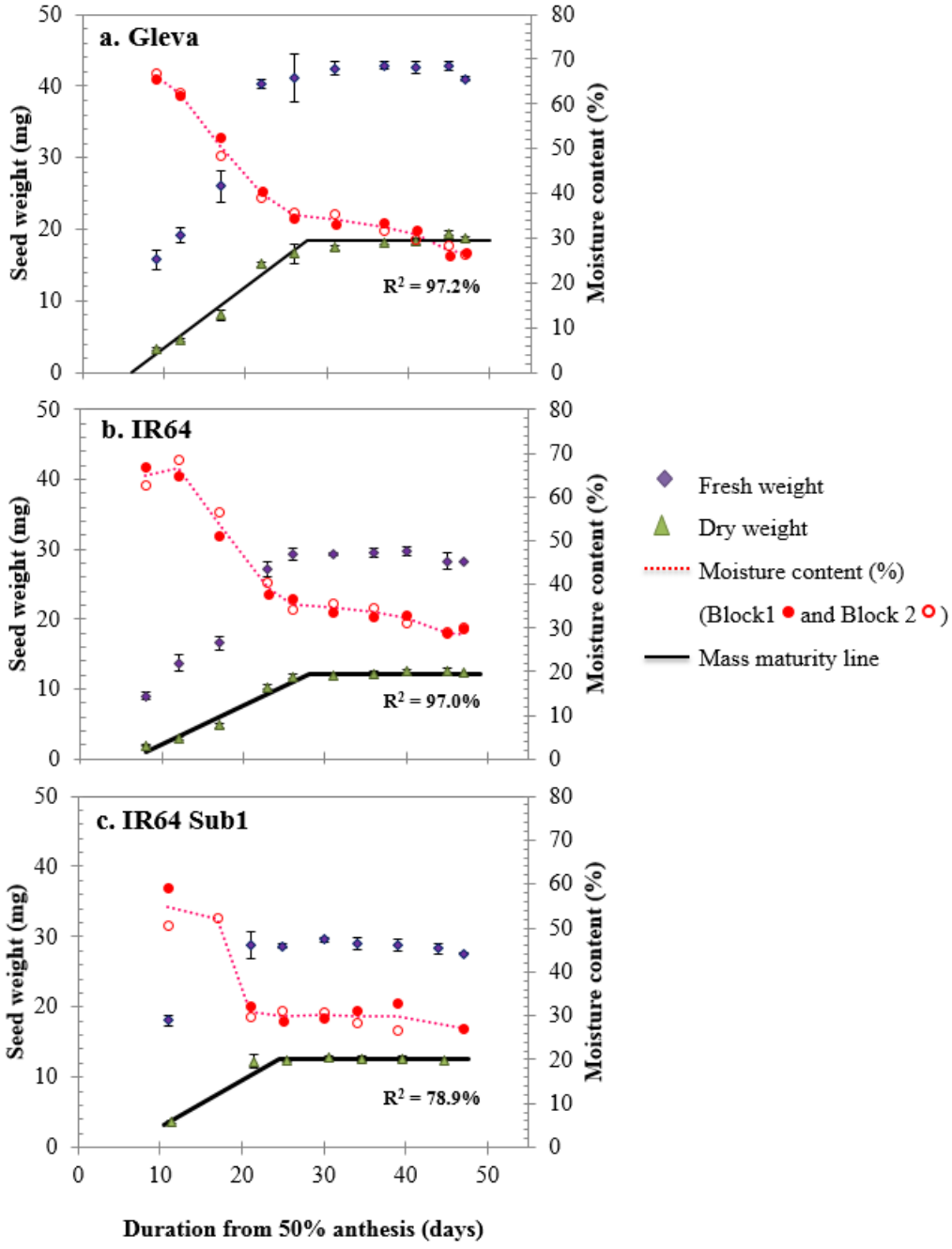

Figure 2.11 Fresh (♦) and dry (▲)seed weight (mean ± s.e.) and moisture content (Block 1;●, Block 2;○) from anthesis until harvest of control (non-submerged) plants of *japonica* rice cv. Gleva (a), *indica* rice cvs IR64 (b) and IR64 Sub1 (c) (2013). The intersect of the solid lines represents the end of grain filling (mass maturity, i.e. 27.6 (s.e. 1.17), 27.5 (s.e. 1.21) and 24.0 (s.e. 3.10) DAA for cvs Gleva, IR64, and IR64 Sub1 respectively; See Appendix 2.20).

Figure 2.12 Colour and size of *japonica* rice cv. Gleva with duration from anthesis (0–35 DAA) during seed development (2013)

2.4.2.2 Development of ability to germinate

Fresh immature seed of *japonica* rice cv. Gleva at 9 DAA showed 36% ability to germinate normally once seed covering structures were removed, but no seed germinated at 9 DAA without this treatment (Fig. 2.13). After that, germinability built up gradually but then plateaued after 26 DAA (> 90% germination). At harvest (47 DAA), 96% of freshly-harvested seed of cv. Gleva were able to germinate normally. Removing the seed covering structures improved germination, but more so earlier in development such that no benefit was detected from 37 DAA onward.

There was, however lower early capability to germinate in *indica* rice cv. IR64 (Fig. 2.13). Ability to germinate (only 3%) was first observed at 17 DAA, but only when the seed covering structures were removed. Without this treatment, germination was not detected until samples were harvested 26 DAA. During subsequent seed development, ability to germinate of IR64 increased to 35-45% between 26 and 45 DAA, and 64% at 49 DAA (harvest maturity) if seed covering structures were removed. The restriction to germination from the seed covering structures was continued throughout until late maturation in cv. IR64, in contrast to cv. Gleva where the effect was lost by 37 DAA.

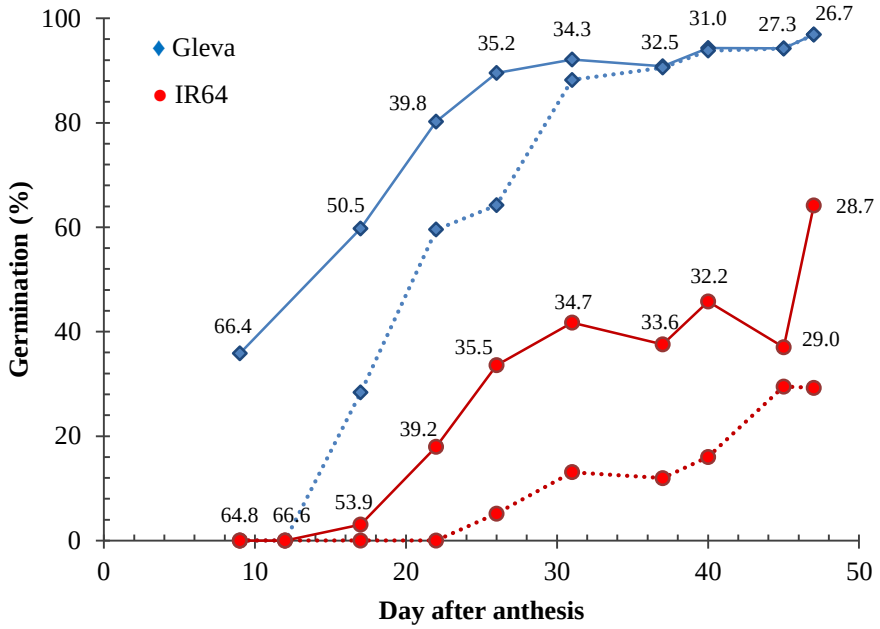

Figure 2.13 Ability to germinate normally of freshly-harvested seed of cvs Gleva (♦) and IR64 (●) (2013) at 21 days in test (.....) and when combined with subsequent removal of seed covering structures from ungerminated seeds (——), respectively. Seed moisture content (%) at harvest is shown for each observation

2.4.2.3 Oxygen dissolved in flood water (2013)

The concentration of dissolved oxygen at the beginning of the experiment was about 10 mg L^{-1} (Fig. 2.14). Then it declined sharply to approximately 7.3 mg L^{-1} within the first day of treatments. Thereafter, dissolved oxygen fluctuated from 6.5-7.5 mg L^{-1} until the end of experiment. Each of the three different treatment times showed a similar pattern.

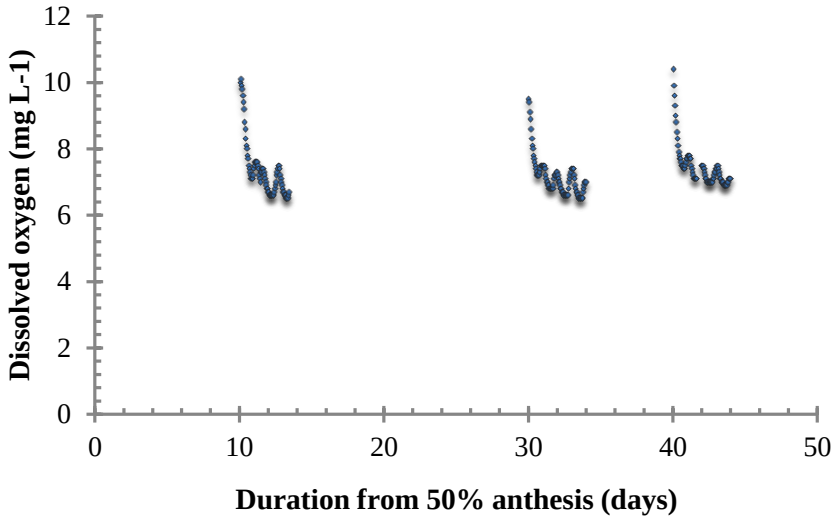

Figure 2.14 Dissolved oxygen concentrations in water when rice plants of cvs Gleva, IR64 and IR64 Sub1 were submerged at 10, 30 or 40 DAA (2013)

2.4.3 The effect of submergence at different seed developmental stages on yield, seed weight, and subsequent longevity in contrasting genotypes of rice (2013)

2.4.3.1 Yield per pot and the occurrence of pre-harvest sprouting

No significant treatment effect on yield per pot was detected (total weight of sprouted and non-sprouted seed at 0 % moisture content; Appendix 2.20). Nevertheless, values ranged from a 43% loss (10 DAA, cv. Gleva) to a 24 % increase (40 DAA, cv. IR64 Sub1) in yield compared to the control from submergence during seed development (Fig. 2.15). The apparent losses tended to be greater the earlier the submergence, and seemed greater in the *japonica* rice cv. Gleva than in the *indica* cvs IR64 and IR64 Sub1; furthermore in the latter case, greater in cv. IR64 than in cv. IR64 Sub1.

Pre-harvest sprouting was detected (P<0.001, Appendix 2.21), but only in seed from cv. Gleva submerged at 30 DAA (7%) or 40 DAA (67%). At 40 DAA, fewer than 1% of seed of the *indica* cvs IR64 and IR64 Sub1 sprouted whilst no sprouted seeds were detected in the earlier treatments to these two cultivars (Fig. 2.16). Analysis of two-way ANOVA confirmed that there was an interaction (P<0.001, Appendix 2.22) between the effects of seed development stage (P<0.001; 40 DAA>30 DAA> 10 DAA or Control) and cultivar (P<0.001; cv. Gleva>cvs IR64 or IR64) on pre-harvest sprouting.

2.4.3.2 Seed moisture content and 1000 seed weight at harvest maturity

2.4.3.2.1 Freshly-harvested moisture content

In the non-submergence treatment (Control), the moisture content of mature seed (non-sprouted seed fraction) at harvest differed insignificantly from 26.6-28.7 % (Table 2.7). Samples of cvs Gleva, IR64, and IR64 Sub1obtained from 4 days' submergence at 10 DAA (the earliest treatment) were 2.0, 1.7, and 1.9 % higher in moisture content than Controls, but without significance. Seed of cv. Gleva from 4 days' submergence at 30 DAA also had (not significantly) higher moisture content than non-submergence. On the other hand, lower moisture content was found (P<0.05) in both *indica* varieties at 30 DAA for cvs IR64 and IR64 Sub1. The moisture content at harvest of the final treatments (i.e. 4 days' submergence at 40 DAA) of all three cultivars was less than non-submergence (Control) seed, with significance in cv. IR64 Sub1.

In 2012 the seed moisture content of both 3 and 5 days' submergence at last treatment (40 DAA) was lower than that for the Control of cv. Gleva. A similar result was detected again in 2013, this time in all three genotypes (Table 2.7). Significant interaction (P < 0.001, Appendix 2.25) within the present study (2013) supports the view that the corresponding differences in seed moisture content were significantly influenced by time of the treatment (P < 0.001; 10 DAA> Control> 30 or 40 DAA, Appendix 2.25). Moreover, varietal differences affected moisture content of mature non-sprouted seeds: cvs Gleva and IR64 had significant higher seed moisture content at harvest maturity than IR64 Sub1 (P < 0.001, Appendix 2.25).

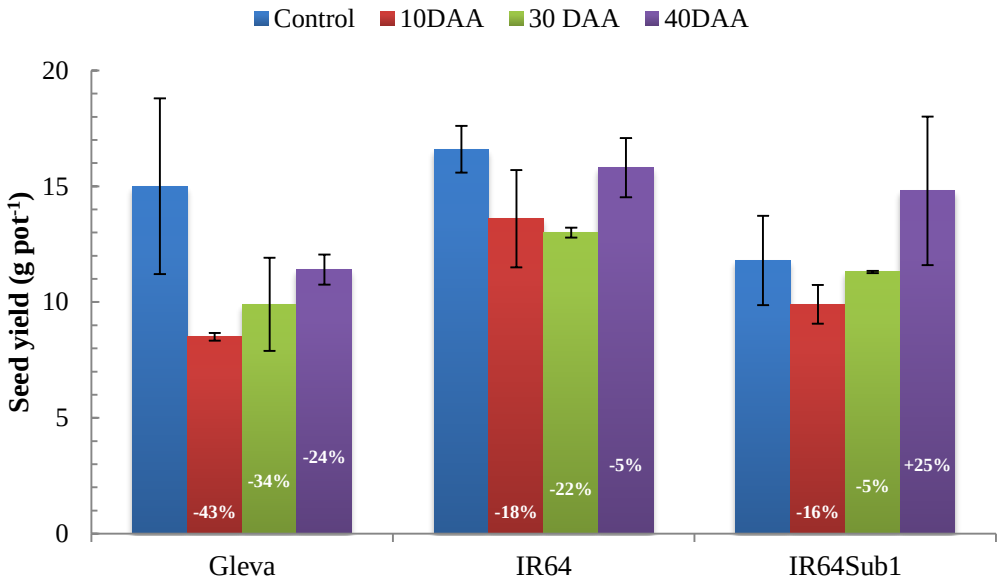

Figure 2.15 The effect of submergence on dry seed yield per pot (at 0 % moisture content) in cvs Gleva, IR64, and IR64 Sub1 (2013). The percentage yield difference of submerged seed (compared with Control) is shown. The vertical bars represent ± s.e. No significant differences amongst cultivar were detected (Appendix 2.20a).

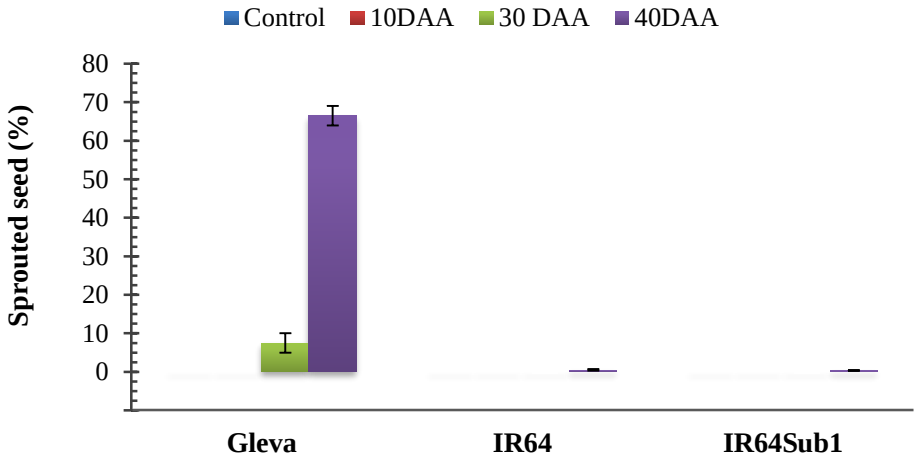

Figure 2.16 The effect of submergence on pre-harvest sprouting in cvs Gleva, IR64 and IR64 Sub1 (2013). The vertical bars represent ± s.e. (Appendix 2.21).

Table 2.7 The effect of treatments on moisture content of freshly-harvested-non-sprouted rice seeds of cvs Gleva, IR64, and IR64 Sub1 (2013). The hand-threshed seed samples were collected at 46 DAA (2 days after the last treatment ended) from non-submergence and 4 days' submergence treatments at different seed developmental stages in 2013.

Treatment	**Moisture content at harvest maturity (%)**		
	Gleva	**IR64**	**IR64 Sub1**
Control	26.6	28.7	27.0
10 DAA	28.6	30.4	28.9
30 DAA	27.6	23.5	21.8
40 DAA	25.9	26.9	20.1
F Test[1] *P*	<0.001	<0.001	<0.001
LSD 0.05	0.740	1.870	1.580
Interaction[2]			
Cultivar (cv)	<0.001		
Seed development stage (DAA)	<0.001		
CV x DAA	<0.001		

[1] One-way ANOVA amongst cultivar, see Appendix 2.24

[2] Interaction analysis between seed development stage and cultivar, see Appendix 2.25

2.4.3.2.2 1000 seed dry weight

Under all simulated flooding conditions for all cultivars, submergence reduced the weight of the non-sprouted seed fraction. Figure 2.17 shows a tendency for the later the submergence the lower the seed weight, but the biggest difference is between the *japonica* cv. Gleva and the *indica* cvs IR64 and IR64 Sub1. Significance differences of dried seed were detected only within cvs Gleva and IR64 Sub1 ($P<0.05$, Appendix 2.26), not in IR64. The effect of submergence on 1000 seed dry weight was subjected to significant interaction ($P<0.001$, Appendix 2.27) between genotype ($P<0.001$: cvs Gleva> IR64 > IR64 Sub1) and submergence at different seed developmental stages ($P<0.001$; Non submergence >10, 30 or 40 DAA).

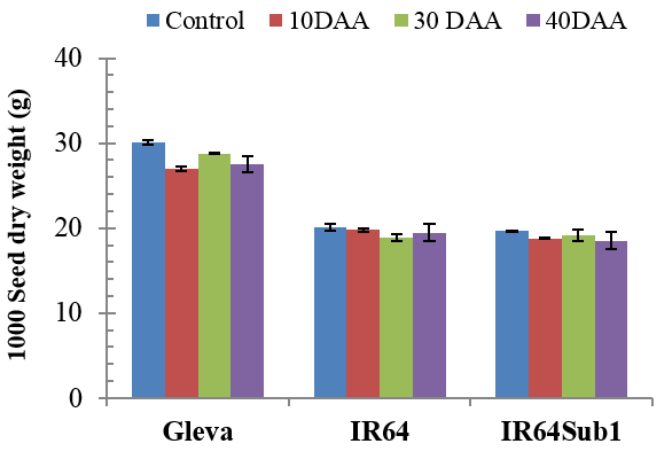

Figure 2.17 The effect of submergence for four days at different seed developmental stages on 1000 seed dry weight (at 0 % moisture content; at harvest maturity (2013). The vertical bars represent ± s.e. Appendices 2.26 and 2.27).

2.4.3.3 Stability of yield per pot and seed dry weight

To assess the impact of submergence at different seed developmental stages on rice seed productivity, interactions between genotypic and environmental effects (i.e. the GxE interaction) were investigated by regression analysis. In these analyses, the "environment" was qualified as the mean performance of all genotypes in that environment, whereby the control regime was the best environment (Fig. 2.18). Fig. 2.18(a) shows the significant linear relation (P<0.001) of genotype yield (g pot^{-1}) in response to environment in the term of simulated flooding treatments (P<0.001, Appendix 2.28.1a), as well as the differences between cultivars (P<0.001, Appendix 2.28.1b). Although, the interaction (i.e. the difference in slopes) was not significant (P=0.427, Appendix 2.28.1c), cv. Gleva did appear to be the cultivar most sensitive (steepest response) to environment, and the separate regression lines are therefore shown.

The main effect of genotype on 1000 seed dry weight was affected by genotypic significant (P<0.001, Appendix 2.28.2b), but not by the mean effect of environment (P>0.05, Appendix 2.28.2a) (Fig. 2.18b). There were significant interactions (P<0.001, Appendix 2.28.2c), however between genetic and environmental factors on 1000 seed weight and these separate lines (differing in slope) are therefore shown. Both regression lines of cvs. IR64 and IR64 Sub1 were parallel and shallow in slope, indicating that seed dry weight of these *indica* cultivars were insensitive to environment (i.e. the flooding), and not differing. On other hand, the mean 1000 seed dry weight of cv. Gleva was much more sensitive to environment, and thus provided a steeper regression slope.

This evidence here, therefore supports the view that yield of the *japonica* rice cv. Gleva is more vulnerable to flooding (late in development) than the two *indica* cultivars. However, there is no evidence from this analysis (Fig. 2.18) of any difference in sensitive between cvs IR64 and IR64 Sub1.

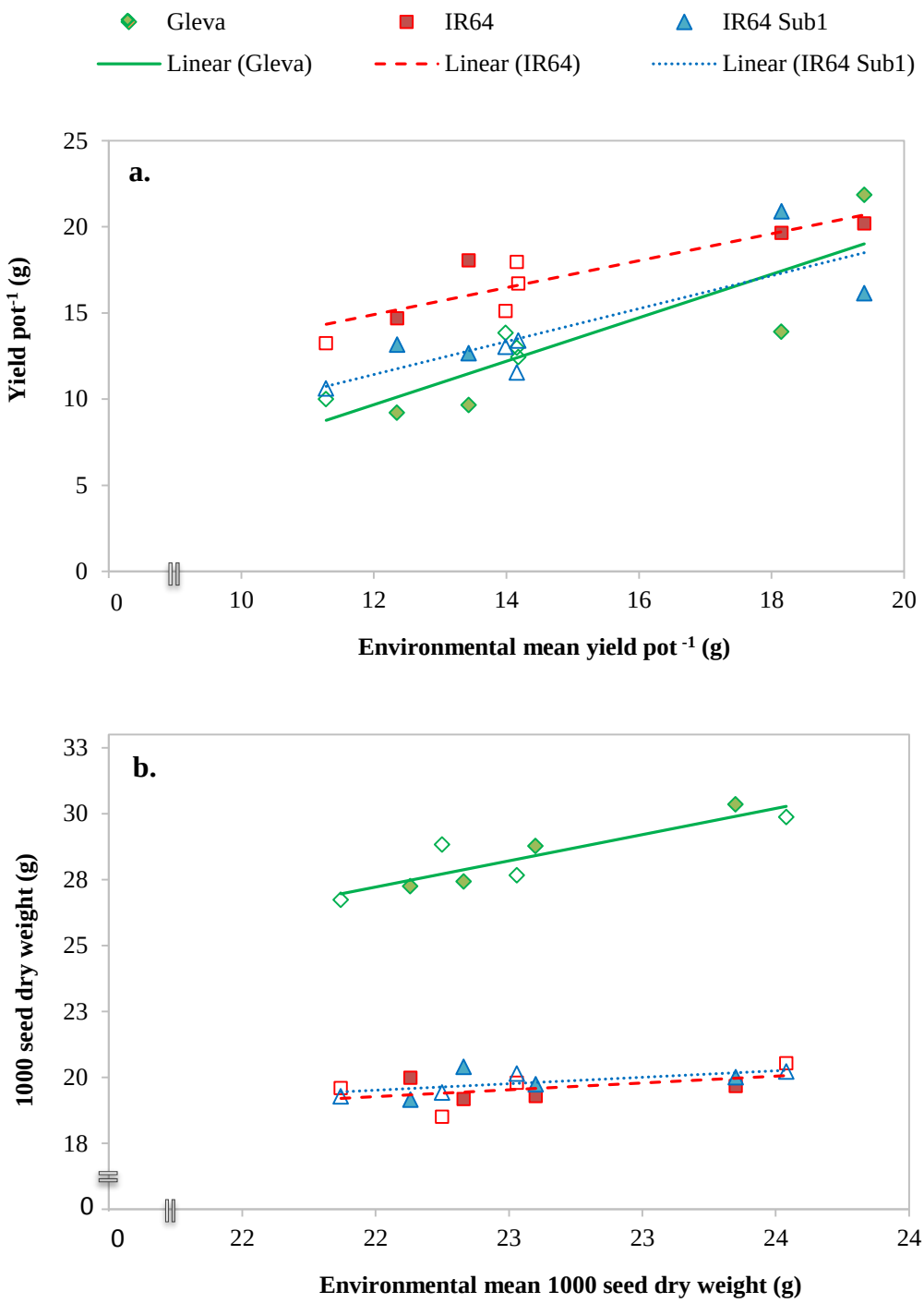

Figure 2.18 Genotype-environment interaction (G x E) for grain yield (g pot^{-1}) (a) and 1000 seed dry weight (b) of cvs Gleva (♦), IR64 (■), and IR64 Sub1 (▲) after submergence for 4 days at 10, 30 or 40 DAA and control. The solid and open symbols represent results from Blocks 1 and 2, respectively (Appendix 2.28).

2.4.3.4 Seed longevity

The influence of submergence at different seed developmental stages and varietal differences on subsequent seed survival during storage were evaluated in 2013. To assess the effect of submergence on subsequent longevity in each rice variety, the data from each treatment combination was first quantified by the best individual fit model shown in Figure 2.19. The ability to germinate normally of all seed lots declined gradually during hermetic storage. All or almost all seeds of cvs Gleva, IR64 and IR64 Sub1 had lost viability after 30, 44, and 41 days in storage, respectively. Overall, there was only small variation caused by block effects in the pattern of seed survival curves within each treatment combination. *Japonica* rice cv. Gleva clearly demonstrated steeper survival curves and had shorter life spans (Fig. 2.19a, b, c, and d) than the two *indica* sub-species IR64 (Fig. 2.19e, f, g, and h) and IR64 Sub1 (Fig. 2.19i, j, k, and l), regardless of whether seeds had been produced from submergence treatments or not.

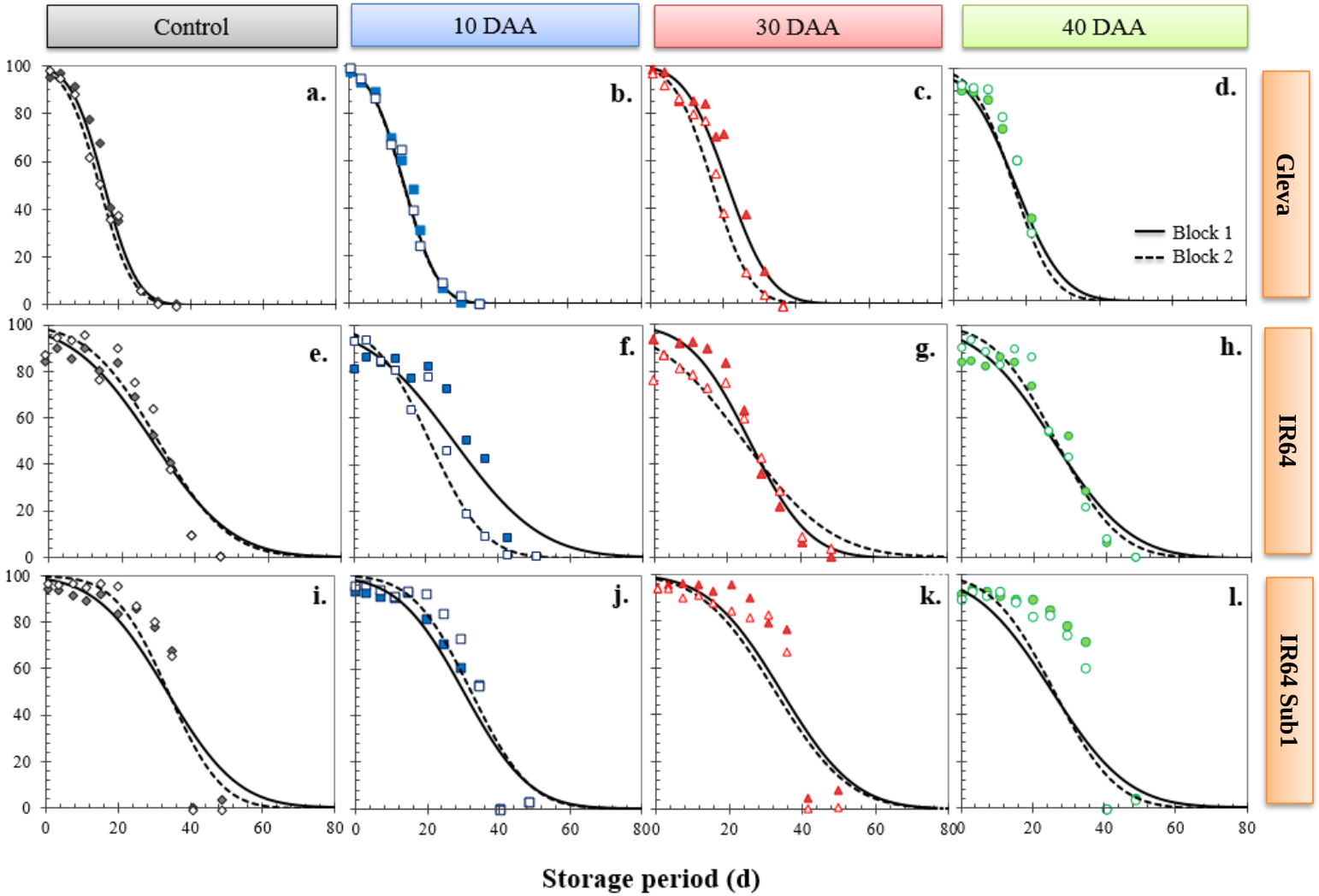
Control
10 DAA
30 DAA
40 DAA
a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
k.
l.
Block 1
Block 2
Gleva
IR64
IR64 Sub1
Storage period (d)

Figure 2.19 Seed survival curves (% normal germination plotted against period in hermetic storage at 40 °C with 14.7 ± 0.2% moisture content) of rice cvs Gleva (a, b, c, and d), IR64 (e, f, g, and h), and IR64 Sub1 (i, j, k, and l) provided by probit analysis for seeds harvested at harvest maturity after no submergence (♦), or submerged for 4 days at 10 (■), 30 (▲), or 40 (●) DAA, respectively (2013). The solid and open symbols show results from Blocks 1 and 2. The different lines represent the fitted-curves for the results from Blocks 1 (——) and 2 (- - - -). The parameters of the fitted-curved are provides in Table 2.8.

Table 2.8 Longevity (parameters of seed viability equation for the best-fit models provided by probit analysis for each treatment combination) for rice cvs Gleva, IR64, and IR64 Sub1 harvested at harvest maturity after submergence for 0 (Control) or 4 days from 10, 30, or 40 DAA (2013) in hermetic storage at 40° C with 14.7 ± 0.2% moisture content. The 95% confidence intervals are shown for p_{50}.

CVs	Treatments	Block	Moisture	K_i		Slope (1/σ)		σ (days)	p_{50} (days)			
			Content (%)	Estimate	s.e.	Estimate	s.e.	Estimate	Estimate	s.e.	Lower 95%	Upper 95%
Gleva	Control	1	14.7	2.20	0.074	-0.138	0.0044	7.2	15.9	0.22	15.50	16.38
		2	14.8	2.01	0.070	-0.137	0.0044	7.3	14.6	0.22	14.22	15.09
	10 DAA	1	14.6	2.06	0.070	-0.135	0.0043	7.4	15.3	0.22	14.83	15.70
		2	14.6	2.05	0.070	-0.136	0.0043	7.4	15.1	0.22	14.69	15.56
	30 DAA	1	14.8	2.29	0.070	-0.108	0.0034	9.3	21.2	0.28	20.68	21.79
		2	14.7	2.08	0.068	-0.122	0.0038	8.2	17.1	0.24	16.63	17.59
	40 DAA	1	14.5	1.66	0.089	-0.098	0.0069	10.2	16.4	0.47	15.57	17.38
		2	14.5	1.94	0.100	-0.119	0.0076	8.4	16.8	0.48	15.97	17.83
IR64	Control	1	14.5	1.63	0.052	-0.058	0.0019	17.3	28.2	0.48	27.29	29.15
		2	14.7	1.96	0.059	-0.066	0.0021	15.1	29.7	0.44	28.87	30.60
	10 DAA	1	14.7	1.46	0.049	-0.053	0.0019	19.0	27.6	0.51	26.65	28.65
		2	14.6	1.76	0.056	-0.084	0.0024	12.0	21.0	0.34	20.38	21.69
	30 DAA	1	14.5	2.03	0.060	-0.077	0.0023	12.9	26.2	0.37	25.49	26.94
		2	14.6	1.28	0.046	-0.053	0.0018	19.0	24.5	0.48	23.53	25.43
	40 DAA	1	14.5	1.51	0.050	-0.060	0.0019	16.8	25.4	0.45	24.56	26.30
		2	14.6	1.91	0.058	-0.073	0.0022	13.7	26.1	0.38	25.32	26.83
IR64 Sub1	Control	1	14.9	2.18	0.065	-0.065	0.0022	15.4	33.6	0.49	32.64	34.57
		2	14.7	2.93	0.092	-0.087	0.0029	11.6	34.0	0.39	33.20	34.75
	10 DAA	1	14.9	2.09	0.062	-0.070	0.0022	14.5	30.2	0.43	29.34	31.03
		2	14.8	2.50	0.075	-0.078	0.0025	12.9	32.2	0.41	31.38	32.98
	30 DAA	1	14.9	2.64	0.082	-0.073	0.0026	13.7	36.2	0.48	35.26	37.16
		2	14.5	2.19	0.065	-0.066	0.0022	15.2	33.3	0.48	32.34	34.24
	40 DAA	1	14.8	2.17	0.065	-0.064	0.0022	15.7	34.2	0.51	33.21	35.21
		2	14.7	2.03	0.061	-0.063	0.0021	15.9	32.4	0.49	31.51	33.44

The estimated parameters of the survival curves from Table 2.8 were analysed by analysis of variance. The results indicated no significant effect of submergence on longevity within variety: there were no significant differences in K_i, σ, or p_{50} amongst cultivars (P>0.05 in cv. Gleva, P>0.25 in cvs IR64 and IR64 Sub1, Appendices 2.29, 2.30, and 2.31). Significant differences (P<0.05) were detected, however, for σ and p_{50} between cultivars (Appendices 2.35.2 and 2.35.3). The duration that initial viability reduced to half was influenced significantly by genotypic factor (P<0.001, cvs Gleva = IR64 < IR64 Sub1), but not by seed development stages (P=0.074) (Appendix 2.32.3b.). Estimates of p_{50} for cv. IR64 Sub1 were double (31-35 days) those for cv. Gleva (15-19 days) (Fig. 2.20). IR64 Sub1 had 5-9 days longer in longevity than the recurrent cultivar IR64 (24-29 days) in all treatments. However, significant differences in p_{50} between these two *indica* varieties were detected only when 4 days' submergence had been applied at 30 or 40 DAA (Appendix 2.32.3a); in the Control (non-submergence), there was no significance between p_{50} of IR64 Sub1 (33.7 days) and IR64 (28.9 days).

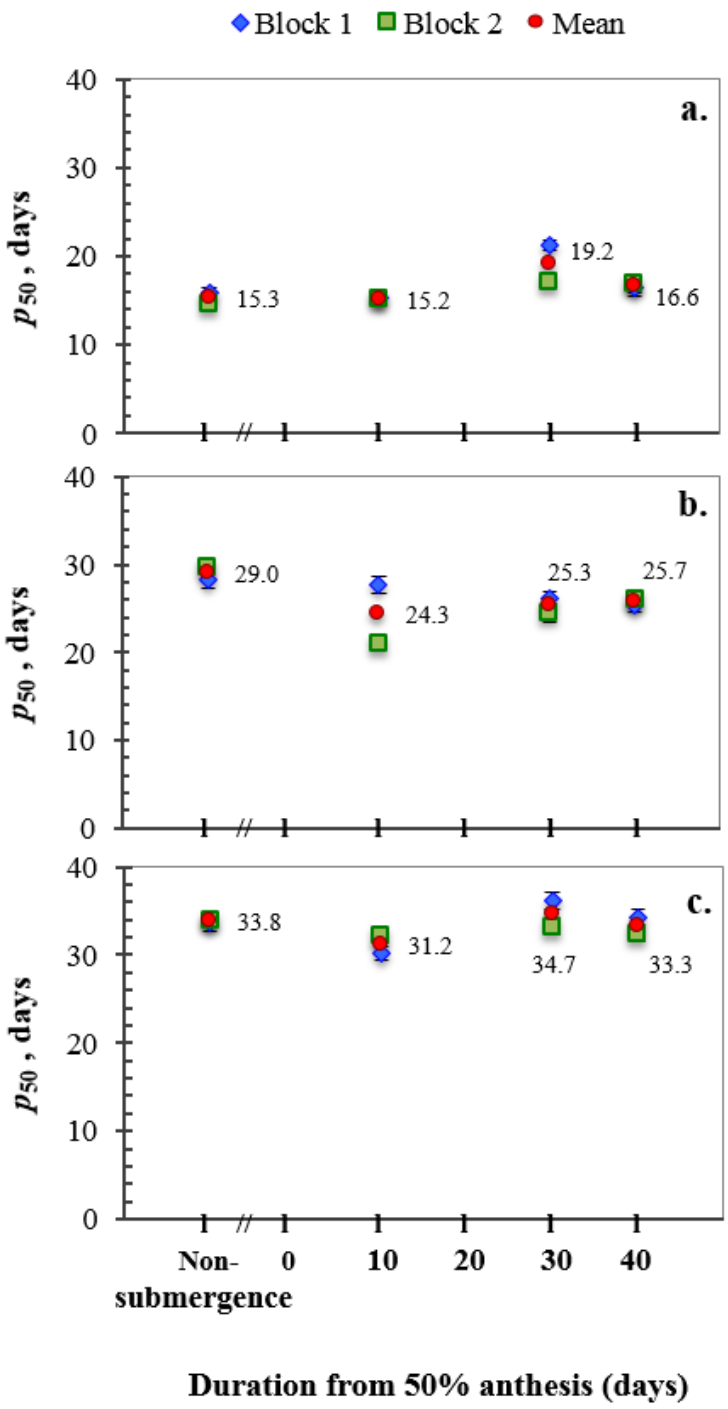

Figure 2.20 Effect of submergence of rice cvs Gleva (a), IR64 (b), and IR64 Sub1 (c) for the longevity (p_{50}) of seeds harvested at harvest maturity after no submergence, or submerged for 4 days at 10, 30, or 40 DAA, respectively (2013).The symbols ♦, ■, and ● represent the values for Blocks 1, 2, and their mean, respectively. The vertical bars represent ± estimates s.e. of each block, when larger than symbol size.

The effect of four days' submergence at different seed developmental stages on seed viability in storage was compared individually within cultivar by using paired F-tests of residual deviance (Appendix 2.33). In cv. Gleva, the control and submergence treatments could be constrained to a common K_i (P=0.156), but σ varied (Fig. 2.21a, P<0.001, Appendix 2.33). In IR64, σ did not vary (P=0.898), but a common line could not be fitted (Fig. 2.21b, P=0.011, Appendix 2.33), and hence K_i varied, whereas in IR64 Sub1 the various treatments could all be constrained to a common line (Fig. 2.21c, P=0.313, Appendix 2.33). Hence, there was no significant effect of 4 days' submergence at any developmental stage on the subsequent longevity of IR64 Sub1. In the two remaining cultivars, significant treatment effects were detected, albeit comparatively small: in cv. Gleva, longevity for submergence of the non-sprouted seed fraction at 30 DAA > 40 DAA > Control or 10 DAA, and in cv. IR64, control > 40 DAA >30 DAA >10 DAA (Figs. 2.21a and b).

The interactions between submergence and cultivar on seed longevity (p_{50}) during storage were evaluated by comparing the reduction in residual deviance (F-Test). The results indicated that 4 days' submergence at any seed developmental stage had no effect on subsequent longevity (P = 0.999, Appendix 2.34.1). On the other hand, submergence tolerance due to varietal differences was the significant influence to maintain seed viability during storage (P<0.001, Appendix 2.34.2): cvs IR64 Sub1 > IR64 >Gleva (Appendix 2.35).

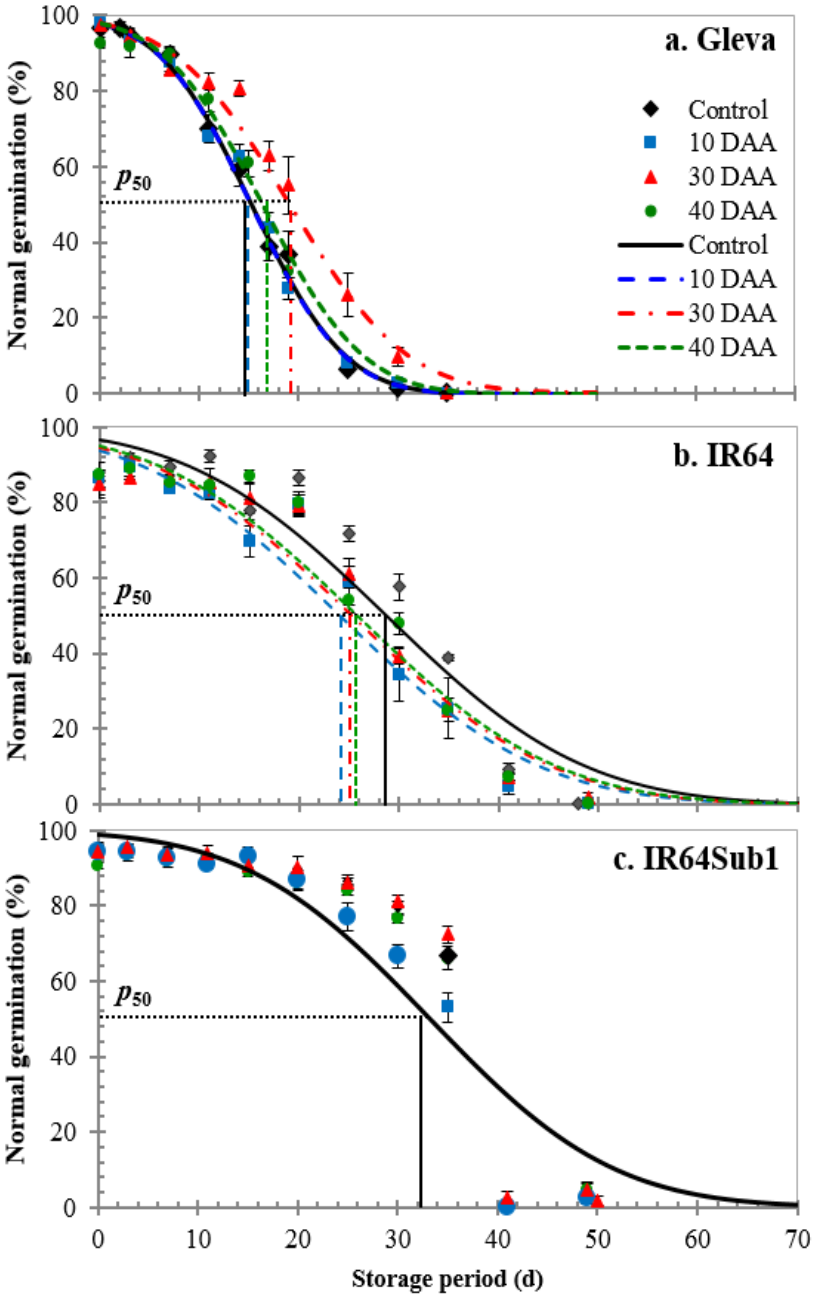

Figure 2.21 Seed survival curves [normal germination (%) plotted against period in hermetic storage at 40 °C with 14.7 ± 0.2% moisture content] of rice cvs Gleva (a), IR64 (b), and IR64 Sub1 (c) justified by the analysis then fitted with following common K_i, common slope and common line for seeds harvested at harvest maturity after no submergence (——), or submerged for 4 days at 10 (– – –), 30 (– · – · -), or 40 (--------) DAA (2013) (Appendix 3.33). The dotted line (.....), shows the point of time that viability fall to 50% (p_{50}). These curves are quantified in Table 2.9.

Table 2.9 Longevity (parameters of seed viability equation fitted by probit analysis) for rice cvs Gleva, IR64, and IR64 Sub1 justified by paired F-test of residual deviance (Appendix 2.34) (2013). The seeds were harvested at harvest maturity after submergence for 0 (Control) or 4 days from 10, 30, or 40 DAA in hermetic storage at 40° C with 14.7 ± 0.2% moisture content. The 95% confidence intervals are shown for p_{50}.

cv.	Treatment	K_i		Slope (1/σ)		σ (days)	p_{50} (days)			
		Estimate	s.e.	Estimate	s.e.	Estimate	Estimate	s.e.	Lower 95%	Upper 95%
Common K_i										
Gleva	Control	2.06	0.026	-0.135	0.0020	7.4				
	10 DAA	2.06	0.026	-0.135	0.0020	7.4				
	30 DAA	2.06	0.026	-0.108	0.0016	9.3				
	40 DAA	2.06	0.026	-0.126	0.0030	7.9				
Common slope										
IR64	Control	1.83	0.025	-0.064	0.0007	15.6	28.8	0.30	28.19	29.36
	10 DAA	1.54	0.024	-0.064	0.0007	15.6	24.1	0.29	23.54	24.66
	30 DAA	1.61	0.024	-0.064	0.0007	15.6	25.3	0.29	24.76	25.90
	40 DAA	1.65	0.024	-0.064	0.0007	15.6	25.9	0.29	25.29	26.43
Common line										
IR64 Sub1	Control, 10 DAA, 30 DAA, 40 DAA	2.29	0.024	-0.069	0.0008	14.5	33.2	0.16	32.92	33.57

2.5 Discussion

2.5.1 Yield and seed weight effects

This study was not designed to investigate the effect of submergence on rice yield, that would have required a much larger scale and different series of experiments. Nevertheless, it was pertinent to collect such data and to compare the results with the literature. The insignificant decrease in yield per pot affected by simulated submergence post-anthesis was detected in 2012 and 2013, although up to 44% yield reduction was observed. It was noted that standard error of yield per pot of some submergence treatments was large (Table 2.3 and Fig. 2.15), and found in both years. Suggesting that substantial yield loss without statistical difference in the present study was relative to the huge variation in standard error observed here, and hence typically lead to non-significant result. The raw data indicated submergence may well have reduced yield per pot. That is, the absence of significance in this investigation does not imply these results contradict studies such as these of Kotera *et al.* (2005) who reported that yield losses of about 40-60% are found with submergence for 5 days one week after 50% anthesis. Further, Kotera and Nawara (2007) observed that the longer the duration (2, 5 or 8 days) of full submergence at 24 days after heading, the greater the yield loss (25, 45 and 55%, respectively). Here, the non-significant difference in yield varied from 12 to 43% depending on duration of submergence, cultivar, and developmental stage when submerged; that is a similar scale of loss. Longer durations of submergence (2012) and earlier submergence in seed development (2012 and 2013) appeared to reduce yield more, with cv. Gleva apparently more susceptible to damage than IR64 or IR64 Sub1 (2013).

In cv. Gleva in both years, seed dry weight (at 0% moisture content) was reduced significantly by submergence (Table 2.5, Fig. 2.17). These effects were highly influenced ($P<0.001$) by seed developmental stage when submergence treatment was imposed. The results from 2013 also indicated significant reductions in seed weight from submergence in all three (for fresh weight) or two (for dry weight) cultivars ($P<0.05$). The exception in 2013 was for dry seed of IR64 (Fig 2.17). Furthermore, the 2013 results showed a strong interaction ($P<0.001$) between cultivars and seed developmental stage at submergence: cv. Gleva was much more sensitive and had a greater reduction of seed weight than cvs IR64 and IR64 Sub1.

Unpredictable flooding during the late reproductive phase could inflict severe damage on yield. Although this study did not demonstrate any significant effects on yield per pot, the raw data for this variable and the detection of significance for 1000 seed weight suggest strongly that such flooding damage is real, and that the current study is not incompatible with those of other researchers. Because developing panicles have been damaged by water directly,

reduction in percentage of ripening grain, number of grain per panicle, percentage of filled-spikelets, thousand grain weight and grain yield per hill have occurred due to submergence during grain filling (Devender-Reddy and Mittra, 1985; Kotera *et al.*, 2005 and Kotera and Nawara, 2007). Moreover, in sensitive cultivars, partial submergence at either anthesis or later during seed development led to increased lodging and hence lower yield (Sharma and Ghosh, 1999).

The reduced grain weight of submerged seed was likely to have been caused by the inefficiency of photosynthesis under water and/or poor carbohydrate translocation during stress. In rice, the upper three leaves including flag leaf blade and sheath is the main source of photosynthate to the developing seed, and hence they contribute strongly to grain yield (Yoshida, 1972; Venkateswaslu and Visperas, 1987; Watanabe *et al.*, 1997; Abou-khalifa *et al.*, 2008; Ambavaram *et al.*, 2014). The majority of starch accumulation in the grain (60-90%) is from photosynthesis after heading and reaches a maximum at the milk-ripe stage (Matsushima and Wada, 1966; Yoshida, 1981b). Matsushima and Wada (1966) highlighted that carbohydrate translocation to and accumulation in the endosperm starts 5-8 days after flowering and ends at around 20 DAA. Therefore, the lower grain weight obtained from submergence at early grain filling (9-10 DAA) in the present investigation may have been caused by poorer starch accumulation.

The decrease in grain weight caused by flooding at late maturity (after 30 DAA) detected was, however, more likely to have been due to the use of stored reserves for the initial germination process (given the observation of sprouted seeds) rather than from the interruption of grain dry matter accumulation. The series of physiological changes of pre-harvest sprouting are similar to those of seed germination *ex planta*, but the seeds are still on the mother plant. After seeds imbibe water and become hydrated, the activation of chemical and physical (expansion of embryo, repair and multiplication of mitochondria, enzyme synthesis and gene transcription) begins and continues thereafter until its culmination with visible germination (radicle emergence) (Bewley *et al*., 2013). Furthermore, the reduction in grain weight of submerged but non-sprouted seeds may have been due to respiration of these seed. This is because cellular respiration commences immediately after imbibition to provide metabolic energy for possible germination (Woodstock and Grabe, 1967; Footitt and Cohn, 1995; Tung and Serrano, 2011).

2.5.2 Varietal differences in submergence tolerance

In the present study, both *indica* rice cvs IR64 and IR64 Sub1 showed significantly greater tolerance to flooding, i.e. yield stability and less negative impact on seed weight, than *japonica* cv. Gleva (Fig. 2.18). The results agree well with the previous findings of Xu *et al.* (2006), Fukao *et al.*(2006), Fukao *et al.* (2009), Hattori *et al.*(2009), Bailey-Serres *et al.* (2010), and Mickelbart *et al.* (2015); all reported that *japonica* cultivars are more vulnerable to flooding than *indica* rice.

Photosynthesis under complete submergence is inefficient due to the lower intensity of light underwater, and respiration can be impaired due to reduced supply of oxygen and greater accumulation of carbon dioxide as well as slower gas diffusion under water (Das *et al.,* 2009; Singh *et al.,* 2009). The plant responses to limited or declining oxygen levels is to switch its carbohydrate catabolism towards anaerobic respiration (Dennis *et al.,* 1992; Perata and Alpi, 1993; Ricard *et al.,* 1994; Quimio *et al.,* 2000; Kato-Noguchi and Morokuma, 2007; Miro and Ismail, 2013). In rice, increase in the activity of ethanol-fermenting enzymes during complete submergence is highly related to susceptibility to submergence and has been shown to account for differences amongst cultivars (Setter *et al.,* 1994; Ellis and Setter, 1999; Quimio *et al.,* 2000; Mohanty and Ong, 2003; Kato-Noguchi and Morokuma, 2007). In many instances, the cultivars that tolerated submergence showed greater tolerance also to anoxia by greater alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) activities (Kato-Noguchi and Morokuma, 2007). Thus, these submergence-tolerant cultivars (cvs FR13A, Kurkurappan, Calrose, Nipponbare, and Yukihikari) produced higher amounts of ethanol with no or less aldehyde accumulation than intolerant ones (cvs IR42, IR22 Leulikelash, and Asahimochi) (Setter *et al.,* 1994; Kato-Noguchi and Morokuma, 2007). Ellis and Setter (1999) have provided supporting evidence that tolerance to anoxia in rice is correlated with ability to activate ADH under oxygen deprivation, which is associated with variation in cultivar tolerance to submergence. The role of PDC in rice under the absence of oxygen is mainly linked to controlling ethanol production rate (Quimio *et al.,* 2000; Mohanty and Ong, 2003). The explanations here suggest that during simulated submergence treatment in the present study, plant tissues of IR64 and IR64 Sub1 might have higher capability in metabolic response through alcoholic respiration than Gleva. By this enhancement of metabolic capacity, both *indica* rices had sufficient energy supply to be able to minimize cellular damage that may subsequently affect growth and impair productivity.

In addition, after the recession of floodwaters, recovery from injury by the vegetative parts of submergence-intolerant cultivars may require the production of new leaves or tillers (Reddy *et al.,* 1985; Singh *et al.,* 2009). This diverts photosynthate or stored assimilate away

from deposition in reproductive organs, thus resulting in decreased carbohydrate accumulation and would correspond with the reduction of grain yield observed in cv. Gleva rather than cvs IR64 and IR64 Sub1. In a preliminary study, plants of cv. Gleva submerged at the booting stage did show subsequent outgrowth of new tillers, whereas, submergence post anthesis did not result in rice tillering.

In previous research Septiningsih *et al.* (2009), Singh *et al*. (2009), Sakar and Bhattacharjee (2011), and Singh *et al.* (2011) claimed that the *Sub1* introgression lines provided yield advantage in completely flooded conditions imposed to 14 days old seedlings because the seedlings survived, whereas less than half of the recurrent mother line's seedlings could survive that treatment. The present study, involving submergence much later in plant development, found that the introgression line IR64 Sub1 provided lower yield per pot overall (in the Control and each submergence treatment) than its parental cultivar IR64, but greater stability in yield with submergence. Notwithstanding the absence of statistical significance, this is compatible with the view that the submergence tolerant introgression line has higher yield stability in flood conditions.

Analysis of variance indicated significant difference in 1000 seed dry weight amongst these two *indica* cultivars, but the difference was small (19.6 and 19.0 g, IR64 and IR64 Sub1) without biological significance. It is possible that a longer submergence duration would have resulted in differences between introgression cultivar IR64 Sub1 and recurrent mother IR64 in yield and seed weight. Similar results have been found by Dar *et al.* (2013). They reported that crop performance between the near-isogenic lines, Swarna and Swarna Sub1 was similar when plants were grown in optimal conditions if flooded for less than five days. The statistical estimation in yield advantage of *Sub1*flood-tolerant rice, Swarna Sub1 was made and compared with sensitive parental cultivar, Swarna. In their study in 2011, the monsoon arrived in the paddy fields of 128 villages of Bhadrak and Balasore Districts, Orissa, India at 60-70 days after transplanting (DAS) when rice was approximately at panicle initiation. The data was obtained from the low-lying areas that are prone to flooding mentioned above, and analysed using nonparametric regressions to estimate loss in rice yield affected by submergence and flood duration. This study revealed that Swarna was predicted to produce 3.5 tonnes ha^{-1}, whereas Swarna Sub1 had been estimated to have 5% lower yield (about 180 kg ha^{-1} less) when these two cultivars were grown in the field without submergence. The former authors proposed that the significant positive impact of using the *Sub1* variety was more noticeable in prolonged flooding durations, 7-14 days: the estimates predicted that yield advantage of Swarna Sub1 was up to 66% (718 kg ha^{-1}) over the parental cultivar Swarna following 13 days of submergence.

Therefore, the marginal differences in yield and seed weight between near-isogenic cultivars IR64 and IR64 Sub1 in the present study may reflect the short submergence period I imposed.

2.5.3 The occurrence of pre-harvest sprouting and seed dormancy

Ellis and Hong (1994) and Ellis (2011) reported that the more mature the rice seed the greater their ability to germinate and survive desiccation. In the current study where water uptake could occur for longer (i.e. 5 days' submergence), more seeds would be able to continue the progress of germination towards radicle emergence. The above could explain why the prolonged duration submergence treatment to the more mature seed lead to the greatest pre-harvest sprouting (Figs. 2.4 and 2.16).

Duration of submergence, the developmental stage at which it occurred, and genotype affected premature sprouting. The interaction between the developmental stage at submergence and flooding duration was significant in 2012, and that of developmental stage and genotype in 2013. Cultivar Gleva had lower pre-sprouting resistance than the *indica* rice cvs IR64 and IR64 Sub1. Nevertheless, some (limited) pre-harvest sprouting was detected in the latter two cultivars when submergence occurred in seed development and maturation (i.e. 40 DAA).

Seed dormancy is the regulation to control (delay or prevent) germination even when favourable conditions are provided (Bewley *et al.*, 2013). This trait is mainly regulated by the genetics of the mother plant, developing embryo, and by environment (Bewley and Black, 1994; Taiz and Zeiger, 2006; Sugimoto *et al.*, 2010). There are two mechanisms of seed dormancy that possibly block germination; first the restraint of seed covering tissues, which prohibit or delay imbibition and physically obstruct radicle growth, second embryo growth restriction by the endogenous hormone abscisic acid (ABA) (Hilhorst, 1995; Sugimoto *et al.*, 2010; Bewley *et al.*, 2013).

In the present study, seeds of the *japonica* cv. Gleva and the *indica* cv. IR64 were first able to germinate *ex planta* (Fig. 2.13) after 17 (28%) and 31 DAA (13%), respectively. Where the seed covering tissues were removed, this was detected earlier on at 9 (36%) and 22 DAA (18%), respectively and before they attained mass maturity (27.6 and 28.1 DAA). Ability to germinate developed further after 30 DAA (Fig. 2.13). This evidence supports the finding of Ellis and Pieta Filho (1992) that seed quality continues to improve after cereal seeds attain maximum weight. Furthermore, the greater germination after removal of seed-covering structures indicates that one block to germination in both cultivars was coat-imposed dormancy. With or without removal of seed-covering structures, cv. IR64 showed greater dormancy than

cv. Gleva, suggesting that the former had greater embryonic dormancy. In cv. IR64 at harvest maturity seeds showed 64% germination and this increased by 20% for samples first dried to 15% moisture content. This effect of desiccation on reducing dormancy and hence promoting subsequent germination is well documented in rice (Roberts, 1973; Kovach and Bradford, 1992; Ellis and Hong, 1994; Still *et al.*, 1994; Zhu *et al*., 2006).

Hence, high dormancy during maturation (as here in cvs IR64 and IR64 Sub1) is helpful to suppress pre-harvest germination where the seeds on mother plants are exposed to warm weather and high humidity and moisture (i.e. from heavy rainfall or floods) in the field before harvest. In the present study, cvs IR64 and IR64 Sub1 had greter dormancy than the submergence-intolerant Gleva. Also, the superior of dormancy in mature seed of cvs IR64 and IR64 Sub1 meant less use of stored carbohydrate for germination and a smaller reduction in seed weight before harvest.

2.5.4. Moisture content of freshly-harvested-non-sprouted seed

Moisture content of non-sprouted seed at harvest varied depending upon preliminary treatment. Surprisingly, previously-submerged seed tended to have lower moisture contents than the Control. This tendency was more apparent in 2012 when the longer duration that seed could dry after submergence before harvest the higher the moisture content of freshly-harvested-non-sprouted seed (Table 2.4). The later in development that submergence occurred, the greater the reduction in seed moisture content at harvest maturity (Appendices 2.6 and 2.24); the reduction was greatest in cv. IR64 Sub1. This result was surprising; I expected the previously submerged seed to show a higher moisture content at harvest maturity. One explanation might be that the submergence stress to the whole plant resulted in more rapid plant senescence, and hence drier seed.

Rice seeds have high hygroscopicity, that is its moisture content varies substantially with ambient relative humidity, with a high capability to absorb moisture (Coleman and Fellows, 1925; Breese, 1955; Juliano, 1964; Roberts, 1972; Lu and Siebenmorgen, 1992; Fan *et al.*, 2000; Prakash *et al.*, 2011). Juliano (1964) and Juliano *et al.* (1990) reported that the hygroscopic equilibria of rice seed was strongly related to its amylose content and so waxy rice was expected to have a higher moisture content than non-glutinous rice. Singh *et al.* (2009) reported 24.9 and 25.6% amylose concentration in grain of cvs IR64 and IR64 Sub1 grown in field trials at IRRI in 2007. A difference in amylose content might explain the effects of submergence and/or the varietal differences, but this was not tested in the present study.

Furthermore, it should be noted that the dates of maturity varied amongst cultivars and hence ambient relative humidity may have differed.

2.5.5 Effect of submergence of rice seed quality

2.5.5.1 The effect of submergence on germinability

In the present study, the submergence treatment mimicked natural flooding during grain filling and maturation drying. The first impact of simulated flooding was that some seeds sprouted on the mother plants. The study of 2012 showed that submergence of the more mature seed (30-40 DAA) of *japonica* cv. Gleva triggered germination with many sprouted seed. The visible sign of germination could be seen by the emergence of the seedling, the radicle, or the tiny open hole of micropyle at the base of the seed (Fig. 2.3). Observations suggest that at the time the seeds experienced flooding, they varied in developmental age within the panicle. Those that sprouted may have been the more mature seed within each panicle. For example, Roberts (1960) and Yoshida (1981b) suggested that all spikelets within a panicle may take 5-10 days to complete anthesis.

Germination of non-sprouted seed from the submerged treatments of cv. Gleva was high, more than 90%, although this was reduced compared to the Control (Fig. 2.5). The latter difference was not detected, however, if seeds were first dried after harvest to 15.0% moisture content. The results described here for submergence might be considered to have received a treatment equivalent to 'priming' even if unintentional (see later).

On the other hand, the ability to germinate of sprouted seeds after harvest was low: only those sprouted seed with minimal visible germination, i.e. open tiny hole at base or less than 5 mm of radicle protrusion, were able to produce normal seedlings subsequently (Fig. 2.8). No sprouted seeds with well-established seedling structures were able to germinate after harvest (and hence desiccation) in the present study. The germination (or not) of sprouted seed post-harvest described above could be explained by "over-priming". Prolonged priming results in the completion of the germination process (Harris *et al.,* 2000), where radicle emergence and seedling structures can be observed. Bewley *et al.* (2013) hypothesized that dehydration of over-primed seed might cause physical damage of radicle tips and hence abnormal growth.

Therefore the maintenance of seed dormancy of rice during seed maturation is a vital trait to prevent pre-harvest sprouting, with tropical rice subjected often to high rainfall and high humidity in the approach to harvest. Pili (1969) reported that the longer the delay to harvest the lower the dormancy. Moreover, early-maturing varieties have reduced dormancy than medium

or late-maturing varieties (Tung and Serrano, 2011). Furthermore, *japonica* subspecies are less dormant than *indica* rice and hence more susceptible to pre-harvest sprouting (Sugimoto *et al.*, 2010). The former findings were confirmed in the present study: *indica* rice were much more dormant than the *japonica* cultivar in later development than earlier, reflecting the negligible pre-harvest germination that was detected in the former. Therefore, to prevent pre-germination during late-grain filling stage in wet environment, genetic control of the character, sprouting in this case, is the main concern for subsequent seed quality.

2.5.5.2 The effect of submergence on longevity

The seed survival curves were generated only from seed that had not sprouted *in planta*. Clearly, those seeds that had sprouted were already damaged and my design avoided confounding that effect with any potential effects on seed apparently undamaged visually at harvest. In general, the effect of submergence on subsequent seed survival was small. For example, in the most flooding-tolerant cv. IR64 Sub1, there were no significant differences in survival curves amongst the various submergence treatments. In cv. IR64, submergence reduced subsequent seed longevity slightly. Whereas, in cv. Gleva significant reductions were detected only after 5 days' submergence at 9 or 40 DAA in 2012, and a significant increase only after 4 days' submergence at 30 DAA in 2013.

It has been reported that priming can be of especial benefit to aged or immature seed of cauliflower, Brussel sprouts, and foxglove (Burgass and Powell, 1984; Powell *et al.*, 2000; Butler *et al.*, 2009a, b). Therefore instead of a detrimental effect of submergence on subsequent longevity, the marginal positive impact found in this study in a few scenarios may be suggesting due to the priming-like treatment enabling repair, which may eliminate stress-induced substances that cause seed deterioration (Burgass and Powell, 1984).

In the present study, the effect of submergence on the subsequent seed storage longevity of non-pre-sprouted seed was small and sometimes nil. This contrast with the large effect of seed storage environment on rice seed longevity, which is an exponential response (Ellis and Hong, 2007), and of temperature during seed development (Ellis *et al.*, 1993). The survival curves in the hermetic air-dry storage environments conformed to negative cumulative normal distribution, as expected (Roberts, 1972).

The seed viability equation developed by Ellis and Roberts (1980a) provides two parameters to assess the effect of pre-storage and storage environment on seed storage longevity. The first parameter is K_i, the value of the seed lot constant, which represents the

initial seed quality. This estimate may vary between seed lots because of internal (genotypic differences and the degree of seed maturity) and external factors (growing conditions, practices at harvest, and drying processes) before storage (Roberts, 1972; Ellis and Roberts, 1980a; Ellis and Roberts, 1981; Pieta Filho and Ellis, 1992). The second parameter is σ, the standard deviation of the distribution of seed deaths in time (days) in storage. This value is affected greatly by storage environment, but is the same in identical storage conditions (i.e. temperature and moisture content) for seed lots within a species (Ellis and Roberts, 1980a). Practically, if the same storage environment is provided for all seed lots, investigations of potential longevity (K_i) can be used to contrast different seed lots that have experienced different production conditions (e.g. high humidity, rainfall, or flood events) during grain filling and maturation.

There is evidence to support the use of the K_i value as the effective criterion to quantify seed quality of crops, for instance barley (Ellis and Roberts, 1980a, and 1981; Pieta Filho and Ellis, 1991; Ellis and Pieta Filho, 1992), wheat (Ellis and Roberts,1980a; Ellis and Pieta Filho, 1992), pearl millet (Rao *et al.*, 1991), rapid-cycling brassica (Sinniah *et al.*, 1998), common bean (Sanhewe and Ellis, 1996; Ghassemi-Golezani and Mazloomi-Oskooyi, 2008), tomato (Demir and Ellis, 1992a), pepper (Demir and Ellis, 1992b), marrow (Demir and Ellis, 1993) and the wild species foxglove (Hay and Probert, 1995; Butler *et al.*, 2009a, b). In rice, seed quality can be limited by environmental conditions through early seed development during the reproductive stage until seeds become mature on the mother plant. In general, hot seed production environments provide poorer rice seed quality than warm regimes (Ellis *et al.*, 1993; Ellis and Hong, 1994; Ellis, 2011), at least for *japonica* rice. The ability to obtain maximum quality, in this case estimated by p_{50} of *japonica* rice seed was reduced slightly when plants were exposed to high temperature after the end of grain filling (Ellis, 2011). On the other hand, Ellis *et al.* (1993) and Ellis and Hong (1994) reported dramatically poorer ability to germinate, potential longevity (K_i) and desiccation tolerance: if seeds were exposed to high temperature from earlier in their development, *japonica* rice was more susceptible to the warmer regime than *indica* and *javanica* rice.

In all assessments for longevity in this Chapter, the seed survival curves were generated only from seed that had not sprouted *in planta*. That is the seed populations had been divided to account for sprouting. Clearly, those seeds that had sprouted were already damaged and my design avoided confounding that effect with any potential effects on seed apparently undamaged visually at harvest. Submergence treatments provided variable impacts on the subsequent pattern of loss in seed viability in storage: depending upon seed development stage when the event occurred (in 2012, Appendix 2.17) or genotype (in 2013, Appendix 2.34). In 2012, significantly poorer potential longevity of cv. Gleva performed by analysis of variance

was observed only in K_i with 5 days' submergence at 9 or 40 DAA. In contrast with 2013, only the estimates of σ for different seed lots differed significantly between cultivars (i.e. Gleva < IR64 Sub1 ≤IR64), without detection of difference in K_i affected by 4 days' submergence treatments. This shows that the adverse impact of submergence during seed development on seed quality at maturity was real and is in agreement with Roberts (1972) and Ellis and Roberts (1980a): pre-storage conditions, i.e. growing environment, affect subsequent seed quality which can be assessed by the differences in potential longevity (K_i of the seed viability equation). Nevertheless, substantial damage from flooding may become more severe in the case that the longer submergence event (e.g. more than 4 days) was imposed during grain filling and maturation. Furthermore, significant difference in σ indicated that *japonica* and *indica* rice were different. This result agreed with Ellis *et al.* (1993) who reported that there was significant effect of rice subspecies on the estimates of σ in the seed viability equation: the greatest value of σ was observed for *indica*, then *javanica*, and finally *japonica* rice.

According to the above variations in σ, the period for viability to reduce to half (p_{50}, the product of K_i σ) was used to assess the pre-storage effect on seed longevity because it is the most accurately estimated viability period (because it is the mean of the distribution of seed deaths in time). In general in the present study, there was the tendency that p_{50} of the control was greater (by up to five days) than other seed lots obtained from flooded treatments. The exceptions were found in some submergence-non-sprouted seed samples of 2013, however; 4 days' submergence at 30 (cvs Gleva and IR64 Sub1) and 40 DAA (cv. Gleva) showed 1-4 days greater seed longevity than the control. In context, these were negligible differences given that longevity depends upon environment and can vary from minutes to thousands of years (Ellis and Roberts, 1980a). Further, the variation in results may be due to variation in anthesis across the plant population; there are about 5-10 days variation in anthesis date within the same rice panicle, and the variable becomes greater between tillers and plants (Robert, 1960; Yoshida, 1981b; Global Rice Partnership, 2013). Moreover, normal flooded seeds may lose their storability that tends to coincide when a germination process has been triggered whether the absence of evidence of seed emergence. In this situation, it is important to note that longevity was determined on the non-sprouted seeds. Del Fueyo *et al.*, 1999 (as cited in Gualana and Benech-Arnold, 2009) suggested that pre-germination takes place when embryo growth begins after imbibition, nevertheless, the radicle emergence is not found because the desiccation interrupted the process before radicle protrusion occurs. This suggested that the flooded seed maintain their viability but longevity may reduce substantially.

The comparison of paired F-Test for fitted models in Figure 2.21 summarizes the findings in the current study (2013) in agreement with the explanations above:

1) Non-submergence (control) and 4 days' submergence at 10 DAA in cv. Gleva provides identical seed survival curves, meanwhile seed lots of submergence treatments at 30 and 40 DAA showed variation in slope (1/ σ)and also better survival in this non-sprouted fraction, but not in K_i (Fig 2.21a).
2) Although control and all submerged seed lots from submergence at 10, 30 or 40 DAA had different initial quality (i.e. K_i), the deviation of distribution of seed deaths in time (days) was the same in cv. IR64 with the control providing the greatest longevity (Fig 2.21b).
3) And finally, there was no significant difference due to submergence on seed longevity observed in cv. IR64 Sub1 (Fig 2.21c).

The apparent contradiction from the effect of flooding amongst genotypes suggested that submergence event during seed maturation encouraged pre-harvest sprouting, and thus provided indirect detrimental effect on subsequent longevity. In cv. Gleva, paradoxical results observed as seed lots had experienced submergence (i.e. 4 days, submergence at 30 or 40 DAA) showed longer longevity (p_{50}) than Control. There was a number of sprouted seed observed from both Control and 4 days' submergence at 10 DAA. This suggests that seed lots of 30 and 40 DAA, in which 7 and 67% of sprouted seeds were observed after submergence treatment, were 'biased samples' because the worst fraction (i.e. sprouted seeds) was removed. Therefore, the seed population in these samples were modified, and I suggest that the sprouted seeds would have been those first to die in the control (or 4 days' submergence at 10 DAA). Furthermore, the most aged seeds at harvest etc. were those which were also the most vulnerable to sprout.

In contrast, in both *indica* rices cvs IR64 and IR64 Sub1, there was almost no sprouted seeds detected, even from 4 days' submergence at 40 DAA (< 1%). The absence of sprouted seed (to be removed) implied that the whole population of each seed lot had been affected by flooding treatment equally or obtaining similar level of damage from flooding, representing by the same σ. The effect of submergence of subsequent seed longevity (p_{50}) of cv. IR64 was following Control > 40 > 30 and > 10 DAA, respectively. Surprisingly, an excellent performance in ability to tolerate submergence, reflected by no damage to subsequent longevity (whether K_i or p_{50}), was found in cv. IR64 Sub1. Hence, the novel finding from the present study confirms that introgression of *Sub1* gene is beneficial for susceptible high yielding rice variety, at least in the cv. IR64 background with no negative effect of submergence on seed storability (or pre-harvest sprouting).

2.5.5.3 Effect of genetic differences on longevity of seed from submerged plants

Chang (1991) and Ellis *et al.* (1993) reported that *japonica* cultivars possess intrinsically poorer seed storage characteristics, as well as being more sensitive to the seed production environment than *indica* cultivars. The finding in this study confirms that, under normal condition, i.e. no submergence, *japonica* rice cv. Gleva had poorer seed longevity reflecting by significantly shorter estimates p_{50} (15.3 days) than both *indica* cvs IR64 and IR64 Sub1 (28.9, and 33.8 days, respectively).

In the circumstance that simulated-flooding was tested, there was significant influence of cultivar on subsequent life span which the longest p_{50} was detected in IR64 Sub1, followed by IR64 and Gleva, respectively. Interestingly, Ikehashi (1973) and Siddique *et al.* (1988) highlighted the issue that dormant rice is likely to provide longer seed viability in storage, whereas cultivars with lower seed dormancy tend to lose viability more rapidly. Roberts (1962), however, found contradictory results to suggest these associations: although dormancy of six rice varieties from three geographical races (*O. sativa* L. sub-species *japonica* and *indica,* and *O. glaberrima* Steud.) produced in identical conditions differed substantially, loss in viability in identical storage conditions were very similar. Here, cv. IR64 Sub1 had about five days longer half-viability period than cv. IR64 under the same production and the same storage environments in controls (i.e. non-submergence treatment), but this difference was insignificant (Fig. 2.20). The possibility that these two cultivars differ in this regard is investigated further in Chapter 3.

The evidence from the present study indicates that introgression of *Submergence 1* gene into the submergence-intolerant-high-yielding-rice variety cv. IR64 had no subsequent negative impact on seed storability. Moreover, the introgression of the *Submergence 1* gene may have or did even provided greater longevity after submergence. The study of QTL mapping revealed that there was relationship between ability to germinate under anoxia, maintaining normal seedling growth, seed dormancy and longevity caused by the co-location of QTLs in rice (Miura *et al.,* 2002; Sasaki *et al.*, 2005; Angaji *et al.,* 2010; Septiningsih *et al.,* 2013). Although the association between the function of the *Submergence1* gene on subsequent longevity remains unclear, further investigation at the molecular level would elucidate the heritability and relevance of submergence tolerance with the seed ageing trait.

It has been mentioned previously that harsh-growing conditions affect subsequent seed quality. The findings in the present study show that submergence at different seed developmental stages encouraged substantial pre-harvest sprouting, thus contributing to a severe yield penalty directly. *Japonica* sub-species clearly showed less seed dormancy and

therefore correspondingly less tolerance to submergence, and hence has significantly higher premature sprouting than *indica* varieties. Nevertheless, there was a negligible effect on subsequent storability of non-sprouted seed caused by submergence during seed development, regardless of differences in cultivar. Moreover, the *Sub1* introgression in variety IR64 Sub1 showed excellent submergence tolerance without a detrimental effect on yield or seed storage characteristics in both normal and flooded conditions.

Overall, the biggest effect of submergence was sprouting and damage to these seed (i.e. many cannot germinate upon reimbibition). The latter indicates that if sprouting can be prevented (by dormancy) then flooding is "survivable", with little or no impact on seed storage survival period.

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Chapter 3

The Effect of Introgression of *Sub1* Gene on Subsequent Storability of Rice

3.1 Introduction

The molecular genetics of submergence tolerance in rice has been elucidated extensively using novel approaches, for instance, screening by segregation pattern, genomic mapping, and DNA sequencing (Mishra *et al*., 1996; Xu *et al.,* 1996; Harushima *et al.,* 1998; Xu e*t al.,* 2000; Xu *et al.,* 2006). Xu *et al.* (1996) found that the major quantitative trait loci (QTL) associated with submergence resistance of rice was mapped on Chromosome 9. Xu *et al.* (2006) later on identified the region and found that the location was near to the centromere. The identification of a submergence tolerance gene, named *Submergence 1 (Sub1)* in the former study explained the variation in submergence tolerance by 70%. Fine mapping was carried out and revealed that the length of this locus was about 0.06 centimorgans (Xu *et al.,* 2006). Nandi *et al.* (1997) and Toojinda *et al.* (2003) reported that there are other QTL on Chromosomes 1, 2, 5, 6, 7, 10, 11 and 12, which are associated with the submergence tolerant trait. Nevertheless, these provided only negligible effects on susceptibility to damage from flooding.

Many submergence related genes in rice belong to the environmental stress responsiveness gene in the group of ethylene response factors (ERF) (Xu *et al.*, 2006). Despite the name, this transcription factor ERF are also regulated by other hormones, for example abscisic, salicylic, jasmonic, and giberellic acids, which are triggered by environmental stress (i.e. drought, anoxia, cold, salinity, and infection by pathogen) (Sakuma *et al.*, 2002; Grennan, 2008; and Pergoraro *et al.*, 2013). The region of *Sub1* designates three polygenic loci that deduce ethylene-responsive element binding proteins; *Sub1A*, *Sub1B*, and *Sub1C*, of which the submergence-tolerant trait regulation is dominated by *Sub1A* (Xu *et al.,* 2006).

The major advantage of the *Sub1A* in submergence tolerance in rice is that the plant has higher efficiency in energy management during submergence: the responsiveness of internode elongation normally triggered by gibberellic acid is suppressed (Fukao and Bailey-Serres, 2008; Singh *et al*., 2011; Bailey-Serres *et al.*, 2010; Singh *et al.,* 2011; Niroula *et al.,* 2012). This helps to retain sufficient carbohydrate reserves to consume during submergence and in recovery post-submergence. Submergence tolerant varieties containing *Sub1A*, moreover, show better performance in energy stabilizing by maintaining total soluble carbohydrate levels and altering

catabolism to alcoholic fermentation (i.e. pyruvate decarboxylase and alcohol dehydrogenase increased, minimizing aldehyde content) (Das *et al.*, 2005; Fukao *et al.*, 2006; Bailey-Serres *et al.* 2010). Furthermore, submergence tolerant rice shows lower oxidative damage with less chlorophyll degradation after the water level subsides (Ella *et al.*, 2003; Jackson and Ram, 2003). Therefore, the energy required to recover from flooding damage (i.e. produce new leaves or tillers) is minimized.

The presence of *Sub1A* in cultivated *O. sativa* and wild rice, for instance *O. nivara* and *O. rufipogon*, however responds differently in terms of submergence resistant trait (Xu. *et al.*, 2006; Fukao *et al.*, 2009; Septiningsih *et al.*, 2009*;* Niroula *et al.*, 2012). Xu *et al.* (2006) found that the variable responses to submergence tolerance in rice were due to allelic variation of *Sub1A*. Two alleles were identified that govern the flooding tolerance trait: tolerance and intolerance-specific allele, called *Sub1A-1* and *Sub1A-2*, of which only the former confers submergence tolerance to rice (Xu *et al.*, 2006). According to allelic differences, there is a significant difference in a single nucleotide polymorphism (SNP) on the open reading frame at 556 base pair (bp) (Xu *et al.*, 2006). At this position, the replacement of a nucleotide base from cytosine (C) to thymine (T) leads to substitution of protein encoding (at position 186) from the amino acid proline (CCG, intolerant, where G is guanine) to serine (TCG, tolerant). In fact, there was one more replacement at 678 bp, in which the nucleotide adenine (A) was replaced by guanine (Xu *et al.*, 2006; Fukao *et al.*, 2009; Septiningsih *et al.*, 2009; Niroula *et al.*, 2012). However, this genomic replacement results in silent substitution because subsequent protein translation encodes no change of amino acid sequence (glutamine; CAA in tolerant and CAG in intolerant cultivars) (Xu *et al.*, 2006).

In rice breeding programmes, the *Sub1* QTL has been exploited to improve submergence tolerance in sensitive-high-yielding rice cultivars through marker-assisted backcrossing (MABC), by using *indica* rice cv. FR13 as a donor for the tolerant gene (Neeraja *et al.*, 2007; Septiningsih *et al.*, 2009: Singh *et al.*, 2009). Six varieties described as submergence tolerant "mega varieties" have been officially released in 2009 from IRRI; Swarna Sub1, Samba Mahsuri Sub1, Thadokkam1 (TDK1) Sub1, BR11 Sub1, CR1009 Sub1 and IR64 Sub1 (IRRI, 2011; Mackill *et al.*, 2012), with two more varieties subsequently released; Ciherang Sub1 and PSB Rc18 Sub1 (Isamil *et al.*, 2013).

The above submergence tolerant cultivars showed robust performance by enduring simulated flooding conditions for approximately two weeks depending upon genotype and quality of flood water (Ram *et al.*, 2002; Das *et al.*, 2009; Sarkar *et al.*, 2009; Singh *et al.*, 2009). Furthermore, these *Sub1* introgression varieties were shown to retain the genome of the original varieties without apparent effect on growth, yield performance, and grain quality in

trials carried out under glasshouse or field environments in India, Bangladesh, the Philippines, and Indonesia. There was, however, linkage of *Sub1* with the neighbouring gene of the inhibitor of brown furrows (*IBF*), which subsequently altered the dark-colour of hulls of the introgressed cvs Swarna Sub1 and TDK1 Sub1 to be lighter-yellow (Neeraja *et al.*, 2007; Shao *et al.*, 2012). Nevertheless, this colour development of the mature seed was considered not to be a disadvantage to economic or agronomic value of introgression of *Sub1*.

To date, the impact of *Sub1* introgression on seed storability from flooding late in development does not appear to have been investigated. It has been reported that the QTLs controlling seed viability in storage are mapped on chromosomes 1, 2, 4, 5, 7, 9, 11, and 12 of rice (Miura *et al.*, 2002; Sasaki *et al.*, 2005; Xue *et al.*, 2008; Jiang *et al.*, 2011; Li *et al.*, 2012). However, the major QTL for storability in those studies was on chromosome 9 explaining 10-60% of the phenotypic variation (Miura *et al.*, 2002; Li *et al.*, 2012). Therefore, introgression of the genomic fragment containing *Sub1A-1* into chromosome 9 of high-yielding rice could affect subsequent seed longevity. In fact in Chapter 2, the impact of simulated flooding during seed development on subsequent longevity was negligible in cvs IR64 and IR64 Sub1: the seed samples of IR64 Sub1 harvested from submergence post-anthesis for 0 or 4 days showed about 5-9 days greater longevity than that of parental cultivar IR64. Thus, the objective of this study was to confirm whether or not there is any effect of the introgression of *Sub1* on the potential longevity of the near-isogenic cultivars, where longevity was assessed by p_{50}.

3.2 Null hypotheses

There is no significant difference in seed quantity and quality postharvest between the original submergence-sensitive, IR64, and introgressed cultivar, IR64 Sub1, produced under controlled non-submergence environments;

1) There is no difference in yield per pot, moisture content at harvest, or 1000 seed weight between this pair of near-isogenic cultivars.
2) There is no difference in seed germinability and longevity during storage between this pair of near-isogenic cultivars determining by ability to germinate normally after harvest and potential longevity (i.e. K_i and p_{50})

3.3 Materials and Methods

3.3.1 The presence of *Sub1* gene and DNA sequencing

3.3.1.1 Plant material

To confirm the presence of the *Submergence 1* gene/allele that confers submergence tolerance in rice, *indica* rice cvs IR64 and IR64 Sub1 were used in the present study, together with the submergence intolerant *japonica* cv. Gleva for comparison as a negative control. The original seed had been obtained from the original providers (cv. Gleva from IART, Spain, and cvs IR64 and IR64 Sub1 from IRRI, Philippines). Seeds produced at PEL from these parent lines in 2013, as well as the original seed lot of parent lines were used for analysis (contributing six samples in total). Twenty-five seeds of each sample of each variety were sown individually in 25 compartmentalised format of square Petri-dishes (Sterilin™ 100 mm, Thermo Scientific, UK). Deionized water (1 mL) was applied to moisten the seed in each compartment. The square Petri-dishes were placed in Seed Laboratory at room temperature.

3.3.1.2 DNA extraction

DNA was extracted from the healthy shoots of five day old seedlings. After cutting, 100 mg of fresh tissue was ground immediately in a mortar and pestle with liquid nitrogen added. The DNA extraction was carried out using DNeasy® Plant Mini Kit (Qiagen, Manchester, UK) according to the manufacture's protocol. The only adjustment in the final steps in this DNA extracting method was as follows: the volume of elution solution (BufferAE) was reduced to 60 µL instead of 100 µL. The final supernatant collected from each extraction was stored in a 2 ml centrifuge tube (Eppendorf AG, Hamburg, Germany) at -20 °C until use.

DNA quantity and quality were determined by NANODROP 2000 (Thermo Fisher Scientific Inc., UK) at the absorbance range of 230, 260, and 280 nm. BufferAE (Qiagen, Manchester, UK) was used as a blank sample in this determination. The quantified amount of DNA present in the mixture and the assessment of nucleic acid purity are shown in Table 3.1.

Table 3.1 Concentration and purity of DNA extracted from five days old shoots of rice cvs Gleva, IR64 and IR64 Sub1 using the seeds provided by IRRI and the seeds produced at PEL. The determination of double strand DNA was conducted by spectrophotometry at the wavelength of 230, 260, and 280 nm.

Samples	DNA (ng mL^{-1})	Purity	
		(A260/A280)	(A260/A230)
1. Gleva (IART)	531	1.86	2.38
2. Gleva (PEL)	723	1.87	2.43
3. IR64 (IRRI)	654	1.87	2.39
4. IR64 (PEL)	519	1.86	2.50
5. IR64 Sub1 (IRRI)	267	1.87	2.55
6. IR64 Sub1 (PEL)	595	1.86	2.42
7. Blank (BufferAE)	0	0.13	0.08

3.3.1.3 PCR amplification

The primers used for amplification were *SUB1A*_1_fw (5'-GATGTGTGGAGGAGAAGTGA-3') and *SUB1A*_1_rev (5'- GGTAGATGCCGAGAAGTGTA- 3') provided by Invitrogen™ (Thermo Fisher Scientific Inc., UK). This pair of primers has been used previously in submergence tolerant rice studies to amplify the locus of submergence tolerant genes on chromosome 9 (*Sub1* QTL), with the expected fragment size of 1015 bp (Xu *et al.*, 2006, Niroula *et al.*, 2012). Amplification of genomic DNA was performed by the polymerase chain reaction (PCR) technique. The total mixture of 40 µL reaction volume for PCR contained 20 µL of REDTaq® ReadyMix™ (PCR Reaction Mix, Sigma-Aldrich Co., LLC., UK), 1.2 µL (0.3 µM) each of *SUB1A_1*_fw and *SUB1A_1*_rev primers, 1 µL DNA template, and 16.6 µL deionized water. This mix was used with all samples, except the genomic DNA of IR64 Sub1 with seeds provided by IRRI, because the DNA concentration of this sample was about 50% lower than other samples in the current study (Appendix 3.1). Therefore, the volume of genomic template for the IR64 Sub1 (IRRI) sample that had been added to the mixture was double, i.e. 2 µL, contributing 41 µL of PCR reaction volume in total.

PCR was performed by using PCR Thermal Cycler (Veriti®, Applied Biosystems, Thermo Fisher Scientific Inc., UK) with initial denaturation of genomic DNA template at 95 °C for 2 min. The PCR conditions for 20 cycles of denaturing, annealing, extension, and final

incubation were 95 °C for 30 sec, 59 °C for 20 sec, 72 °C for 30 sec, and 72 °C for 7 min, respectively. The PCR product was kept at -20 °C for subsequent screening of *Sub1A-1* allele and DNA sequencing.

3.3.1.4 Identification of *Sub1A* allele

The presence of *Sub1A* gene in *indica* rice demonstrates variable submergence tolerant levels, i.e. susceptible, moderate, and insusceptible to flooding. Xu *et al.*, (2006) investigated the differences in flooding tolerance and found that only the rice varieties that possess *Sub1A-1* allele can withstand submergence stress. Yuanxin *et al.* (2003) reported that there was a specific sequence that the restriction *BseNI* enzyme can digest DNA amplicons and can be used efficiently as a screening tool in rice and *Arabidopsis*. Furthermore, Niroula *et al.* (2012) found that *BseNI* enzyme with the recognition site with the DNA sequences of 5'-ACTGG-3' is able to cut those specific sequences on DNA strands found identically in the *Sub1A-1* allele (at 678-682 bp of *Sub1A* locus), and hence allelic differences relating to ability to tolerate submergence could be distinguished. Thus PCR work was carried out to confirm the efficiency of using the restricted enzymes *BseNI* as a submergence tolerance screening tool in rice. Because the enzyme can cut the base at the specific sequence at 678 bp of *Sub1* region, and hence positive result with *BseNI*, two fragments with approximate genome sizes of 400 and 600 bp were expected in the submergence-tolerant rice that possesses the *Sub1A-1* allele.

The reaction was carried out in the volume mixture of 20 µL containing 7 µL nuclease-free water, 1 µL enzyme *BseNI* (Thermo Fisher Scientific Inc., UK), 2 µL 10X Buffer B (Thermo Fisher Scientific Inc., UK), and 10 µL PCR product from Section 3.3.1.3. The solution was incubated for digestion at 65 °C for 90 min (Thermo Mixer C, 1.5 mL with 24 wells, Eppendorf AG, Hamburg, Germany). The final product containing genomic fragment(s) was then visualized by electrophoresis and staining.

The loading sample for gel electrophoresis was prepared from 5 µL of genomic fragment products after digestion by *BseNI* and 2 µL loading dye. Then, 5 µL of this digest mixture was loaded onto the gel. To prepare 1% (w/v) agarose gel, 1.0 g of agar was weighed and dissolved in 100 mL of 1X TAE Buffer including 1µg mL^{-1} of 0.05% ethidium bromide for staining. Electrophoresis was performed for 45 min at 80 V (PowerPac™ Basic, Bio-Rad, Herculus, USA). The gel was visualized under ultraviolet light and photographed by gel imager (GelDoc-It®TS2 Imager, Ultra-Violet Products Ltd., UK).

The comparison of the effectiveness of the enzyme *BrseNI* was made by comparing the results on the gel for the PCR product without adding *BrseNI* in the procedure described above.

A blank sample was prepared similarly according to the protocols of PCR amplification and screening of *Sub1A-1* allele, except that the genomic template was not added into PCR reaction mixture. A DNA ladder (100 Lanes HyperLadder™ 1kb, Bioline Reagents Limited, UK) for 3 µL was used to give an estimate of genomic fragment size after gel electrophoresis.

3.3.1.5 DNA sequencing

The sample of PCR product that provided the positive band on purified gel at approximately 1000 bp was selected for further determination of DNA sequencing. The original PCR product obtained from Section 3.3.1.3 was sequenced on both strands. DNA sequencing was performed to source BioScience (Cambridge, UK). The nucleotide sequences were aligned using CodonCode Aligner 5.0 (Release date March 2014, CodonCode Corporation, Massachusetts, USA). Where the results of sequences showed multiple peaks, the base was represented as N instead of nucleobase's abbreviations (A = Adenine, T = Thymine, C = Cytosine, and G = Guanine). To identify the actual position of nucleotides between samples, the alignment was inspected visually and adjusted manually.

3.3.2 Determination of seed longevity

To examine the impact of introgression of *Sub1* allele into a submergence intolerant rice cultivar on subsequent longevity, seeds of the two near-isogenic cultivars i.e. cvs IR64 and IR64 Sub1 were grown and their seed viability in storage compared after harvest. Plant culture was carried out as described in Chapter 2 by using the seed provided by IRRI. The experimental design in the current study was based on a RCBD of three blocks with 8 replicate pots of each cultivar, therefore the total number of pots was 48.

Sowing and seedling emergence dates were 2 and 8 June 2014. The pots were transferred from the glasshouse to a Saxil growth cabinet (R.K. Saxton, ARC works, Bredbury Cheshire, UK) (maximum capacity was 48 pots) when the first leaf appeared on 11 June 2014. This date was also the first day that the nutrient-drip-feed irrigation system (Chapter 2) was provided instead of tap water. The temperature inside the cabinet was maintained at 28/20 °C day/night. The photoperiod of 11 hours was obtained from cool white fluorescent tubes that provided approximately 700 µmol $m^{-2}s^{-1}$of light intensity. The concentration of carbon dioxide in the cabinets was maintained at 385 µmol mol^{-1}. Relative humidity by day and night was maintained at 60 ± 5% and 80 ± 5%, respectively. To obtain uniform plants, thinning was conducted 70 days after sowing by reducing to the four strongest plants per pot. Cultivar IR64

produced flag leaves on 81 DAS, with flowering (50% anthesis) and harvest 96 and 140 (13 October 2014) DAS; in the case of cv. IR64 Sub1 these development stages were 90, 105, and 149 (22 October 2014) DAS, respectively. Irrigation provided continuously till harvest.

Seeds were threshed by hand from panicles and combined from all plants within each cultivar within each block. Determination of moisture content of freshly-harvested seed, 1000 seed fresh and dry weight, and ability to germinate, were carried out according to procedures described in Chapter 2. The practices for seed dying, moisture content adjustment for longevity, and determination of seed viability in storage, including test conditions, were also given in the former chapter.

Experimental seed storage began on 2 December 2014 with everage seed moisture contents of 13.7±0.2% (Block 1, 2, and 3 of IR64 was 13.8, 13.5, and 13.8%, and IR64 Sub1 was 13.8, 13.9, and 13.6%, respectively). Samples were withdrawn from storage at intervals of 1-5 days 15 times: after 0, 1, 3, 7, 10, 15, 20, 22, 24, 26, 28, 31, 34, 38, or 43 days in storage. These serial results for estimate of normal germination during seed storage were fitted by probit analysis in accordance with the seed viability equation developed by Ellis and Roberts (1980a) described in Chapter 2.

3.3.3 Statistical analyses

A two samples *t*-test was performed to compare varietal differences between IR64 and IR64 Sub1 for each of yield per pot, seed weight, and moisture content at harvest. Statistical analyses were carried out using GenStat (17th edition, 2014, VSN International Ltd., UK).

3.4 Results

3.4.1 Genotyping rice lines for the *Sub1A* gene and *Sub1A-1* allele

Seedlings of *japonica* cv. Gleva and two near-isogenic *indica* cvs IR64 and IR64 Sub1 were genotyped for the *Sub1A* locus on chromosome 9, which confers submergence tolerance in rice. There were six genomic samples from the two sources of seeds examined in the present study: the seeds provided by original producers, IART for Gleva, or IRRI for IR64 and IR64 Sub1, and seeds of all three cultivars produced at PEL. The primer pair, *SUB1A_1*_fw and *SUB1A_1*_rev were used to amplify the region of the *Sub1A* submergence gene, with expected fragment size of about 1000 bp.

Positive bands with an amplicon size of approximately 1000 bp were observed in both *indica* rice cultivars (Fig. 3.1a, columns 4-7). Neither samples from the *japonica* rice cv. Gleva obtained from IART nor offspring produced at PEL produced any amplification with submergence tolerant primers (Fig. 3.1a, columns 2 and 3). PCR products were further digested by the restriction enzyme, *BseNI*, to identify the presence of the submergence tolerant *Sub1A-1* allele (Fig 3.1b). Only IR64 Sub1 showed two distinct positive bands at approximately 400 and 600 bp (Fig 3.1b, columns 6 and 7), while a single band remained at 1000bp in cv. IR64 (Fig 3.1b, columns 4 and 5). The digested genomic fragments obtained from *BseNI* of cv.IR64 Sub1 from both samples at 600 bp were comparatively thicker and brighter than the remainder at 400 bp. Moreover, some leftover PCR products that *BseNI* had not digested completely during the incubating time remained: the very light bands shows at 1000 bp (Fig 3.1b columns 6 and 7).

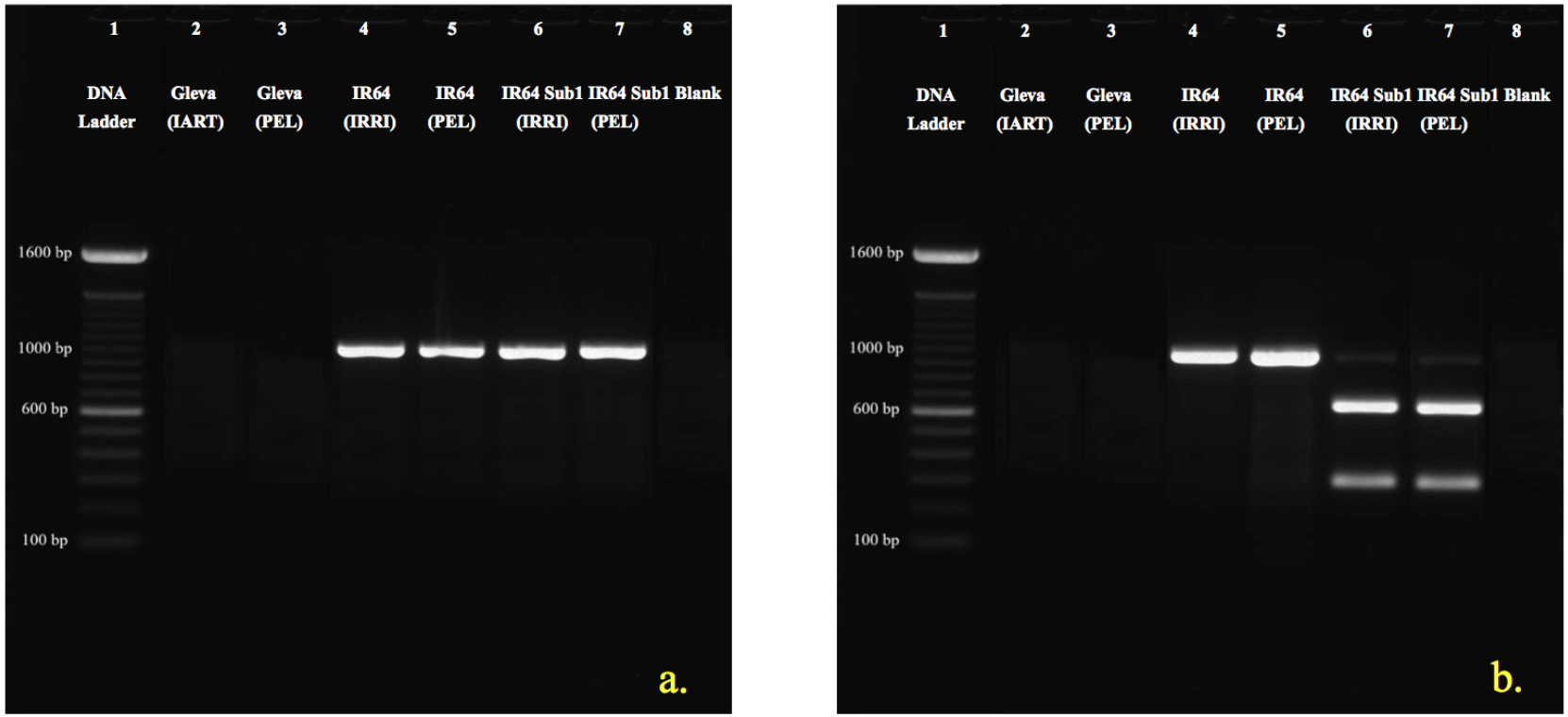


Figure 3.1 Genomic screening of *Sub1* gene in cvs Gleva, IR64, and IR64 Sub1 using the seeds provided by IART, IRRI, and the seeds that had been reproduced at PEL in 2013 (2014). PCR products amplified by using specific primers (*Sub1A_1*_fw and *Sub1A_1*_rev) to identify the submergence tolerance region on chromosome 9 (a). Allelic differences reflecting variation in submergence tolerance levels of near-isogenic genotypes were identified using restriction enzyme *BseNI* (b).

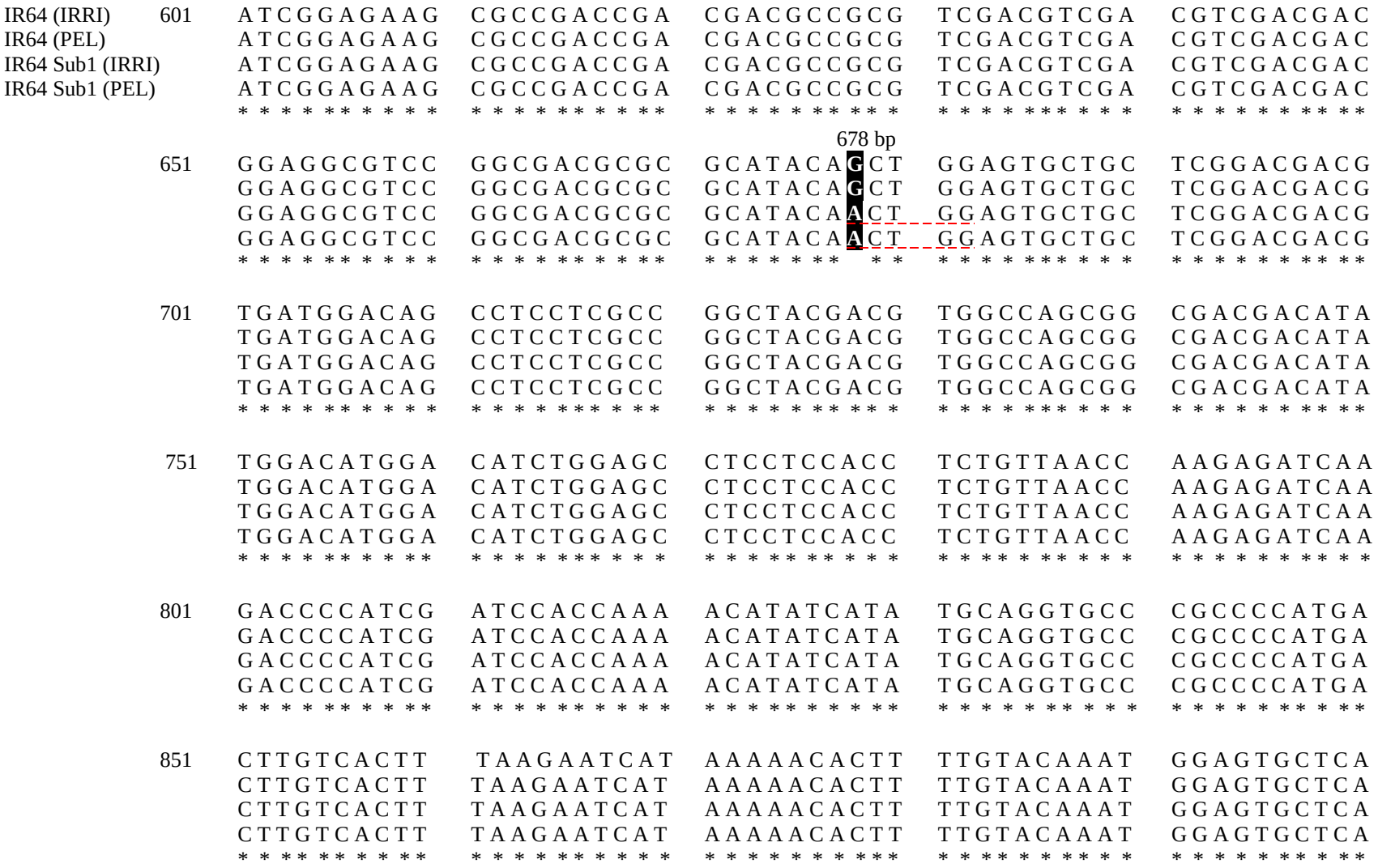
3.4.2 Differences in nucleotide alignment between the near-isogenic cultivars

Since the presence of *Sub1A* gene that confers the submergence tolerant trait was found only in *indica* rices (i.e. IR64 and IR64 Sub1, Fig. 3.1a), genomic samples of *japonica* cv. Gleva were excluded from further investigation. The differences in allelic versions responsible for differing ability to tolerate submergence between these two cultivars were verified comprehensively by DNA sequencing.

Identical DNA sequencing within each cultivar was observed from samples derived from seed provided by IRRI and that reproduced at PEL (Fig. 3.2). High similarity in nucleic acid sequence between IR64 Sub1 and its recurrent cultivar IR64 was also observed. Two nucleotide positions within the fragment showed differences in nucleotide alignment between IR64 and IR64 Sub1: at 556 and 678 bp, where there was substitution of bases from C to T and from G to A, respectively (Fig. 3.2).

```
IR64 (IRRI)          51  GCCACGCCAC  GGCGACGGCG  AGACATGGGT  TGACAGAAAG  AGGAGGAACA
IR64 (PEL)               GCCACGCCAC  GGCGACGGCG  AGACATGGGT  TGACAGAAAG  AGGAGGAACA
IR64 Sub1 (IRRI)         GCCACGCCAC  GGCGACGGCG  AGACATGGGT  TGACAGAAAG  AGGAGGAACA
IR64 Sub1 (PEL)          GCCACGCCAC  GGCGACGGCG  AGACATGGGT  TGACAGAAAG  AGGAGGAACA
                         ********** ********** ********** ******* *** ** ********** **

                     101 AGAAGAAGAG  GAAGCGCGGC  GCCGACGAAG  AATGGGAGGC  CGCCTTCCAG
                         AGAAGAAGAG  GAAGCGCGGC  GCCGACGAAG  AATGGGAGGC  CGCCTTCCAG
                         AGAAGAAGAG  GAAGCGCGGC  GCCGACGAAG  AATGGGAGGC  CGCCTTCCAG
                         AGAAGAAGAG  GAAGCGCGGC  GCCGACGAAG  AATGGGAGGC  CGCCTTCCAG
                         ********** ********** ********** * ********** ********** **

                     151 GAGTTCATGG  CTGCTGACGA  CGACGACGAC  GGCGGCGGAC  TCGTGTTAAG
                         GAGTTCATGG  CTGCTGACGA  CGACGACGAC  GGCGGCGGAC  TCGTGTTAAG
                         GAGTTCATGG  CTGCTGACGA  CGACGACGAC  GGCGGCGGAC  TCGTGTTAAG
                         GAGTTCATGG  CTGCTGACGA  CGACGACGAC  GGCGGCGGAC  TCGTGTTAAG
                         ********** ********** ********** * ********** ********** **

                     201 TAGTAAATCT  TTGGTGTTGA  GGTCACCAGG  TGAAAATGAT  GCAGGCCGGG
                         TAGTAAATCT  TTGGTGTTGA  GGTCACCAGG  TGAAAATGAT  GCAGGCCGGG
                         TAGTAAATCT  TTGGTGTTGA  GGTCACCAGG  TGAAAATGAT  GCAGGCCGGG
                         TAGTAAATCT  TTGGTGTTGA  GGTCACCAGG  TGAAAATGAT  GCAGGCCGGG
                         ********** ********** ********** ********** ********** *

                     251 GCGCCGCCGC  CACCATGTCC  ATGCCGCTGG  ACCCCGTGAC  CGAGGAGGCC
                         GCGCCGCCGC  CACCATGTCC  ATGCCGCTGG  ACCCCGTGAC  CGAGGAGGCC
                         GCGCCGCCGC  CACCATGTCC  ATGCCGCTGG  ACCCCGTGAC  CGAGGAGGCC
                         GCGCCGCCGC  CACCATGTCC  ATGCCGCTGG  ACCCCGTGAC  CGAGGAGGCC
                         ********** ********** ********** ********** ********** *
```

```
IR64 (IRRI)          301  GAGCCGGCGG  TGGCTGAGAA  GCCTCGCCGG  CGCCGGCCGA  GGCGGAGCTA
IR64 (PEL)               GAGCCGGCGG  TGGCTGAGAA  GCCTCGCCGG  CGCCGGCCGA  GGCGGAGCTA
IR64 Sub1 (IRRI)         GAGCCGGCGG  TGGCTGAGAA  GCCTCGCCGG  CGCCGGCCGA  GGCGGAGCTA
IR64 Sub1 (PEL)          GAGCCGGCGG  TGGCTGAGAA  GCCTCGCCGG  CGCCGGCCGA  GGCGGAGCTA
                         ********** ********** ********** ********** **********

                     351 CGAGTACCAC  GGCATCCGGC  AGCGGCCGTG  GGGGCGGTGG  TCGTCGGAGA
                         CGAGTACCAC  GGCATCCGGC  AGCGGCCGTG  GGGGCGGTGG  TCGTCGGAGA
                         CGAGTACCAC  GGCATCCGGC  AGCGGCCGTG  GGGGCGGTGG  TCGTCGGAGA
                         CGAGTACCAC  GGCATCCGGC  AGCGGCCGTG  GGGGCGGTGG  TCGTCGGAGA
                         ********** ********** ********** ********** **********

                     401 TCCGCGACCC  CGTCAAGGGC  GTCCGCCTCT  GGCTCGGCAC  CTTCGACACC
                         TCCGCGACCC  CGTCAAGGGC  GTCCGCCTCT  GGCTCGGCAC  CTTCGACACC
                         TCCGCGACCC  CGTCAAGGGC  GTCCGCCTCT  GGCTCGGCAC  CTTCGACACC
                         TCCGCGACCC  CGTCAAGGGC  GTCCGCCTCT  GGCTCGGCAC  CTTCGACACC
                         ********** ********** ********** ********** **********

                     451 GCCGTCGAAG  CCGCGCTCGC  CTACGACGCC  GAGGCCCGCC  GCATCCACGG
                         GCCGTCGAAG  CCGCGCTCGC  CTACGACGCC  GAGGCCCGCC  GCATCCACGG
                         GCCGTCGAAG  CCGCGCTCGC  CTACGACGCC  GAGGCCCGCC  GCATCCACGG
                         GCCGTCGAAG  CCGCGCTCGC  CTACGACGCC  GAGGCCCGCC  GCATCCACGG
                         ********** ********** ********** ********** **********

                     501 CTGGAAAGCC  CGGACAAACT  TCCCACCCGC  CGATCTTTCT  TCGCCGCCGC
                         CTGGAAAGCC  CGGACAAACT  TCCCACCCGC  CGATCTTTCT  TCGCCGCCGC
                         CTGGAAAGCC  CGGACAAACT  TCCCACCCGC  CGATCTTTCT  TCGCCGCCGC
                         CTGGAAAGCC  CGGACAAACT  TCCCACCCGC  CGATCTTTCT  TCGCCGCCGC
                         ********** ********** ********** ********** **********

                                556 bp
                     551 CGCCGCCGCA  GCCGCTCTGC  TTCTTGCTCA  ACGACAACGG  CCTCATCACA
                         CGCCGCCGCA  GCCGCTCTGC  TTCTTGCTCA  ACGACAACGG  CCTCATCACA
                         CGCCGTCGCA  GCCGCTCTGC  TTCTTGCTCA  ACGACAACGG  CCTCATCACA
                         CGCCGTCGCA  GCCGCTCTGC  TTCTTGCTCA  ACGACAACGG  CCTCATCACA
                         *****.**** ********** ********** ********** **********
```


IR64 (IRRI)
IR64 (PEL)
IR64 Sub1 (IRRI)
IR64 Sub1 (PEL)
678 bp

```
IR64 (IRRI)       901   A C C A T G C T A A   A C T T A C T C A A   A G G C C A C A A A   C A A T A A T A T A   A T T C T T T T T C
IR64 (PEL)              A C C A T G C T A A   A C T T A C T C A A   A G G C C A C A A A   C A A T A A T A T A   A T T C T T T T T C
IR64 Sub1 (IRRI)        A C C A T G C T A A   A C T T A C T C A A   A G G C C A C A A A   C A A T A A T A T A   A T T C T T T T T C
IR64 Sub1 (PEL)         A C C A T G C T A A   A C T T A C T C A A   A G G C C A C A A A   C A A T A A T A T A   A T T C T T T T T C
                        * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *
```

Figure 3.2 Nucleotide alignment of *Sub1* gene in the pair of near-isogenic cultivars, IR64 and IR64 Sub1, using the seeds provided by IRRI and the seeds produced at PEL. The results were obtained from contiguous assembly of PCR products from the primers in forward (*Sub1A_1_fw*) and reverse (*Sub1A_1_rev*) directions. The transcript sequences of nucleotide bases are indicated by abbreviations of A = Adenine, T = Thymine, C = Cytosine, and G = Guanine (Appendix 3.1). The asterisks indicate similarity in nucleotide base at the same position, whereas differences in genomic sequence are indicated by highlight. The recognition site of the restricted enzyme, *BseNI*, on amplicon at 678-682 bp is marked with a dashed underline.

3.4.3 Differences in seed yield between pair of near-isogenic cultivars

Because of differences in flowering time and other developmental periods, mature seeds of IR64 and IR64 Sub1 were harvested at 44 DAA with different harvest-moisture contents of 30.9 and 29.6%, respectively (Table 3.2, $P<0.001$, Appendix 3.2). IR64 Sub1 had significantly lighter 1000 seed fresh and dry (0% moisture content) weights than IR64 (27.9 and 30.5 g for fresh weight, and 19.7 and 21.0 g for dry weight) (Table 3.2, $P<0.01$, Appendix 3.2). On the other hand, grain yield (dry matter) of IR64 Sub1showed significantly greater than that obtained from IR64 ($P = 0.011$, Appendix 3.2).

Table 3.2 Comparison of seed yield and weight of IR64 and IR64 Sub1 produced in growth cabinet at PEL in 2014

Parameter	IR64		IR64 Sub1		Probability[4]
	Mean	s.e. (n=3)	Mean	s.e. (n=3)	
Moisture content at harvest (%)[1]	30.9	0.13	29.6	0.15	<0.001
1000 Fresh seed weight (g)[2]	30.5	0.25	27.9	0.03	0.009
1000 Dry seed weight (g)[2,3]	21.0	0.19	19.7	0.06	0.002
Yield (g pot^{-1})[2, 3]	102.2	9.24	146.4	3.55	0.011

[1] The mean was taken from six replicates (two per block)

[2] The mean was taken from three replicates (one per block)

[3] at 0% moisture content

[4] Differences of means analysed using two samples *t*-test, see Appendix 3.2

3.4.4 Ability to germinate and longevity

3.4.4.1 Germinability of fresh and dried seed

In the present study, mature freshly-harvested seed of IR64 showed lower ability to germinate than IR64 Sub1, which did not contradict with the results observed in 2013 (Fig. 3.3). After drying to equilibrium with the ambient environment of the laboratory, both cultivars produced in 2014 showed near identical ability to germinate normally, and hence

considered dormant (Fig. 3.3b). These seeds of IR64 and IR64 Sub1, also remained dormant during initial seed storage (see later in Section 3.4.3.2). This contrasted with the limited effect of drying in 2013 (Fig. 3.3a): in fact there was some loss in dormancy in both cultivars with drying in 2013, but with similar estimates for both cultivars for ability to geminate after drying.

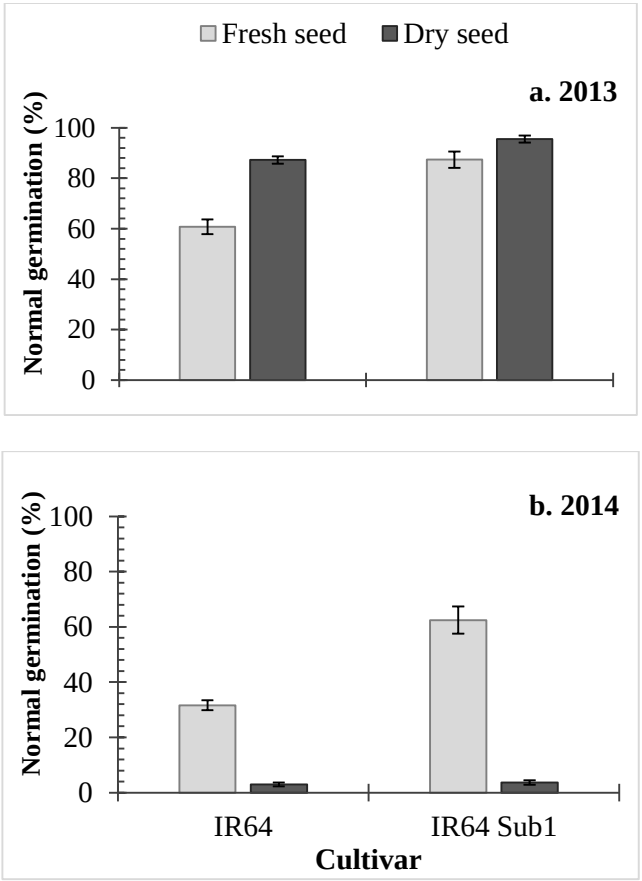


Figure 3.3 Ability to germinate normally of mature freshly-harvested (■) and dry seed (13-15% moisture content) (■) of IR64 and IR64 Sub1 observed in 2013 (a) and 2014 (b). The vertical bars represent ± s.e. (n=3). The results for 2013 are repeated from Section 2.3.4.3, Chapter 2.

3.4.4.2 Longevity

Considerable loss of seed dormancy was observed in both cultivars during the first seven days of storage (Fig. 3.4a). Thereafter, the two patterns of loss in seed viability followed the negative cumulative normal distribution (Fig. 3.4b) explained by Ellis and Roberts (1980a). Therefore, the multiplicative model combining loss in dormancy and viability recommended by Whitehouse *et al.* (2015) was applied to quantify the responses observed:

$$g = (K_d + \beta_1 p) \times (K_i - (p / \sigma))$$

where g is ability to germinate normally, converted to normal equivalent deviates (NED; same as probits where 50% equals zero), K_d is initial germination percentage (non-dormant seed) in NED units, β_1 is loss in dormancy (NED day^{-1}), while K_i, p and σ are the values as defined in the seed survival equation 1.4 in Chapter 1. The equation used by Whitehouse *et al.* (2015) was developed from the models originally introduced by Kebreab and Murdoch (1999). This combined probit analysis procedure was performed using the FITNONLINEAR directive in GenStat (17th edition, 2014, VSN International Ltd., UK).

During the first seven days of storage at 40°C with 13.7±0.2% moisture content, the rate of loss of dormancy in IR64 was similar to that of IR64 Sub1, in which the latter showed a little lower dormancy (i.e. higher germination ability, Fig. 3.4a). Thereafter, both cultivars had similar seed viability and germinability until 22 days in storage (Fig. 3.4b). The germination ability of IR64 Sub1 then declined slightly more rapidly, reflected by shorter estimated p_{50} of 26.4 days, compared to IR64 (28.1 days) (Fig 3.4c). The small variations of seed survival curves between blocks within pair of this near-isogenic cultivars are shown in Appendix 3.3, in which within-cultivar variation is of a similar magnitude to that between cultivars (Fig. 3.4c).

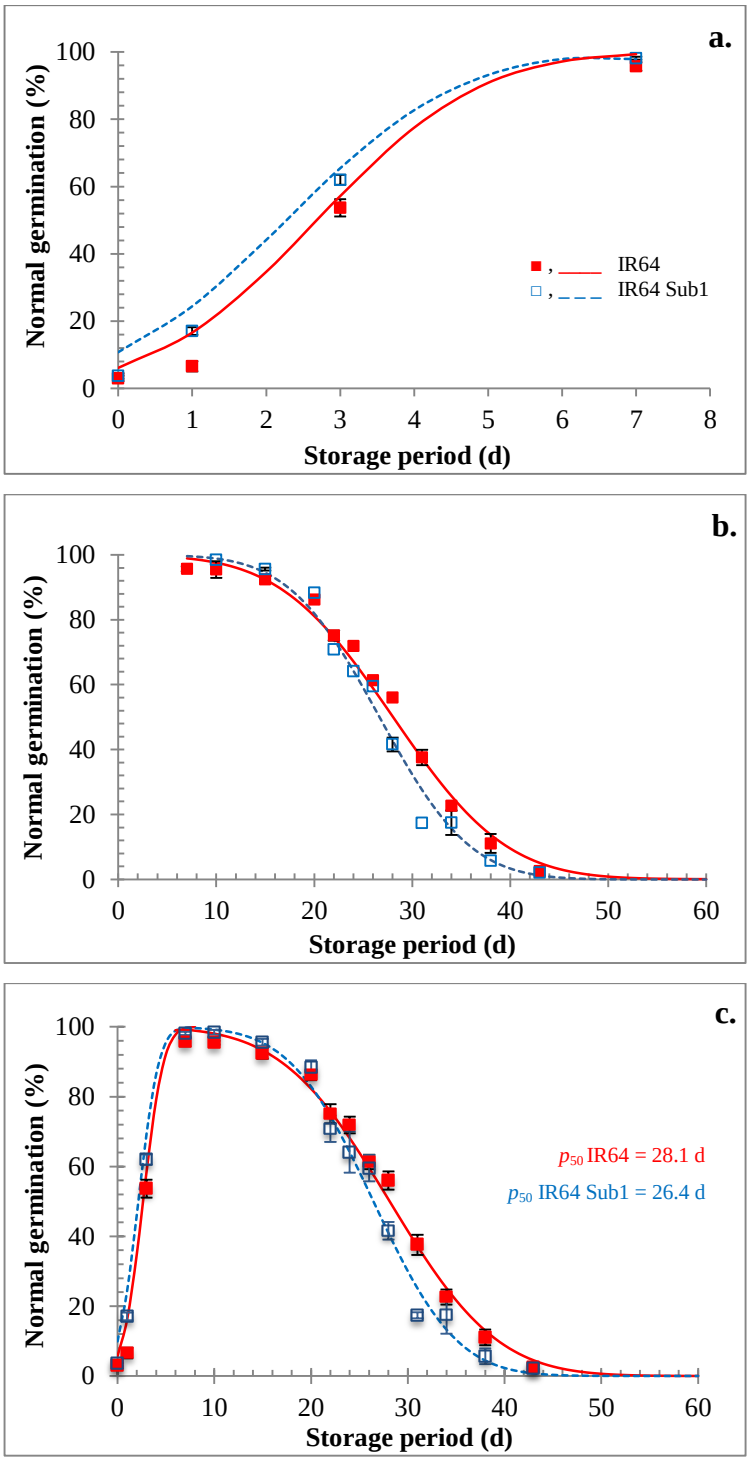


Figure 3.4 Loss of seed dormancy during the first seven days in storage (% normal germination plotted against period in hermetic storage at 40 °C with seed of 13.7 ± 0.2% moisture content) (a) and subsequent seed survival curves (b) of *indica* rice cvs IR64 (■) and IR64 Sub1 (□) (2014). The probit model combining loss in dormancy comprises a common slope for loss in dormancy with different slopes for loss in viability (c) (Appendix 3.5). These fitted models are quantified in Table 3.3. Observations are means of germination percentage ± s.e. (n=9, three replicates each from three blocks).

Table 3.3 Parameters of a probit model combining loss in dormancy with loss in viability of *indica* rice cvs IR64 and IR64 Sub1 harvested at harvest maturity in subsequent hermetic storage at 40 °C with 13.7 ± 0.2 % moisture content (2014). The 95 % confidence intervals are shown for p_{50} where applicable.

Cultivar	**K_d**		**β_1**		**K_i**		**Slope (1/σ)**		**σ (days)**	**p_{50} (days)**			
	Estimate	**s.e.**	**Estimate**	**s.e.**	**Estimate**	**s.e.**	**Estimate**	**s.e.**	**Estimate**	**Estimate**	**s.e.**	**lower 95%**	**upper 95%**
Loss of dormancy during the first 7 days of storage (Model: Kebreab and Murdoch, 1999): Fig. 3.4a													
IR64	-1.78	0.047	0.545	0.0155									
IR64 Sub1	-1.55	0.045	0.575	0.0181									
Loss of seed viability after 7 days of storage (Model: Ellis and Roberts, 1980a): Fig. 3.4b													
IR64					3.08	0.069	-0.110	0.0025	9.1	28.0	0.18	27.7	28.4
IR64 Sub1					3.66	0.080	-0.137	0.0030	7.3	26.7	0.14	26.4	26.9
Combining of loss in dormancy with loss in viability (Model: Whitehouse *et al*., 2015): Fig. 3.4c													
IR64	-1.29	0.129	0.599	0.0423	3.21	0.276	-0.114	0.0102	8.8	28.1			
IR64 Sub1	-1.58	0.120	0.599	0.0423	3.90	0.216	-0.147	0.0008	6.8	26.4			

The model combining loss in dormancy with loss in viability could not be constrained to a common line for both cultivars [F (4, 84), P<0.001)] nor a common slope for loss in seed viability [F (2, 80), P=0.008)] (Appendix 3.5). However, loss in dormancy (β_1) did not differ amongst the cultivars [F (1, 80), P=1.000), Appendix 3.5]. Hence, different fitted lines are shown for the two cultivars, where IR64 Sub1 showed slightly lower dormancy initially and slightly more rapid loss in viability than IR64 subsequently, but the slope for loss in dormancy is the same (Fig. 3.4c)

3.5 Discussion

In the previous work of Xu *et al.* (2006), submergence-tolerant-associated gene was identified and high-resolution mapped using the recombinant rice (DX 202) of 4,022 derived from a cross between tolerant *indica* FR13 and intolerant *japonica* M-202. The former authors proposed that the enhancement of the submergence-tolerant character in the *Sub1*-transgenic *japonica* rice was due to up-regulation of the gene encoding alcohol dehydrogenase, the vital catabolic enzyme in anaerobic metabolism. The presence, absence, or difference in allele of *Sub1A* on Chromosome 9 was linked to ability to tolerate submergence in rice. According to allelic survey in contrasting rice genotypes carried out by Xu *et al.* (2006), the submergence-tolerant phenotype (i.e. possessed *Sub1A-1* allele) was found only in *indica* rice (cvs. FR13, IR40931-26, DX18-121, IR48930, Goda Heenati, and Kurkaruppan). On the other hand, the intolerant attribute was observed in both *indica* (i.e. lack of *Sub1A*; cvs IR24, IRBB21, Swarna, IR50, and Habiganj aman, or contained *Sub1A-2* allele; cvs LMNIII, Teqing, CO39, IR64, IR64-M6D6-933-1-2, and 93-11) and *japonica* rice (i.e. lack of *Sub1A* allele; cvs Nipponbare, Liaogeng, M-202, and Taipei 309).

The impact of the introgression of the *Sub1A* gene into high-yielding rice variety (i.e. IR64) on seed weight, yield, and subsequent ability to germinate and survive in storage was studied. The presence of the introgressed *Sub1A* gene was determined using primers designed by Xu *et al.* (2006). Thereafter, the identification of *Sub1A-1* allele was carried out using the restriction enzyme (*BseNI*) recommended by Niroula *et al.* (2012). The results from screening of *Sub1A* gene in the present study were in agreement with Xu *et al.* (2006): the submergence-tolerance related genes were absent in the *japonica* rice cv. Gleva, but were present in the two *indica* cultivars (Fig. 3.1a).

There were two differences in a single nucleotide polymorphism (SNP) at 556 and 678 bp where bases in IR64 and IR64 Sub1 were replaced from C →T and G → A, respectively (Fig. 3.2). These findings confirm the conclusion for the effect of allelic

differences on susceptibility to submergence of rice reported by Xu *et al.* (2006). The substitution of nucleotide base at 556 bp leads to misencoding of protein from proline (intolerant) to serine (tolerant), but the second point of replacement (678 bp) results in no change in protein translation (Xu *et al*., 2006). The confirmation from DNA sequencing of the present study supports the efficiency of *BseNI* enzyme (Fig. 3.1b) as suggested by Niroula *et al.* (2012): this enzyme has a unique recognition site, which match with the area of the SNP at the location of synonymous substitution. Hence, *BseNI* can be used successively as an identifying tool for rapid screening approach in submergence tolerant rice studies. Gel electrophoresis (Fig 3.1), together with nucleic acid sequencing (Fig 3.2) revealed identical results within each cultivar, even though the plant materials were from different generations (i.e. original and new seed reproduced from the stock provided by IRRI). These observations agree with the previous reports of Xu *et al.* (2006) and Niroula *et al.* (2012): after introgression, the *Sub1* gene was successfully harboured on allele *Sub1A-1* in IR64 Sub1, and this locus was heritable to offspring.

In the present study, substantial dormancy was observed in both cultivars at harvest (Fig. 3.3b) and at the beginning of storage, whereas it was largely absent in 2013 once seeds were dried (Fig. 3.4a). In *indica* subspecies, strong dormancy was observed in rice provided in rainy weather (Sircar, 1963, as cited in Takahashi, 1984). Moreover, rice responded to high temperature and relative humidity during seed maturation by increasing seed dormancy (Takahashi, 1975 as cited in Takahashi, 1984). The occurrence of rainfall close to harvest also affects dormancy levels; rice seed harvested in high-humidity weather showed subsequently higher seed dormancy after harvest compared to harvests in a warm and dry area (Araullo *et al.*, 1976; Dev, 1981; Halimathul Saadiah, 1992). This suggests that the differences in dormancy of rice between the years may have been due to the effect of different growing conditions (2013, glasshouse; 2014, growth cabinet) on postharvest dormancy. Whilst temperatures were similar, relative humidity and irradiance were not controlled in the glasshouse.

Furthermore, there were differences in the pre-storage period before the determination of longevity began; 150 and 50 days after harvest in 2013 and 2014, respectively. Crocker (1919) stated that rice seeds may remain dormant after harvest, and a drying period is required for these seeds to release germination ability. Moreover, the pattern and duration of after-ripening vary depending upon rice species (Veasey *et al.*, 2004). Thus, the shorter period of "after-ripening" of rice in 2014 may also explain the greater dormancy detected during initial storage. In the current study, dormancy was released after about seven days in hermetic storage at 40 °C with 13.7 ± 0.2 % moisture content. This is in

approximately agreement with the practical recommendation of Roberts (1962): sun dry the seed to 11% moisture content, then incubate at 47 °C for seven days to break dormancy of rice seed.

There are several reports which confirm the benefits of introgression of *Sub1* into submergence-intolerant rice cultivars, Ideta *et al.* (1995), Neeraja *et al.* (2007), and Shao *et al.* (2012) however found side effects of *Sub1* introgression: mature seed of Swarna and TDK1, have golden yellow hulls, which are straw-coloured in the *Sub1* introgressed varieties. This difference, however, is of little importance compared to the benefits. Introgression of *Sub1* gene into domesticated rice varieties can confer submergence tolerance for up to 17 days depending on genetic background of the original variety and flooding conditions (Ram *et al.,* 2002; Das *et al.,* 2009). To date, furthermore, there is no report of an adverse impact on crop productivity and grain quality due to introgression of *Sub1* gene under flooding environment as well as normal (i.e. non-submergence) conditions (Siangliw *et al.,* 2003; Xu *et al*., 2006; Neeraja *et al.,* 2007; Sarkar *et al.,* 2009; Singh *et al.,*2009). Siangliw *et al.* (2003), moreover supported the advantages of this introgressive hybridization: introgression of *Sub1* into cv. KDML 105, Thai jasmine variety (intolerant), improved submergence tolerance with no negative impacts on subsequent cooking quality. In their study, the recurrent mother cultivar completely died after flooding, whereas the four weeks old introgressed plants survived 8 days of submergence, and were able to retain agronomical traits without deleterious effect on aroma and flavour of the original variety. Comparison of grain productivity between near-isogenic cultivars of IR64 and IR64 Sub1 with no submergence treatment in the present investigation showed similar results to those previously published of Singh *et al.* (2009) and Lang *et al.* (2010). A significant increase (P=0.011) of 48 % in yield pot^{-1} of IR64 Sub1 compared with the original cultivar was detected. However, this pot-grown plant study in a growth cabinet above is not a suitable design to make any conclusion about grain yield. It is also noted that seed weight of IR64 Sub1 was significantly lighter than IR64 (P<0.01). The differences were small, however: an 8 and 6 % reduction in weight of 1000 fresh and dry seeds, respectively (Table 3.2).

A small impact of only Control (non-submergence) results for the two cultivars on seed longevity was reported in Chapter 2 (Sections 2.4.1.4 and 2.4.3.4). The present study, therefore, validated and reconfirmed the effect of introgression *Sub1* gene on subsequent seed storability of a pair of near-isogenic rice cultivars. Although dormancy was detected in both cultivars during initial storage (Fig. 3.4a), these seed lots thereafter lost its viability providing negative cumulative normal distribution of seed death in time (Fig. 3.4b), which is in agreement with Ellis and Roberts (1980a) and Whitehouse *et al.* (2015). There were

significant but small differences in the seed survival curves of IR64 and IR64 Sub1, with the same rate of loss of dormancy (Fig. 3.4c, Appendix 3.5). In 2014, slightly longer p_{50} detected in IR64 with estimates of p_{50} of 28.1 day, and 26.4 days for IR64 Sub1 (Fig. 3.4c), whereas in 2013, cv. IR64 Sub1 showed marginal greater longevity in the control treatments (Fig. 2.20). Besides variations between the two years, the results here for seed longevity (p_{50}) reconfirm that the introgression of the *Sub1* gene does not reduce this aspect of seed storability. Thus, the introgression of *Sub1* gene did not affect seed survival in storage.

Over the last two decades, the molecular basis of the genetic control of many aspects of plant genotype responses to agronomic and economic traits has been elucidated. Harrison *et al.* (1987) proposed that the successful practical implication of introgression may reduce or reinforce, deduced by genetic linkage between selected loci and neighbouring regions. In rice, there were reports of genetic linkage between *Sub1* locus and adjacent loci on chromosome 9 (Ideta *et al.*, 1995: Neeraja *et al.*, 2007; Shao *et al.*, 2012). In fact, QTLs related to seed germinability and viability also located on chromosome 9, meant that it was important to consider potential effect of introgression of the *Sub1* gene on seed storability. The results of the current study showed comparatively small differences of longevity (p_{50}) between these near-isogenic cultivars (i.e. about 1.5 days, Fig 3.4c). Giving that the effect of storage environment in exponential with longevity varying from minutes to thousands of years (Ellis and Roberts, 1980a). The above effect is negligible and within experiment error.

In conclusion, the *Sub1* introgression variety in the IR64 background, has no negative effect on grain yield, seed weight, or subsequent seed storability.

Chapter 4

The Effect of Foliar Applications of Molybdenum (Mo) on Pre-harvest sprouting of *japonica* Rice under Submergence Conditions

4.1 Introduction

Pre-harvest sprouting is primarily influenced by the genotype and the environment experienced by the mother plant (Bewley and Black, 1994; Taiz and Zeiger, 2006; Sugimoto *et al.,* 2010; Bewley *et al.,* 2013). In rice, considerable yield losses due to sprouted grain were reported in developing seeds subjected to prolonged spells of rain close to harvest time (Wan *et al.,* 2006). In southern China, more than 6% of the rice area was subjected to pre-harvest sprouting in the case when wet conditions occurred during maturation (Guo *et al.,* 2004). This problem is more severe in modern rice varieties because they are likely to be more susceptible to pre-harvest sprouting. For example, one fifth of the rice-growing area in south China (i.e. Sichuan and Yangtze River Valley) has shown damage due to pre-harvest sprouting when modern hybrid rices were selected for cultivation (Hu *et al.*, 2003 as cited in Guo *et al.,* 2004), which led to 10-50% yield losses (Tao *et al.,* 2007; Gao *et al.,* 2008). Fan *et al.* (2007) proposed that the sensitivity to pre-harvest sprouting of modern rice varieties was due to the missplicing of *Rice Viviparous 1* (*OSVp1*), the gene associated to seed maturation and seed dormancy. The risk of pre-harvest sprouting in cereal crops exposed to variable rainfall during later stages of grain development is not limited to China. Economic loss due to the former problem is a global concern, affecting cereal production in Japan, Europe, Canada, United States of America, Iran, South Africa, Kenya, Australia, and New Zealand (Tavakkol-Afshari, 2006; Ogbonnaya *et al.*, 2007; Zhang *et al.,* 2014).

Since the occurrence of pre-harvest sprouting is controlled by genetic and environmental factors, i.e. rainfall and warm field temperature, the estimation of yield losses is erratic and varies year by year (Mahbub *et al.,* 2005). Adequate seed dormancy during grain maturation is one of the most important factors which prohibits pre-harvest sprouting responsiveness under wet and warm weather close to harvest time. This resistant character in cereals is associated with the greater ABA content in developing seed than that of sensitive cultivars in maize (Hole, *et al.,* 1989), barley (Gubler *et al.,* 2008; Howard *et al.,* 2012), wheat (Walker-Simmons, 1987; Yang *et al*., 2007; Schramm *et al*., 2010; Keiko *et al*., 2015), and sorghum (Benech-Arnold *et al.,* 1991). In rice, some varieties (e.g. cvs IR24, N22, Fuhui838, and Lemont) have been developed with high resistance to pre-germination

that farmers can utilize to avoid losses from pre-harvest sprouting grain (Dong *et al.*, 2003; Fan *et al.*, 2007; Durantini *et al.*, 2008; Sugimoto *et al.*, 2010; Liu *et al.*, 2014). However, these cultivars provided lower benefit in terms of agronomic and economic performance compared with sensitive high-yielding domestic rice varieties (e.g. cvs Mahsuri, Asominori, G46A, and HeiB). Genetic resistance to pre-harvest sprouting can be manipulated by breeding, and research in rice is now underway to elucidate and fine-tune the major quantitative trait loci (QTL) associated with pre-harvest sprouting resistance (Lin *et al.*, 1998; Dong *et al.*, 2003; Gu *et al.*, 2004; Gao *et al.*, 2008; Hori *et al.*, 2010; Sasaki *et al.*, 2013). This approach may take considerable time to introgress the genes responsible for resistance to pre-harvest sprouting into commercial rice varieties and evaluate them under field conditions. Therefore, under poor seed production environments, such as unpredictable rainfall or flash-floods during seed maturation, providing a chemical treatment to alleviate pre-harvest sprouting may be an appropriate solution in the current context.

Several reports have shown applications of molybdenum (Mo) can contribute to greater crop performance and enhanced seed dormancy. Corresponding yield and yield component improvements were found in Mo-treated crops, for example brassica vegetables (Hewitt and Bolle-Jones, 1952; Fido *et al.*, 1977), cereals (Agarwala *et al.*, 1978; Chatterjee *et al.*, 1985; Yaneva *et al.*, 1996; Chatterjee and Nautiyal, 2001; Bala and Hossain, 2008), legumes (Anderson, 1956; Farooq *et al.*, 2012) and grapevines (Mullins *et al.*, 2000; Williams *et al.*, 2004) in both normal and Mo-deficient soils. In terms of seed quality, supply of exogenous Mo was likely to have played a role in inducing dormancy. The observation that Mo is involved in controlling seed dormancy and vivipary was first reported in maize (Tanner, 1978). According to the studies of Walker-Simmons (1989) and Modi and Cairns (1995), spraying Mo at flag leaf stage could induce a wheat embryo to produce more endogenous ABA, with ABA increasing parallel with Mo spray doses (0-600 mg L^{-1} supplied in sodium molybdate). These authors also found that the inhibition of germination in Mo-treated seed increased with Mo spray rates. Thus, under poor seed production environments, such as unpredictable rainfall or flooding that may occur during seed maturation, foliar application of Mo before grain-filling stage may both increase plant growth and alleviate the loss of seed quality caused by seed sprouting pre-harvest.

Improving crop yields after Mo supply may result from greater NR activity and/or abundance (Kaiser *et al.*, 2005). The increased activity of NR may enhance nitrate reduction, in which nitrate is catalysed into nitrite, then nitrite into nitric oxide, respectively. The former biochemical processes are essential for the biosynthesis of amino acids, necessary for subsequent plant metabolism and development (Yamasaki *et al.*, 1999; Rockel *et al.*, 2002; Alboresi *et al.*, 2005). In terms of inducing seed dormancy after Mo application, Mo is

involved in the ABA biosynthesis pathway. This relationship was investigated in different ABA mutants of *Arabidopsis*, tobacco, tomato, maize, barley, and rice (Walker-Simmons *et al.*, 1989; Leydecker *et al.*, 1995; Bittner *et al.*, 2001; Sagi *et al.*, 2002; Singh *et al.*, 2003; Porch *et al.*, 2006; Fang and Chu, 2008). In maize and rice, viviparous or pre-harvest sprouting mutants were associated with; 1) induction and maintenance of dormancy, and 2) contained mutations in enzymes involved in the ABA biosynthesis pathway, which have been classified into three classes (Fig. 4.1); Class I mutants that affect the early carotenoid biosynthesis pathway, however, there was negligible effect on ABA synthesis in these mutants, Class II mutants participated in the precursors of ABA biosynthesis, and Class III mutants were involved in the synthesis of Moco, which is required to complete the final step of ABA biosynthesis (McCarty, 1995; Singh *et al.*, 2003; Porch *et al.*, 2006; Suzuki *et al.*, 2006; Fan and Chu, 2008). Moco combines with ABA aldehyde oxidase (AAO), the enzyme for the oxidation process that converts abscisic aldehyde into ABA (Mendel and Hänsch, 2012; Porch *et al.*, 2006).

Bewley and Black (1994), Alboresi *et al*. (2005), and Arc *et al*. (2013a, b) remarked that although nitrogen compounds were not involved in ABA synthesis directly, molecules of nitrate and nitric oxide may act as signalling substances for maturation and maintaining of dormancy. The high concentration of nitrates or low nitric oxide (i.e. presumably because of low NR activity) in the mother plant, as well as developing seed, may alter the balance of ABA/GA biosynthesis, and hence dormancy release. Therefore, in this study the fundamental objective was to investigate the possible role of Mo in terms of ABA inducible dormancy in non-dormant rice, i.e. cv. Gleva. The present experiment has not been designed to investigate the interactions between nitrogen assimilation and ABA biosynthesis as a result of Mo foliar applications, nevertheless the possible role of Moco in these two pathways on seed dormancy was discussed.

4.2 Null hypotheses

4.2.1 Foliar applications of Mo or ABA have no effect on preventing pre-harvest sprouting after simulated flooding treatments.

4.2.2 Different concentrations of Mo or ABA have no effect on subsequent pre-harvest sprouting damage (i.e. seed yield per plant, 1000 seed dry weight, and sprouted seed) after simulated flooding treatments.

4.2.3 Applying Mo at flag leaf stage has no effect on Mo concentration in mature seed at harvest.

4.2.4 Applying Mo at flag leaf stage has no effect on seed ABA concentrations.

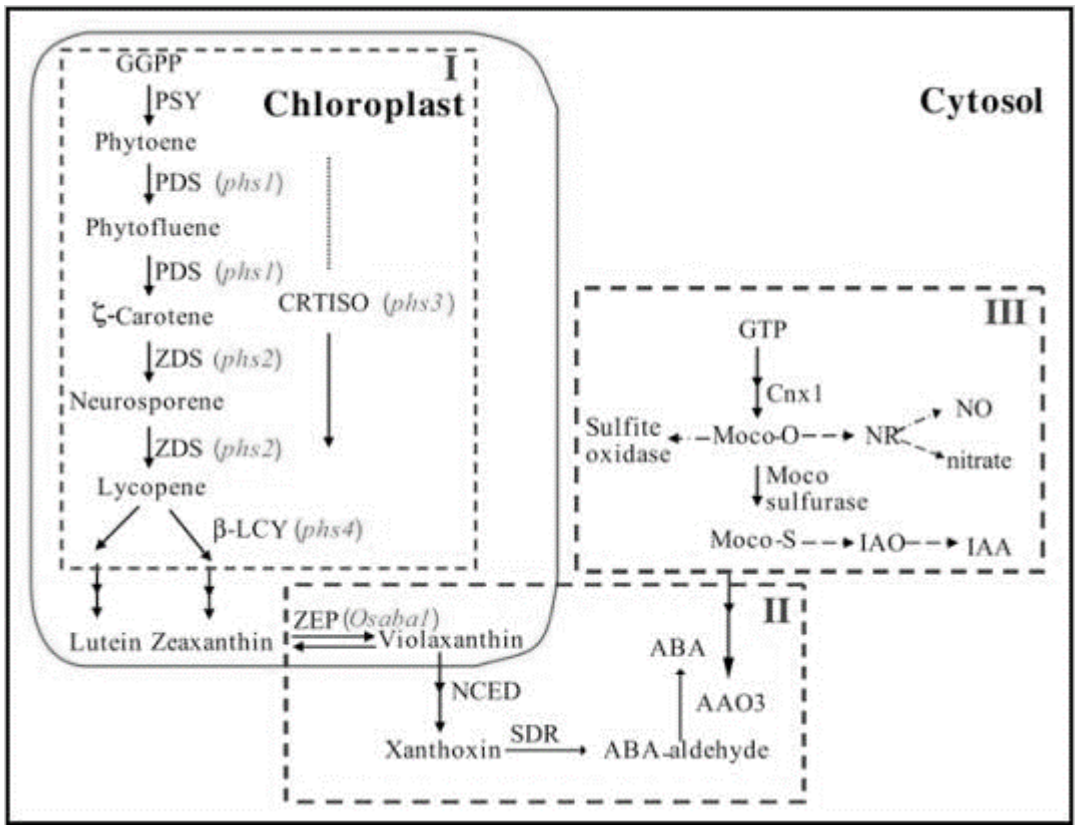


Figure 4.1 *Pre-harvest sprouting* (*phs*) rice mutants in ABA production and their associations in ABA biosynthesis pathway (from Fan and Chu, 2008). The mutants with deficient genes participated in ABA synthesis in three steps: Class I, early reaction of carotenoid synthesis, in which the products thereafter were ABA-initial precursors, Class II, the final step of biosynthesis, for which a molybdenum-containing aldehyde oxydase (AAO) is needed, and Class III, the Mo-cofactor synthesis pathway, which is required for the former conversion step. The abbreviations stand for; GGPP, Geranylgeranyl pyrophosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, z-carotene desaturase; β-LCY, lycopene β-cyclase; ZEP, zeaxanthin epoxidase; NCED, 9-cis-epoxycarotenoid dioxygenase; SDR, short-chain dehydrogenase/reductase; AAO3, Abscisic aldehyde oxidase 3; IAO, Indole-3-acetaldehyde oxidase; NR, Nitrate reductase; Cnx1, cofactor for nitrate reductase and xanthine dehydrogenase 1.

4.3 Materials and methods

4.3.1 Plant culture

The study was carried out in 2013-2014 and followed the procedures for pot-grown-plant culture described in Chapter 2, Section 2.3.1. Due to the occurrence of pre-harvest sprouting (6-67%) and yield loss in simulated flooding treatments of previous experiments (Figs. 2.4 and 2.16, Chapter 2), *japonica* rice cv. Gleva was selected for the current experiment.

Seeds were sown on 3 December 2013. Seedling emergence for the majority of seed occurred nine days thereafter. Pots of plants were transferred from glasshouse to Saxil growth cabinets 10 DAS. The growing conditions as well as irrigation supply were those described previously in Chapter 3 (Section 3.3.2). To ensure that all plants in this experiment had uniform growth, thinning from seven to four plants per pot was conducted when plants produced their 6^{th} leaf at 36 DAS (8 January 2014) by cutting all the plant's parts above ground level. Flowering date (day of anthesis) of the main tiller of all plants in this experiment was recorded, using criteria described in Chapter 2, Section 2.3.1. The date that half of the population of main tillers reached anthesis was 11 Mar 2014 (98 DAS), which is referred to as the zero day of anthesis for this experiment.

4.3.2 Foliar applications and simulated flooding treatments

4.3.2.1 Foliar sprays

Five foliar spray treatments were applied to plants when they produced fully-open flag leaf at 84 DAS on 25 February 2014; Mo (at 100, 600, and 3,000 mg L^{-1}), ABA at 50 μM, or deionised water (DI water), as the negative control and referred to as Mo 0 mg L^{-1}). Three growth cabinets (maximum capacity was 48 pots/cabinet) were used and represented three blocks of the experiment. In each growth cabinet, there were eight pots per foliar treatment, thus 40 pots in total. A further eight pots were included in each cabinet for observation (without either foliar spray, or submergence), and this group of plants was not used in statistical analyses. Therefore, the total pot number of this experiment was 144. To determine flag leaf stage, 48 plants from 12 pots (4 plants/pot) were selected randomly from each growth cabinet (i.e. 144 plants in the three blocks) to observe and record regularly about the number of leaves produced during the vegetative phase.

Mo solutions were prepared the same day just before foliar treatments. Mo was supplied as Sodium Molybdate (VI) Dihydrate (99+%, $Na_2MoO_4 \cdot 2H_2O$, molecular weight

241.95, Acros Organics, New Jersey, USA). In each foliar treatment, there were 24 pots (18 cm in diameter) in total from three blocks, contributing approximately 1 m^2 (0.18 x 0.18 x 24 pots = 0.776 m^2) of spray area. The spray rate was 200 L ha^{-1}, therefore the application rate of each Mo concentration was equal 20 mL m^{-2}, and hence 0.002, 0.012, and 0.060 g of $Na_2MoO_4 \cdot 2H_2O$ was required to prepare Mo at 100, 600, and 3000 mg L^{-1}, respectively. The $Na_2MoO_4 \cdot 2H_2O$ in each solution was firstly dissolved in 50 mL of DI water, then the solution was transferred to 500 mL volumetric flask to adjust the final volume to 500 mL by the addition of DI water. For the preparation of 50 μM of ABA in 500 mL, 0.00607 g of ABA (grade ≥ 98%, Sigma Aldrich, UK) was weighed and dissolved in 5 mL of 1N NaOH. The dissolved solution was poured into a 500 mL volumetric cylinder where DI water was added to 500 mL. This prepared solution was kept in the glass bottle and stored at -20 °C until use (at the same day of preparation), however it was left for 1 hour under room temperature before spray.

To provide greater contact of the spray treatments with the plant parts, 5 mL of Tween 20 (PBS Tween-20, Thermo Scientific, UK) surfactant, which helps to reduce surface tension between droplets, was added and mixed to each 500 mL prepared-solution. The foliar solution was applied outside the growth cabinets. Pressure sprayer (2 L Pressure Sprayer, Cocraft Clas Ohlson, UK) was used to spray a solution throughout the upper parts. The pots were left to dry for 2 hours, after that they were returned to their previous position in the growth cabinets until the submergence treatment began.

4.3.2.2 Submergence treatments

Simulated flooding treatments for four days was imposed on 20 (31 Mar 2014) or 30 DAA (11 April 2014), with four pots of each foliar application from each cabinet at each time. All pots from growth cabinets were moved to the glasshouse four days before the first submergence treatment began on 27 Mar 2014. Pots from each growth cabinet were placed together in a trolley (trolley numbers 2, 3 and 4 were used, Appendix 2.1), which represented blocking according to the experimental design. The controlled environments of glasshouse and simulated flooding conditions were as described in Chapter 2, Section 2.3.2.

4.3.2.3 Experimental design

The current study consisted of five foliar treatments applied with two timings of simulated flooding, corresponding to ten treatment combinations (Table 4.1). The experimental design was based on RCBD with three blocks (using three growth cabinets, and three trollies) of

four pots as replicates. Thus, the number of pots for all treatment combinations was 120, plus the extra 24 pots (8 pots per growth cabinet), in which the latter were grown under normal growing conditions without foliar spray or submergence for observation purposes.

Table 4.1 Details of foliar application of Mo or ABA on preventing pre-harvest sprouting of *japonica* cv. Gleva during 2013 to 2014

Four days' submergence	Foliar treatment
20 DAA	Deionized water (Mo 0 mg L^{-1})
	Mo at 100 mg L^{-1}
	Mo at 600 mg L^{-1}
	Mo at 3,000 mg L^{-1}
	ABA 50 μM
30 DAA	Deionized water (Mo 0 mg L^{-1})
	Mo at 100 mg L^{-1}
	Mo at 600 mg L^{-1}
	Mo at 3,000 mg L^{-1}
	ABA 50 μM

4.3.3 Harvest

The drip irrigation system was stopped 34 DAA (15 April 2014), the final day of the second submergence treatment. Harvest was carried out three days later, 37 DAA (18 April 2014). Filled seeds from each treatment were threshed out from panicles that were harvested from the same block. Sprouted seeds were separated using the method described in Chapter 2, Section 2.3.4.4 and counted and kept individually as the fraction of sprouted seed sample.

4.3.4 Assessment

4.3.4.1 Yield per plant, seed weight, and percentage of pre-harvest sprouting

After harvest, the seed from each block was kept separately in muslin bag and air-dried at 20 °C in controlled-temperature room, Seed Laboratory. Yield per pot, thousand dry seed weight, and number of sprouted seed were determined on 15 May 2014. Yield $plant^{-1}$ in this experiment represented total weight of seeds produced from each treatment, in which the moisture content of sprouted and non-sprouted seed was about 11 % (water activity of all

seed samples was 0.500±0.05). Determination of 1000 seed dry weight and percentage of pre-harvest sprouting were conducted based on the methods described in Chapter 2, Sections 2.3.4.1 and 2.3.4.4, respectively.

4.3.4.2 Analysis of Molybdenum

4.3.4.2.1 Sample preparation

Given the limited amount of sprouted and non-sprouted seed obtained from submergence treatments, 5 g of dry seed (at 0 % moisture content) from each treatment combination of each block were ground separately in a grain mill (Laboratory Mill 3303, Perten Instruments Ab, Stockholm, Sweden). Between samples, the mill was brushed-clean and the fine dust removed by vacuum cleaner (Aztec 1300 Goblin, Fareham, The British Vacuum Cleaner & Engineering Co. Ltd., UK) and aerosol spray (Air Duster, Office Depot Europe B.V., Venlo, The Netherland) to prevent cross-contamination between samples. The ground samples were kept in plastic screw cap vials (Sterilin™ 7 mL Polystyrene Bijou Containers, Fisher Scientific UK Ltd., Loughborough, UK) and stored in the Seed Laboratory at room temperature until analysis.

4.3.4.2.2 Determination of Mo

Mo content of rice seed samples was determined at Central Laboratory Co, Ltd., Bangkok, Thailand, the accredited laboratory under the standard of ISO 17025, who collaborated in this work. The procedure for analysis of Mo followed the methods of in-house validation of the Association of Analytical Communities (AOAC), Official Method 999.10 (AOAC, 2005).

A subsample of 0.5 g of rice flour was taken from the total sample for quantitative analyses of Mo. The weight of each subsample (*m*) was recorded for final calculation of the result. The sample was placed inside a 100 mL digestion vessel (SK-10 Medium/High Pressure Rotors, Milestone, Italy), which had been washed with 10 % v/v nitric acid (HNO_3; Merck, Darmstadt, Germany) and rinsed two times with purified water (18.2 MΩ, Milli-Q system, Millipore Co., Bedford, USA) before use. Then, 7 mL 0.1M HNO_3 (Analytical grade 69-70%, Merck, Darmstadt, Germany) and 1 mL H_2O_2 (30% m/v, Merck, Darmstadt, Germany) were added to the sample. The vessel was closed firmly with the cap and placed in a microwave oven (Ethos One, Milestone, Italy), which had been programmed to heat the sample at 200 °C for 20 minutes. The samples were cooled using the fan system of the

microwave oven to approximately 30 °C. The blank sample was prepared as above, except no ground rice seed subsample was added. A vessel with the blank sample was included in each batch of microwave digestion.

The solution in the digestion vessel was transferred to a 25 mL volumetric flask. Deionized water was used to rinse the inner wall of the digestion vessel and lid and this was added to the 25 mL volumetric flask. The final volume was adjusted to 25 mL using deionized water. The solution was kept in a plastic vial with closed-cap and stored at 20 °C.

To detect the amount of Mo in solution from the digested samples (*a*) and blank samples (*b*), Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; 7700x ICP-MS, Agilent Technologies, US) with 0.040 mg kg^{-1} limit of detection (LOD) and 0.075 mg kg^{-1} limit of quantification (LOQ) was used in the present study. The technical details of instrument and operation conditions of the instrument are reported in Table 4.2. The final concentration of Mo (*C*; mg kg^{-1}) in each sample was calculated based on the equation below;

$$C = \frac{(\mathrm{a} - \mathrm{b})\ DF\ x\ 25}{m}$$

where *a* and *b* are the concentration of Mo mg L^{-1} in solution from digested samples and blank samples respectively, DF is dilution factor; which equalled 1 in this experiment (no dilution was employed), 25 is the volume of sample solution that had been adjusted after microwave digestion, and *m* is the original weight of the subsample.

4.3.4.3 Analysis of Abscisic acid

4.3.4.3.1 Sampling

For each treatment, sampling of panicles was conducted just before and immediately after submergence treatments: hence, 20, 24, 30 and 34 DAA. Panicles with the same anthesis date were sampled in this experiment. At each sampling date, one panicle per block was cut, then snap-frozen instantly for 10 minutes using dry-ice and ethanol (99.5 %, Sigma-Aldrich, UK). After snap freezing, individual panicles were kept in centrifuge tubes with flat screw caps (15 mL, VWR International, Leuven, Belgium). The samples were placed inside a safe-flask containing liquid nitrogen during transportation from PEL to Molecular Laboratory, University of Reading, where the panicle samples were then stored at -80 °C.

Table 4.2 Operation conditions for the ICP-MS measurements

Parameter	Value
RF power	1500 W
Plasma gas flow-rate	0.91 L min^{-1}
Intermediate gas	0.32 L min^{-1}
Nebulizer	Babington Nebulizer
Nebulizer sample uptake	0.10 mL min^{-1}
Type of spray chamber	Quartx
Spray chamber temperature	2 °C
Acquisition mode	- points peak jump
Total acquisition	1 min.
Dwell time	0.1 Sec.
Resolution	0.8 amu
Mo standard solutions	0.5, 1, 5, 10, 25, 50 µg L^{-1} prepared from stock solution of 1000 mg L^{-1}, Merck, Darmstadt, Germany

4.3.4.3.2 Sample preparation

Panicle samples were removed from the -80 °C refrigerator just before processing. Forty filled-seeds were threshed out from each panicle and ground to a fine powder in a pre-chilled mortar and pestle with liquid nitrogen. Ground samples were stored in new 15 mL screw-cap centrifuge tubes at -80 °C. The samples were then freeze-dried (Freeze Dryer GAMMA 1-16 LSC, Freeze Drying Solutions, UK) at the Department of Food and Nutritional Sciences, University of Reading. The cap of each sample tube was removed before freeze-drying and re-capped again after four days when the drying process was finished. Freeze-dried samples were kept in sealed-aluminium bags and stored in the Seed Laboratory at room temperature.

The extraction of ABA from rice seed samples was performed according to Forcat e*t al.* (2008). A subsample of 50 mg of freeze-dried-rice-flour was weighed (Sartorious 1601 A MP8-1, 0.1 mg accuracy, Data Weighing System, Inc., USA.) and placed in 2 mL safe-lock microtube (Eppendorf AG, Hamburg, Germany). To this, 400 µL of the extraction solvent containing 10 % methanol (v/v; Methanol, > 99.95 %, HPLC Grade, Fisher Scientific UK

Ltd.; Water, OptimaTM, LC/MS Grade, Fisher Scientific UK Ltd.), 1% acetic acid (Acetic acid, 100%, VWR PROLABO®, VWR International Ltd., UK) and 5 ng of internal ABA standard (Stable isotope-labelled compound, $^{2}H_{6}$, ABA-D6, 0.5 µg mL^{-1}). A Tungsten-carbide bead (3 mm diameter, Qiagen, UK) was placed in each microtube, and shaken (Tissuelyser Retsch MM301, Qiagen, UK) at 25 Hz s^{-1} for 3 min. Samples were then placed in an ice bath for 30 min. Samples were then centrifuged at 13,000 g (Eppendorf 5702, Eppendorf AG, Hamburg, Germany) at 4 °C for 10 min. The supernatant was collected, and the pellet was re-extracted again as above. The second supernatant was mixed with the first, then centrifuged again for 10 min at 4 °C. In order to obtain a pure extraction, the final supernatant was filtered through 1 mL sterile syringe (Plastipak, Becton, Dickinson and Company, USA), fitted with a filter (30 mm Syringe Filter 0.45 µm Cellulose, Chromacol Ltd.,UK), which had been wetted prior to use with LC/MS grade water (OptimaTM, LC/MS Grade, 2.5 L, Fisher Scientific UK Ltd.). The extracted solution after filtration was kept in a glass vial (300 µL glass insert, fused into a 2 mL screw top vial, Chromacol Ltd.,UK) then capped. The samples were stored at -20 °C until analysis of ABA concentration. Two blank samples were prepared as above without adding any freeze-dried tissue sample.

4.3.4.3.3 Sample analysis

The ABA was measured in collaboration with Prof. Colin Turnbull and Dr Mark Bennett at the Department of Life Sciences, Faculty of Natural Sciences, Imperial College, London. ABA detection was carried out using high performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) technique with 50 µL of extracted sample. HPLC (Agilent 1100 HPLC, Agilent Technologies, Inc., USA) was performed using Phenomenex column (3 µm C18(2) 100 mm × 2.0 mm, Luna Aschaffenburg, Germany) at 35 °C and a solvent flow rate of 200 µL min^{-1}. Analysis of ABA content was based on the mass transition weight of endogenous ABA inside the sample (263 >153 m/z) and the labelled ABA internal standard ($^{2}H_{6}$ ABA 269 > 159 m/z, Olchemim Ltd., Czech Republic). Mass spectrometer (Applied Biosystems Q-TRAP 2000, Applied Biosystems, California, USA) was coupled after chromatographic separation using negative mode, where TurboIonSpray™ was the ion source. The optimised conditions for mass analyser are shown in Table 4.3. The results were interpreted using Analyst 1.4.2 software (released on May 2008, Applied Biosystems).

4.3.4.3.4 ABA content of developing seed

To investigate the pattern of ABA accumulation in developing seed, the panicles of cv. Gleva from the determination of seed development and maturation of rice (Chapter 2, Section 2.3.4.3) were used. During the serial harvests at various stages, i.e. 8, 11, 16, 22, 26,

31, 37, and 45 DAA, fresh-cut panicles were frozen immediately and subsequently processed as described above (Section 4.3.5.4). Each sample contained four rice panicles from two replicates pots of two blocks. In cases where assessments were carried out during early seed development with no filled seed, the unfilled or half-filled immature seeds were selected for ABA analysis instead.

4.3.5 Statistical analyses

These were carried out according to the statistical analyses introduced in Chapter 2, Section 2.3.5.

Table 4.3 Operation conditions for the mass spectrometry

Parameter	Value
Capillary temperature	400 °C
Ion spray voltage	4.5 kV
Ion sources	gas 1 50 psi gas 2 60 psi
Curtain gas	40 psi
CAD gas setting	2; DP (-25 V), EP (-9) and CEP (-2) were constantly held
Collision energies and dwell times	CE-17 and DT 250 ms
ABA standard solutions	0.5, 1, 5, 10, 25, 50, and 100 ng mL^{-1} (Abscisic acid, ≥98%, $C_{15}H_{20}O_4$, Sigma-Aldrich, UK)

4.4 Results

4.4.1 Yield per plant, seed weight, and pre-harvest germination

Simulated flooding treatments caused substantial reduction in seed yield (at 0% moisture content) per plant (down to 4.4-7.8 g pot^{-1}) compared with non-submerged plants

(approximately 16 g pot^{-1}, data not shown) by 50-73%. There were no significant differences in seed yield amongst the five foliar applications (Fig. 4.2) whether 4 days' submergence occurred at 20 (P = 0.148, Appendix 4.1.1) or 30 DAA (P = 0.402, Appendix 4.1.2). Submergence 20 DAA resulted in lower seed yield per plant across all foliar treatments. The lowest seed yield per plant was obtained from foliar application of DI water, followed by Mo at 3000 mg L^{-1} < 50 μM of ABA, < Mo at 600 mg L^{-1} and < Mo at 100 mg L^{-1}: the main effect of foliar application approached significance at 5% (P=0.065, Appendix 4.1.3).

Simulated flooding treatments significantly increased the number of sprouted seed pre-harvest (Fig. 4.3). Submergence for four days 20 DAA and treatment with DI water at flag leaf stage resulted in 27% sprouted seed, whereas Mo or ABA treatments were lower at 11-15%. The differences between foliar applications at 20 DAA were not significant (P=0.116, Appendix 4.2.1). The percentage of sprouted seed was higher for 30 DAA treatments with plants sprayed with DI water having the highest (75%; Fig. 4.3). The percentage of sprouted seed was lower with Mo treatments of 100 (53%), 600 (53%), and 3000 (67%) mg L^{-1}, or 50 μM of ABA (68%), with almost significant differences at P=0.055 (Appendix 4.2.2). Although the number of sprouted seed was affected by foliar application (P=0.004; DI water ≥ Mo at 3000 mg L^{-1} or 50 μM ABA ≥ Mo at 100 or 600 mg L^{-1}) and timing of submergence (P<0.001; 30 > 20 DAA), there was no interaction between the two factors (P=0.286, Appendix 4.2.3). Moreover, the main effects of the foliar application treatments and of different durations of flooding were both significant (Appendix 4.2.3).

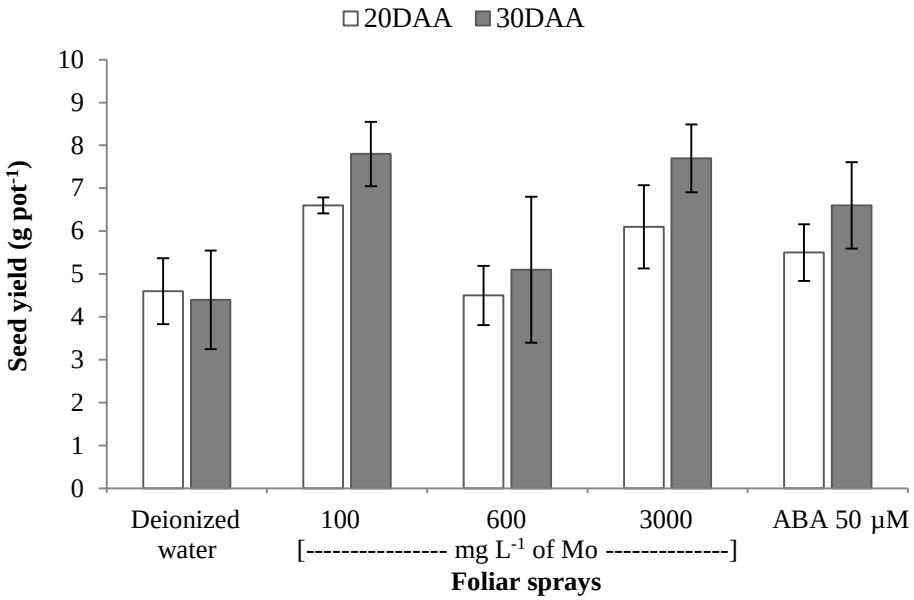


Figure 4.2 Effect of foliar applications of deionized water, molybdenum (Mo at 100, 600, 3000 mg L^{-1}), and 50 µM abscisic acid (ABA) and submergence for four days at 20 (□) or 30 (■) DAA on seed yield dry matter (0% moisture content, g pot^{-1}) of *japonica* rice cv. Gleva (2014). The vertical bars represent mean ± s.e. (n=3) (Appendix 4.1).

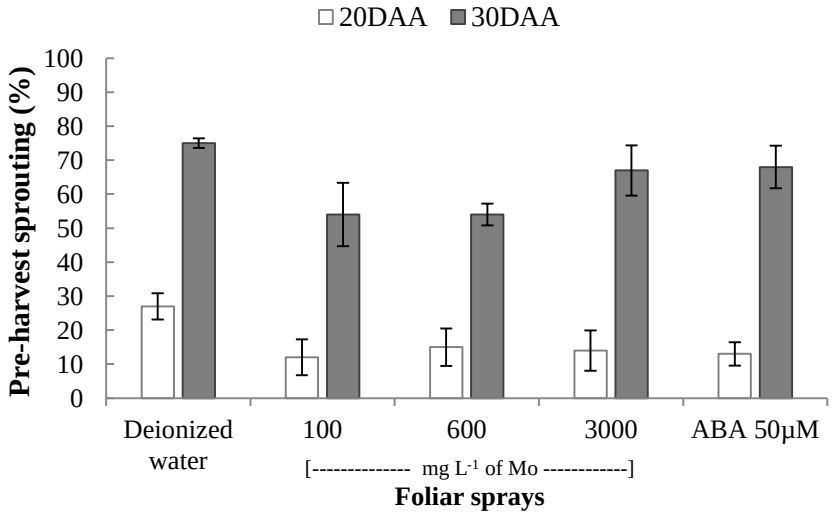


Figure 4.3 Effect of foliar applications of deionized water, molybdenum (Mo at 100, 600, 3000 mg L^{-1}), and 50 µM abscisic acid (ABA) and submergence for four days at 20 (□) or 30 (■) DAA on pre-harvest sprouting percentage of *japonica* rice cv. Gleva. Plants were harvested 37 DAA. The vertical bars represent mean ± s.e. (n=3) (Appendix 4.2).

Since simulated flooding treatments led to pre-harvest sprouting, seed samples were divided into sprouted and non-sprouted seed fractions. The dry weight of one thousand non-sprouted seeds for plants grown under normal conditions provided greater seed weight (31.3 g, data not shown) than non-sprouted seed samples obtained after submergence treatment of all foliar applications (26.8-27.2 g). There were no significant differences amongst spray treatments in the thousand seed weight of sprouted and non-sprouted seed samples obtained from 4 days' submergence at 20 and 30 DAA (Fig. 4.4; Appendices 4.3 and 4.4). Sprouted seeds were heavier than non-sprouted seeds. Furthermore, treated-seed samples with Mo or ABA solutions had slightly greater seed weight than DI water treatment.

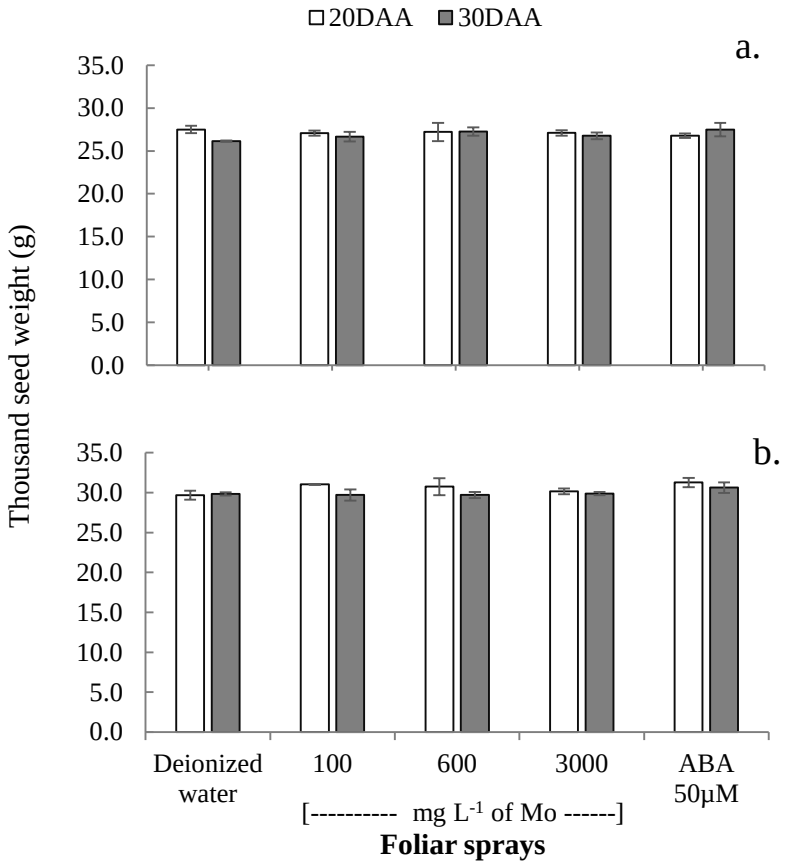


Figure 4.4 Effect of foliar applications of deionized water, molybdenum (Mo at 100, 600, 3000 mg L^{-1}), and 50 µM abscisic acid (ABA) and submergence for four days at 20 (□) or 30 (■) DAA on dry weight (% moisture content) of a thousand non-sprouted (a) or sprouted seeds (b) of *japonica* rice cv. Gleva. Plants were harvested 37 DAA. The vertical bars represent mean ± s.e. (n=3) (Appendices 4.3 and 4.4)

4.4.2 Mo concentration in harvested seed

The response of Mo concentration in mature seed at harvest and applied Mo at flag leaf stage showed significant positive relations in both sprouted and non-sprouted seed samples ($P < 0.001$, Appendix 4.5). Seed Mo concentration increased with increasing Mo concentrations in the foliar applications, with significant differences between the highest concentration of applied Mo (i.e. 3000 mg L^{-1}) and non-Mo pre-treatments (i.e. spraying of DI water or 50 µM ABA) (Table 4.4, Appendices 4.6 and 4.7). The maximum Mo concentration in seed was detected from foliar applications at 3000 mg Mo L^{-1}: with 1.67 and 2.12 mg kg^{-1} Mo detected from sprouted and non-sprouted seed samples obtained after submergence at 20 DAA, and 1.49 and 2.27 mg kg^{-1}, from submergence at 30 DAA, respectively. The Mo concentration in seed samples from non-Mo pre-treatments varied from 0.65-0.81 mg L^{-1}, and mature seed from plants with no foliar treatments or submergence also had a similar Mo concentration of 0.75 mg L^{-1} (data not shown).

Interestingly, Mo concentrations in sprouted seed samples were always higher than non-sprouted seeds amongst foliar treatment in all cases, but there was no interaction between foliar treatment and whether the seed was sprouted or not for both submergence treatments at 20 (Appendix 4.6.4) or 30 DAA (Appendix 4.7.4). The interaction between foliar application and type of seed was only detected when differences in Mo content performed using three-two-way ANOVA model with one more factor; time of submergence. Nevertheless, the latter had no significant influence (Appendix 4.8b, c). A significant difference amongst sprouted and non-sprouted seed samples was only detected in seeds sprayed with 3000 mg L^{-1} Mo followed by 4 days' submergence at 30 DAA: Mo concentrations in sprouted and non-sprouted seed were 2.27 and 1.49 mg kg^{-1} respectively ($P<0.001$, Appendix 4.7.3).

Table 4.4 Molybdenum concentrations in sprouted or non-sprouted seed samples of *japonica* rice cv. Gleva after 4 days' submergence at 20 or 30 DAA (2014).

Submergence	Treatments	Foliar spray	Seed Mo concentration (mg kg^{-1})			
			Non-sprouted seed	s.e.	Sprouted Seed	s.e.
At 20 DAA	Submergence					
		Mo at 0 [1] mg L^{-1}	0.74 [2]	0.027	0.76 [3]	0.063
		100 mg L^{-1}	0.98	0.098	1.08	0.143
		600 mg L^{-1}	1.25	0.195	1.52	0.135
		3000 mg L^{-1}	1.67	0.291	2.12	0.438
		ABA at 50 µM	0.71	0.444	0.75	0.053
At 30 DAA	Submergence					
		Mo at 0[7] mg L^{-1}	0.72 [4]	0.086	0.80 [5]	0.081
		100 mg L^{-1}	1.01	0.070	1.09	0.105
		600 mg L^{-1}	1.11	0.174	1.33	0.143
		3000 mg L^{-1}	1.49	0.376	2.27	0.284
		ABA at 50 µM	0.65	0.074	0.81	0.064

		P	**LSD**	**Appendix**
At 20 DAA	Non-sprouted seed	0.003 [2]	0.4076	4.6.1
	Sprouted seed	0.001 [3]	0.5269	4.6.2
At 30 DAA	Non-sprouted seed	0.017 [4]	0.4516	4.7.1
	Sprouted seed	<0.001 [5]	0.4578	4.7.2

[1] Deionized water
[2] Appendix 4.6.1
[3] Appendix 4.6.2
[4] Appendix 4.7.1
[5] Appendix 4.7.2

4.4.3 Seed ABA concentration

4.4.3.1 Seed ABA concentration during seed development and maturation

The highest concentration of ABA during seed development was 355 ng g^{-1} fresh tissue weight, detected at the first sampling time (Figure 4.5). The ABA concentration in immature seeds then declined rapidly within the next seven days to 105 ng g^{-1} at 16 DAA. No significant difference in seed ABA concentrations was observed thereafter as seed matured (Figure 4.5). At harvest maturity (46 DAA), the seed ABA concentration was 86 ng g^{-1}.

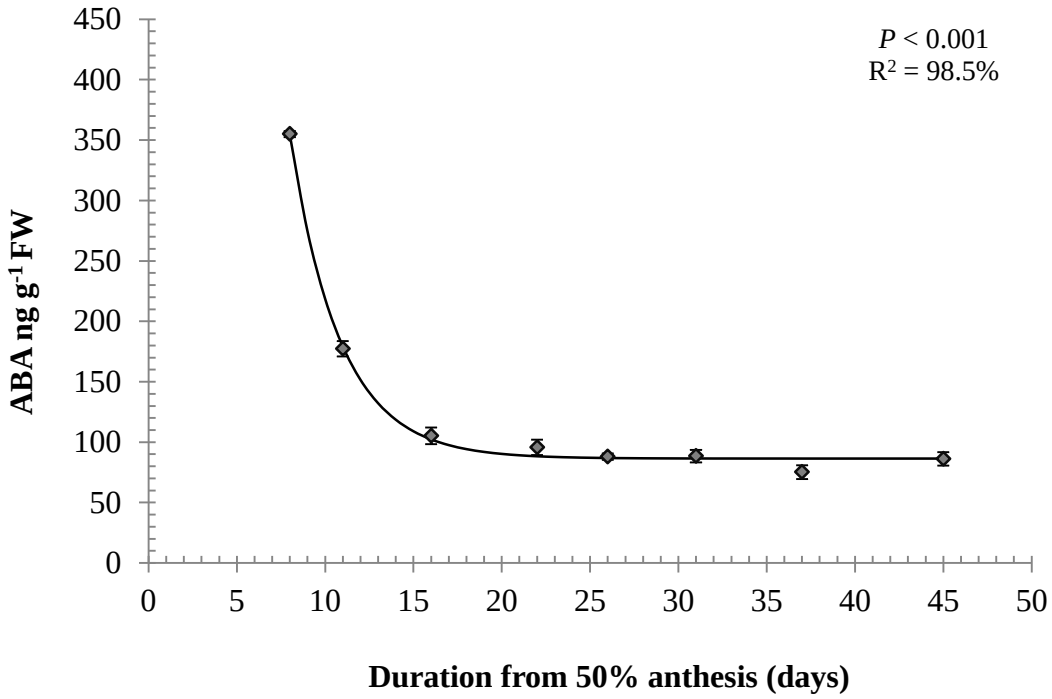


Figure 4.5 Abscisic acid concentration during seed development and maturation of rice cv. Gleva (2012). The vertical bars represent mean ± s.e. (n=4) of each sampling date (data from two replicates of each block, i.e. four samples in total from two blocks). The fitted curve is quantified by the exponential equation $Y = A + B.R^X$). Parameter values are provided in Appendix 4.9.

4.4.3.2 Seed ABA concentrations in harvested seed after foliar applications

The relationship between application of Mo and endogenous ABA was investigated before and after the submergence treatments (Table 4.5). The seed ABA concentrations after submergence were considerably reduced. Nevertheless, the concentration amongst five foliar sprays was not significantly different (Appendix 4.10), either before (i.e. 20 or 30 DAA) or after (i.e. 24 or 34 DAA) submergence. Further, the effect of Mo applications on seed ABA concentrations gave mixed results. In most cases foliar application of Mo increase ABA concentrations in seeds compared to seeds treated with DI water. The exceptions were treatment combinations of submergence at 20 and 30 DAA of Mo at 100 and 3000 mg L^{-1} respectively, which had lower seed ABA concentrations than seeds treated with DI water (Table 4.5). Spraying exogenous ABA did not increase ABA concentrations in developing seed when the submergence treatment was applied 20 DAA (113.8 ng g^{-1}). In contrast, in the more mature seed, treated at 30 DAA, ABA foliar treatment resulted greater in seed ABA concentrations than some Mo treatments (i.e. 100 and 3000 mg L^{-1}) and DI water.

In pot-grown plants under normal growing conditions (no submergence and no foliar spray), ABA concentrations in seed at 20 and 30 DAA were 165.4 and 112.6 ng g^{-1}, respectively, with a small decline (about 12%) observed between 20-24 and 30-34 DAA (data not shown). Submergence caused a significant decrease in seed ABA concentration (Appendix 4.11.3). Reductions in ABA concentration after four days submergence were lowest in plants submergence at 20 and 30 DAA and treated with Mo foliar applications of 100 and 600 mg L^{-1}, respectively (Table 4.5). There was no relationship between Mo application and seed ABA concentration at 20, 24, 30, or 34 DAA, but positive relation was detected at 24 DAA (Fig. 4.6, Appendix 4.12.2).

Table 4.5 Abscisic acid concentrations in seed samples of rice cv. Gleva before (20 and 30 DAA) and after (24 and 34 DAA) submergence (2014) following treatment with different foliar applications of deionized (DI) water, molybdenum (Mo) or abscisic acid (ABA) (Appendices 4.10).

Submergence	Foliar sprays		Seed ABA concentration (ng g^{-1}fresh weight)				Reduction of ABA content after submergence (%)
			Before submergence	s.e.	After Submergence	s.e.	
At 20 DAA	Submergence						
	Mo at	0[1] mg L^{-1}	136.3	8.74	26.0	4.56	81
		100 mg L^{-1}	134.1	23.54	42.6	20.90	68
		600 mg L^{-1}	151.7	17.63	20.4	3.52	87
		3000 mg L^{-1}	150.9	20.82	14.3	0.64	91
	ABA at	50 µM	113.8	8.57	30.6	8.53	73
At 30 DAA	Submergence						
	Mo at	0[1] mg L^{-1}	122.4	10.40	9.3	2.21	92
		100 mg L^{-1}	126.4	16.96	10.1	0.81	92
		600 mg L^{-1}	134.2	8.26	48.4	35.94	64
		3000 mg L^{-1}	114.9	17.75	9.5	2.35	92
	ABA at	50 µM	128.1	6.98	14.3	1.97	89

[1] Deionized water

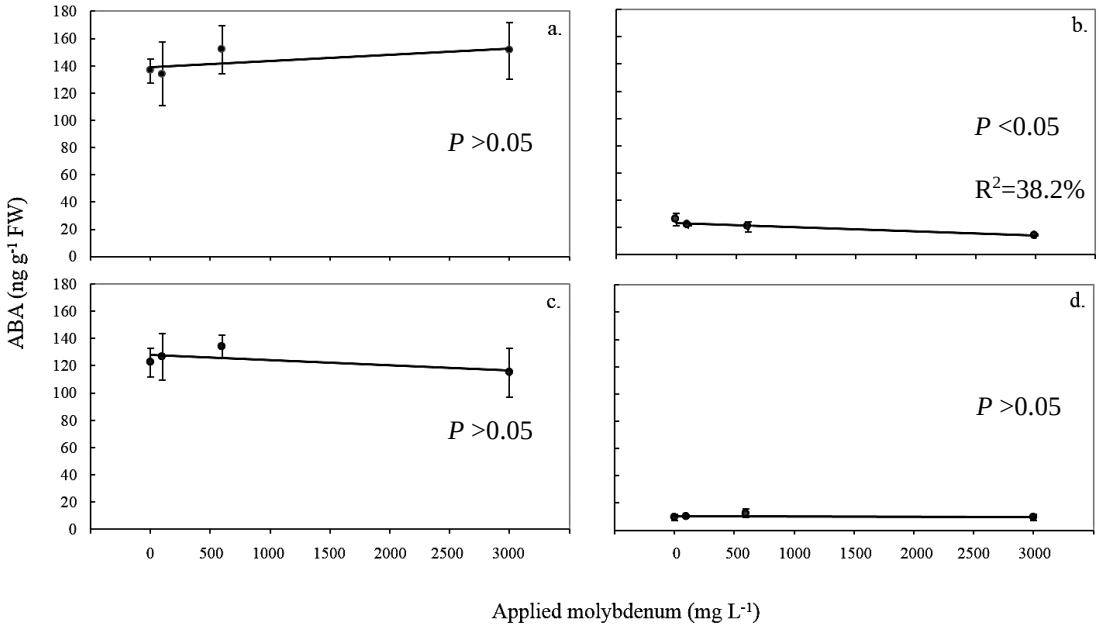


Figure 4.6 Relationships between foliar applied molybdenum and seed abscisic acid concentrations of *japonica* rice cv. Gleva before submergence (a, 20 DAA; c, 30 DAA) or after 4 days' submergence (b, 24 DAA; d, 34 DAA). The symbols represent means ± s.e. (n= 3). Only the regression in b. (24 DAA) was significant (Appendix 4.12).

4.5 Discussion

In this Chapter, the efficiency of foliar applications at flag leaf stage on mitigation of pre-harvest sprouting after simulated flooding (four days' submergence at 20 or 30 DAA) was investigated. Based on its susceptibility to submergence, the significant number of sprouted seed and the absence of submergence tolerance gene in the former studies (Chapters 2 and 3), *japonica* rice cv. Gleva was selected to be representative of a less dormant and so more sensitive to flood cultivar in the current study. Comparisons of the effect of Mo, ABA, or DI water foliar applications on subsequent submergence damage (Figs. 4.2, 4.3 and 4.4) were investigated. These experiments were designed to determine the most effective foliar treatment for preventing agronomic losses due to the varying occurrences of submergence, and the interaction of Mo in inducing seed dormancy (i.e. inducible ABA biosynthesis) at harvest time (Table 4.5).

Unsurprisingly, submergence in the pot-culture experiment of the current study provided poorer yield per plant, seed dry weight, and greater pre-harvest sprouting in all cases

whether prior to Mo or ABA foliar applications were applied (Figs. 4.2, 4.3, and 4.4) compared to non-submergence plants. These results were in agreement with the findings from Chapter 2. Nevertheless, substantial variations between this experiment and that in Chapter 2 in yield per pot and the occurrence of sprouted seed were observed in results obtained from plants submerged 30 DAA. In 2013, there was approximately 12 g pot^{-1} of Gleva seed produced from plants submerged 30 DAA. In contrast, the yield in the current experiment was approximately 40% of this for the comparable treatment (1.2 g $plant^{-1}$, or 4.8 g pot^{-1}). The observed number of sprouted seed was significantly greater in the current experiment at 75%, compared with 7.3% in 2013.

These differences above suggest that the severity of subsequent submergence damage may be due to the physical growing environment of the mother plant on vigour, embryogenesis, and regeneration ability. The interaction between genotype and environment is a vital element of the susceptibility to premature sprouting. Deep dormancy was found in *indica* rice produced in rainy weather (Sircar, 1963, as cited in Takahashi, 1984), whereas high temperature (30 ºC) together with a humid climate during grain filling and maturation strengthens dormancy (Ikehashi, 1973; Takahashi, 1975, as cited in Takahashi, 1984). Takahashi (1962) and Ota and Takeichi (1966), as cited in Takahashi (1984), reported that prolonged day length during grain formation stimulated greater dormancy. The former authors also found a similar trend for germination ability post-harvest: the dormancy pattern of early- or medium-maturity varieties was released faster than late-maturity cultivars. Dormancy is lost during post-harvest storage (after ripening) at a rate dependent upon temperature (Roberts, 1965) and moisture content (Ellis *et al.*, 1983). Furthermore, Pili (1968) found that the longer delay to harvest the shorter the dormancy period of grain in storage.

In the presentstudy, rice plants in 2013 and 2014 were grown in a glasshouse and a controlled environment cabinet, respectively, although the seeds of them both were produced in the glasshouse. There were, however, differences in relative humidity and light intensity during the vegetative growth: these two growing parameters were controlled in controlled environment cabinet, but were not controlled in glasshouse. Dunwell *et al.* (1985) and Guan (1995) reported that plants grown under controlled conditions had more favourable conditions for flowering, and thus microspore and somatic embryo yield was higher than plants grown in less controlled growing environments. Pratap *et al.* (2009), however, recommended that some plant species may require specific environmental factors for better embryogenic microspores. Ziska *et al.* (1997) reported the effect of difference in light quality on rice productivity. They found that controlled environment chamber grown rice had 15% lower photosynthetically active radiation (PAR) compared to the outside ambient, therefore resulting in a reduction in total biomass and

grain yield. The greater pre-harvest sprouting observed in 2014, may also have been affected by the above factors, as Bewley and Black (1994), Sugimoto *et al.* (2010), and Bewley *et al.* (2013) proposed that the degree of seed dormancy and responses of sprouted seed to wet weather could be influenced by the growing environment the mother plant had experienced previously.

The application of Mo has the potential to reduce losses from pre-harvest sprouting. Foliar application of exogenous Mo before submergence reduced yield loss by up to 25 % (Fig. 4.2), nevertheless this observation was not significant. Foliar applications of Mo also reduced pre-harvest sprouting by 12-15% and 8-21% for submergence at 20 or 30 DAA, respectively (Fig. 4.3). Although, significant differences in these agronomic traits were not observed amongst the different concentrations of Mo, the results suggest that Mo solutions at 100 or 600 mg L^{-1} were the most efficient resulting in the least pre-harvest sprouting and yield loss, and providing greater thousand seed dry weights.

In the present study, seed Mo concentrations in both sprouted and non-sprouted seed samples determined at harvest maturity did relate positively and significantly with the Mo concentration applied (Table 4.4, $P < 0.001$, Appendices 4.5 and 4.6). This is in agreement with Walker-Simmons (1989) and Modi and Cairns (1995). This suggests that the Mo (i.e. in molybdate form) applied as a foliar spray at flag leaf stage did translocate through the plant and accumulate in the developing seed. A similar redistribution pattern of Mo in plants was reported previously by Jongruaysup *et al.* (1994 and 1997): the relationship between Mo supply in growing media and Mo content in black gram seed was positive. In plants, molybdate (MoO_4^{2-}) is the common anion form that can be utilized for growth and development processes (Lindsay, 1979; Mengel *et al.*, 2001; Kaiser *et al.*, 2005). After uptake, the soluble molybdate anion is incorporated into Moco, the vital cofactor for molybdenum-requiring enzymes; for example in nitrate reductase, xanthine dehydrogenase, aldehyde oxidase, and sulphite oxidase (Kaiser *et al.*, 2005). It is possible that these crop improvements may have been due to the increase of Moco after spraying, which encouraged the activity of molybdoenzymes, and/or benefits to protein and abscisic acid synthesis.

In addition, it was noted that Mo content in sprouted seed of this study was higher than non-sprouted seed in all cases within each treatment combination. In black gram and common bean, Mo is redistributed from roots to the developing seeds during early grain filling until maturity (Jongruaysup *et al.*, 1994 and 1997), with the main sources of Mo in seed being translocation from the mid stem, nodules and pod wall (Brodick and Giller, 1991; Jongruaysup *et al.*, 1997). The higher amount of Mo in sprouted seeds (Table 4.4) may have been more

mature and incorporated more Mo. Since I did not determine variation amongst individual seed in Mo of dry weight, I cannot resolve this speculation.

ABA is involved in seed development, induction of dormancy, as well as plant stress responses (Bewley and Black, 1994; Sugimoto *et al.*, 2012; Bewley *et al.*, 2013). In this investigation, exogenous ABA was applied to investigate the impact on seed ABA concentrations and compare with the effect of foliar Mo applications on seed ABA concentrations. No significant differences in grain yield per plant, 1000 seed dry weight, sprouted seed number, or seed ABA concentrations were observed in plants treated with ABA compared to those treated with DI water (Table 4.5). Applying ABA as a foliar treatment prior to submergence, however reduced loss in yield with fewer sprouted seed than spraying with DI water. The foliar application of ABA had a similar efficiency of reducing pre-harvest sprouting as Mo at different concentrations in the 20 DAA submergence treatment. In plants submerged at 30 DAA, nevertheless, Mo applied at 100 or 600 mg L^{-1} was the most effective (54% sprouted seed), whereas ABA showed lower ability to prohibit pre-harvest sprouting (about 68%), similar to Mo treatment of 3000 mg L^{-1}.

Observed ABA concentrations in developing seed of the current study (Fig. 4.5) were in agreement with most previous observations: ABA concentration peaked during early grain filling, thereafter declined continuously over later seed development and maturation (Hilhorst, 1995; Bewley *et al.*, 2013). Seed ABA content is synthesized by the developing embryo (*de novo* ABA synthesis) and delivery from the maternal plant (Bewley *et al.*, 2013). The latter source may contribute to early seed development and suppress pre-harvest sprouting, meanwhile the former induced and maintained seed dormancy during seed maturation (Bewley *et al.*, 2013). In the present study, some agronomic traits showed improvements as a consequence of Mo or ABA foliar spray treatments. One potential mechanism noted already could be an increase in Moco, which may result in greater ABA aldehyde oxidase activity, and hence enhance ABA biosynthesis in Mo-treated seed samples. However, determination of seed ABA concentrations in this study was inconsistent and no firm conclusions could be drawn.

In ABA production, the primary rate-limiting stage is associated with the carotenoid pathway (Bewley *et al.*, 2013; Endo *et al.*, 2014). In this step, zeaxanthins (i.e. violaxanthin and neoxanthin) are converted by 9-cis-epoxycarotenoid dioxygenase (NCED) to xanthoxin, and thus provide the precursor for ABA synthesis. The molybdenum cofactor (Moco), necessary for aldehyde oxidase activity, catalyses ABA aldehyde to active ABA. Synthesized ABA has three possible destinations; remain active and function in plant stress responses or control seed development programme, conjugated with a monosaccharide, to an inactive form, or degraded into phaseic acid (PA) and 4'dihydrophaseic acid (DPA) via oxidation (Taiz and Zeiger, 2006).

In the present study, the proportion of ABA biosynthesis and catabolism might regulate the seed ABA concentrations. Seed ABA concentrations before and after simulated-flooding treatments and in non-submerged plants (at 20, 24, 30, and 34 DAA) declined gradually (about 13% reduction) (Table 4.5). Nevertheless, significant reductions in seed ABA concentrations were observed in seeds treated with all foliar sprays. No significant increases in seed endogenous ABA concentrations were observed with increasing applications of Mo. However, foliar applications of low Mo concentrations (i.e. 600 mg L^{-1} or lower) did increase seed ABA concentrations. This may result in greater availability of internal Moco. Consequentially, the greater Moco level may have contributed to Mo enzyme (i.e. abscisic aldehyde oxidase) activity in ABA synthesis, and hence ABA concentration in seeds increased. Similar observations to these have been reported in maize (McCarty, 1995; Singh *et al.*, 2003, Porch *et al.*, 2006, Suzuki *et al.,* 2006, Fan and Chu, 2008). These authors demonstrated that the absence of available cytosolic Moco was consistent with the lack of ABA production, and resulted in strong premature germination as well as seedling lethal mutations.

Interestingly, excessive foliar applications of Mo, such as 3000 mg L^{-1}, did not increase seed ABA concentrations and may have contributed to stress or created toxic conditions, although no visual symptoms were observed. Plant Mo toxicity is seldom reported in the field, however poor plant growth was observed in laboratory conditions (Liphadzi and Kirkham, 2006). Plants grown with high concentrations of Mo commonly show yellow tips, light-green shoots, and leaf malformation. Liphadzi and Kirkham (2006) suggested that Mo toxicity symptoms under extreme Mo supplied might have been caused by high accumulation of molydo-catechol complexes in vacuoles of plant cell.

Because Moco cofactors are also necessary for the correct functioning of NR, other factors may contribute to dormancy induction and maintenance. Previous reports have indicated that high concentrations of initial substrate (i.e. NO_3^-) and nitrogen-end-products of nitrogen assimilation could regulate seed germinability by inducing GA production or reducing active ABA. Karssen and Lacka (1986), McIntyre *et al.* (1996), McIntyre (1997), and Alboresi *et al.* (2005) proposed that high nitrate content in maternal and embryonic tissue benefits germination processes by 1) acting as an osmoticum, therefore water uptake during germination phase I is increased, 2) reducing seed GA requirements for germination, and 3) acting as a signalling molecule for GA synthesis for embryo growth, thus breaking dormancy. Furthermore, it is likely that nitric oxide also contributes to ABA antagonism in the seed. Bethke *et al.* (2007), Liu *et al.* (2009), Simontacchi *et al.* (2009), and Arc *et al.* (2013a, b) found that ABA catabolism can be triggered by nitric oxide. Moreover, this nitrogen compound acts upstream of GA production, which can induce the release of protein stored in aleurone cells. Hormonal

interactions between ABA and GA are pivotal in the subsequent phenotypic responses i.e. dormancy maintenance or release (Bewley *et al.,* 2013, Liu *et al.,* 2014).

In the current investigation, besides favourable physical conditions (e.g. water, temperature, and oxygen) for germination provided by simulated flooding, supplying Mo might influence the hormonal balance between ABA and GA: Mo-treated seed might not only have had greater ABA synthesis, but also better stability in the ABA/GA balance.

In this study, foliar applications of ABA did not improve seed ABA concentrations in seed submerged 20 DAA, compared with foliar application of DI water. However, foliar applications of ABA in plants submerged 30 DAA showed greater seed ABA concentrations following submergence compared to the DI water treatment. Exogenous ABA may enhance the stability of this phytohormone post submergence: there was higher ABA content (i.e. lower ABA reduction) than DI water sprayed seed both times after submergence (Table 4.5). In contrast to Mo spray treatments, in which Moco may contribute to ABA synthesis, exogenous ABA supplied may remain functional or may be oxidised and degraded, or may be transformed into inactive-ABA forms. The function of ABA in the last case may be reversible later on, and may contribute to seed ABA concentrations detected in this foliar spray treatment. The instability of ABA may also have been affected by timing, concentration of supplied solution, and the timing of submergence.

This study showed that foliar applications of Mo or ABA reduced submergence damage on yield losses (i.e. seed yield plant^{-1}, thousand seed weight, and sprouted seed percentage), although significant differences were not always detected. The different foliar treatments did show differences in their effectiveness on mitigation of flood damage post-submergence: Mo at 100 or 600 mg L^{-1}> Mo at 3,000 mg L^{-1} or ABA 50µM> Mo at 0 mg L^{-1} (DI water). Regression analysis clearly confirmed that Mo concentrations in mature seed at harvest was the result of Mo uptake by plant due to applied Mo at the flag leaf stage. For some treatments, plants treated with Mo did have higher seed ABA concentrations, but the relationship between applied Mo and measured ABA before and after submergence was unclear. Mo applied at 100 and 600 mg L^{-1} had similar low rates of ABA decline following submergence as the ABA foliar treatment. However, amongst the three foliar treatments above, 100 and 600 mg L^{-1} Mo sprays resulted a slightly greater responses (i.e. grain yield and the number of sprouted seed) than the foliar application of ABA. The greater performances of these Mo treatments were clearer when plants were submerged at 30 than at 20 DAA. Since similar agronomic responses to submergence were observed from both 100 and 600 mg L^{-1} Mo foliar sprays, the lower dose (i.e. Mo at 100 mg L^{-1}) would be the most efficient solution in the current context as it would provide the agronomic benefit but have lower cost implications [approximately 134 GBP per 100g of

Ammonium molybdate (99.98% trace metals basis, price from Sigma Aldrich, December 2015) or 26.8 GBP ha^{-1} based on 200 L ha^{-1} applied at 100 mg L^{-1}]. However, there was significant variation in the experiments, and more research in controlled and field conditions would be needed to validate these conclusions.

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Chapter 5

General Discussion and Conclusions

This thesis investigated whether submergence during grain development and ripening affects subsequent rice seed quality postharvest. Simulation of natural flash-flooding of pot-grown rice plants was provided by submergence in a water tank. The experimental approach and equipment proved effective in altering subsequent seed characteristics, such as the relative high number of pre-harvest sprouting detected in cv. Gleva, without severely damaging the plant structurally or in terms of accelerated senescence.

To examine the damage from flooding on rice seed production, first of all the effect on seed physiological quality due to submergence at different seed developmental stages was determined (Chapter 2), in which flooding duration (0, 3, 4, or 5 days) and variety (*japonica* cv. Gleva and *indica* cvs IR64 and IR64 Sub1) were the variables investigated. The high-yielding-submergence-tolerant-rice IR64 Sub1 is the result of introgression of the *Submergence1* (*Sub1*) gene into the parental cultivar IR64. The impact of the *Sub1* gene on subsequent storability had not been tested up to the present. Therefore, the second investigation was the examination of the pattern of loss in seed viability during storage of the above two near-isogenic cultivars (Chapter 3). Finally, evaluation of alternative methods to lessen pre-harvest sprouting of seeds on mother plants after flooding was carried out using foliar application of molybdenum as a potential sprouting inhibitor (Chapter 4).

Climate model projections tend to predict that extreme future climates could affect agricultural production (Parry and Ruttan, 1991; Mirza, 2011; Knox *et al.*, 2012; Ranuzzi and Srivastava, 2012; Mohanty *et al.*, 2013; Wheeler and von Braun, 2013; Porter *et al*., 2014) in which the rice crop, for example, is under threat from variable rainfall and inundation. Previous studies have investigated the loss due to submergence that occur at different growth stages of rice; complete crop failure or considerably less yield were the most common detrimental impacts in the case that paddy fields were flooded for longer than seven days during the seedling stage (Shama and Ghosh, 1999; Das *et al.*, 2009; Singh *et al.*, 2009; Singh *et al.*, 2011), tillering (Devender-Reddy and Mittra, 1985; Reddy *et al.*, 1985), flowering (Devender-Reddy and Mittra, 1985; Kotera and Nawata, 2007), and beginning of seed development (Kotera *et al.*, 2005). The results from the current study of the effects of flooding post-anthesis are in agreement with the eight reports above: there was subsequent loss of rice seed quality due to simulated flooding at different seed developmental stages.

In Chapter 2, it was concluded that the severity of yield loss and detriment to seed quality due to mimicking submergence after anthesis depended upon when it occurred. This is summarised in Figure 5.1. Vulnerability to flooding begins at the early stage of grain-filling and sensitivity to flood damage through pre-harvest sprouting increased thereafter to a maximum in late maturation drying. Submergence at the early seed filling stage of *japonica* rice cv. Gleva (i.e. milk stage; 9 or 10 DAA) reduced grain weight (Fig. 5.1a). On the other hand, flooding at mid- (20 DAA) to late- (40 DAA) maturity provided two kinds of results (Fig. 5.1b); light-weight-non-sprouted or sprouted seeds. Prolonged submergence for five rather than three days increased the number of seeds that sprouted, but only when submergence occurred at late maturity (40 DAA). These investigations were carried out at the pot-experimental scale and may not be appropriate for extrapolation to the field scale for yield. Nevertheless, the results of the current study showed that simulated flooding at early grain filling (9 or 10 DAA) caused greater yield loss (i.e. the total sprouted and non-sprouted seed yield per pot), up to 32%, in cv. Gleva than submergence later in seed development (Table 2.3). In terms of ability to germinate normally in storage after flooded stress of cv. Gleva, the results here were a little surprising; there was a negligible effect on subsequent longevity (p_{50}) caused by simulated flooding amongst the non-sprouted seed fractions. Submergence at early grain filling reduced p_{50} slightly, but later in seed development had little detriment or sometimes the estimates of p_{50} were increased (Fig. 2.9).

Pre-harvest sprouted seeds were not detected when submergence occurred at the beginning of grain filling. The number of such seeds increased the later during seed development that submergence occurred: around 40 DAA was the worst developmental stage for submergence resulting in sprouted seeds. Sprouted seed had poor germinability postharvest, with up to 60% reduction in ability to germinate if this fraction was included within the seed lot (i.e. sprouted and non-sprouted seed within the sample) (Fig. 2.7). This thesis did not examine the storability of sprouted seeds, but this was not necessary as many sprouted seed were unable to survive desiccation. The most vulnerable seed developmental stage to simulated flooding therefore, was submergence at late maturity in terms of the number of sprouted seeds and seed quality (i.e. germinability): the more the mature seed at simulated flooding the lower the non-sprouted seed fraction, and hence the greater severity of damage to seed quality postharvest.

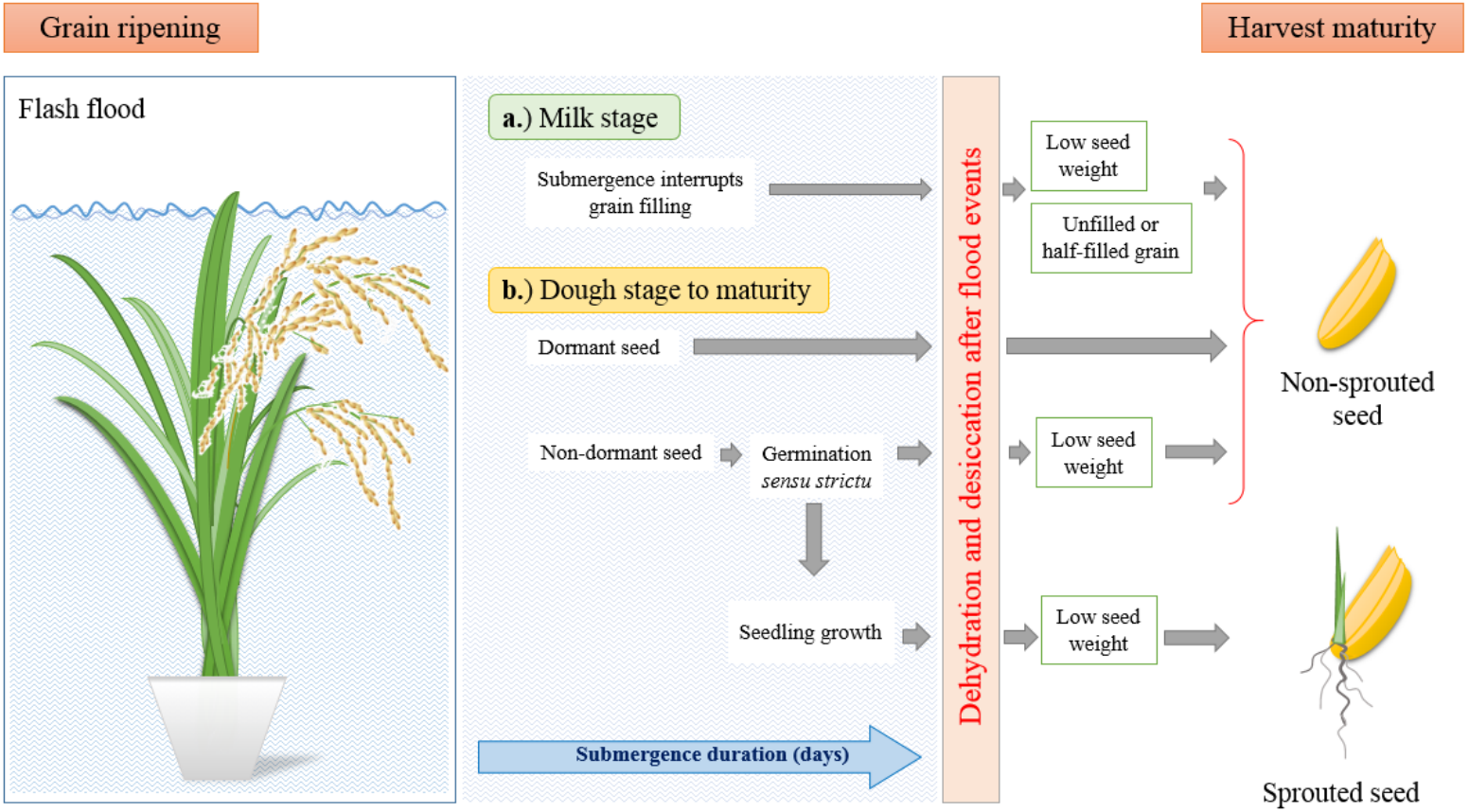

Figure 5.1 Summary of the effect of submergence during grain ripening on seed physiological quality. Simulation of flooding during early grain filling (i.e. milk stage, 9-10 DAA) may interrupt seed dry matter accumulation, and thus lead to unfilled or light-weight filled seed (a). Flooding at later seed developmental stages (i.e. dough and maturity stages, 14-40 DAA) may trigger the germination process to begin, and hence pre-harvest sprouting may be observed (b). Rice cultivars with high seed dormancy during maturation, for example cvs IR64 and IR64 Sub1 were better able to cope with flood conditions than cv. Gleva, with less reduction in 1000 seed weight, and lower percentage sprouted seed detected.

Selecting rice cultivars that tolerate submergence during grain ripening and maturation is the most suitable crop production practice to reduce flood damage. Although reduced yield per pot (Fig. 2.15) and thousand seed weight (Fig. 2.17) were detected in submergence treatments of all three cultivars, *indica* rice cvs IR64 and IR64 Sub1 were far less sensitive to submergence than *japonica* cv. Gleva (Figs 2.18 and 5.1). The former two *indica* rices, moreover, showed greater dormancy (i.e. less than 1% of pre-harvest sprouted seeds, Fig. 2.16) even when submergence occurred at late maturity (40 DAA). Further, there was a less negative impact on subsequent seed longevity (p_{50}) due to submergence treatments in cvs IR64 and IR64 Sub1 than in cv. Gleva (Fig. 2.21).

Seed storage longevity post-harvest is influenced by the environment pre-harvest (Roberts, 1972), in which extreme growing conditions (e.g. drought, heat, cold, heavy rainfall) during seed development and maturation can reduce subsequent seed longevity (Ellis *et al.,* 1993; Ellis and Hong, 1994; Kochanek *et al.,* 2010; Ellis, 2011; Kochanek *et al.,* 2011; Mondoni *et al.,* 2011; Gajender, 2013). The findings in this study are broadly similar to those findings, but the impact on longevity from submergence was mild for non-sprouted seeds. Other examples reported earlier show limited or no environmental effects on longevity included shading after anthesis of spring barley (Pieta-Filho and Ellis, 1991) and high carbon dioxide (mean concentration of 684 µmol CO_2 mol^{-1} air) in winter wheat (Sanhewe *et al.,* 1996); in both cases, little or no effect on subsequent ability to germinate normally or on longevity were detected. Thus factors that affect crop production (in this case, incident radiation, and carbon dioxide concentration) may not necessarily affect seed quality, and *vice versa.*

Ellis and Hong (2007) proposed constant values for the estimation of rice seed viability in hermetic air-dry storage for different periods as quantified by the seed viability equation of Ellis and Robert (1980a). Comparison of those predictions with the results of this study showed that the estimated standard deviation of the frequency distribution of seed death in time (σ, days) in the current study were generally smaller than the estimates provided by the constants suggested by the above authors (Fig. 5.2). Nevertheless, overprediction occurred mainly in cv. Gleva, whereas the observations for cvs IR64 and IR64 Sub1 showed closer agreement with the estimates from Ellis and Hong (2007). This suggests that besides the effect of inter-specific differences in longevity shown, for example by Ellis and Hong (2007), intra-specific differences in the relation between σ and seed storage moisture content were evident here with the two *indica* cultivars showing greater longevity than the *japonica* cultivar.

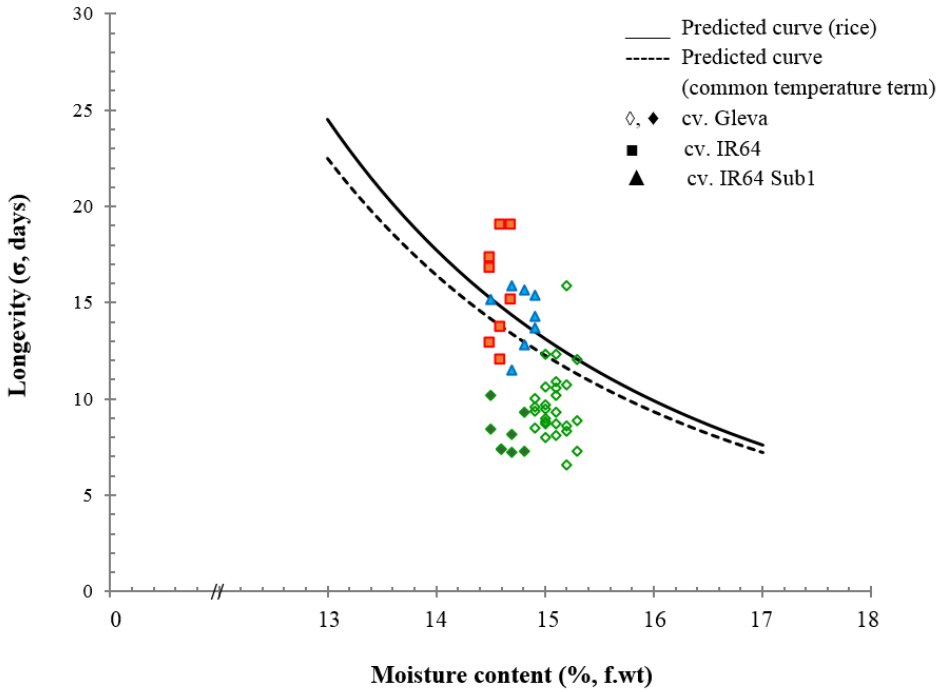

Figure 5.2 Relationship between the standard deviation of the frequency distribution of seed death in time (σ, days) and seed moisture content in hermetic storage at 40°C. The continuous and broken curves are derived from the seed viability equation and the estimates of the viability constants K_E, C_W, C_H, and C_Q for rice (*Oryza sativa*) provided by Ellis and Hong (2007), where the broken curve is derived from constants constrained to a common temperature term for all 12 crops investigated and the continuous curve with temperature term estimated fron the data for rice alone. The observations are the estimates for σ, provided by probit analysis, reported for the current studies in 2012 (open symbols) and 2013 (solid symbols) for rice cvs Gleva (◊, ♦), IR64 (■), and IR64 Sub1 (▲).

From the results in this thesis, it is possible to answer several of the general hypotheses posed at the beginning of the research, as follows;

1) Submergence during seed development has no effect on yield loss and subsequent seed quality

a.) Submergence at all seed developmental stage results in similar impact on yield loss and subsequent seed quality

b.) Flooding duration variation results in similar impact on yield loss and subsequent seed quality

c.) Flooding susceptibility of indica and japonica rice is the same

Simulated flooding at early grain filling lead to yield loss (in the sense of mean thousand seed weight) due to the reduction of filled seeds. The loss was more severe if flooding occurred at late maturity because the wet conditions encouraged pre-harvest sprouting, thus contributing to loss in assimilates. The loss in yield was also greater the longer the duration of submergence, because more seed sprouted. Vulnerability to submergence of *indica* rice cvs IR64 and IR64 Sub1 was less than *japonica* rice cv. Gleva, resulting in greater yield stability and very few sprouted seeds even when submergence occurred at harvest maturity. In contrast to the substantial effect on yield loss, there was only a small impact on longevity of all three genotypes caused by submergence at different seed developmental stages: cv. Gleva showed poorer p_{50} than cvs IR64 and IR64 Sub1 after control and flooded seed production environments. Thus, the core problem from flood events during grain ripening was yield and subsequent seed quality losses due to pre-harvest sprouting of seeds.

Using submergence-tolerant rice, especially varieties with *Submergence1* gene, is helpful for farmers in flood-prone areas. The study here confirms that there was no deleterious impact on seed storability affected by introgression of the submergence-tolerant gene into the elite rice cultivar IR64. The studies in Chapter 3 provide assurance that the gene *Submergence1* with allele *Sub1A-1* was present (Figs 3.1 and 3.2) and cv. IR64 Sub1 showed a similar pattern of seed loss viability in hermetic storage as the parental line cv. IR64 (Fig. 3.4). Therefore, in response to the following hypothesis;

2) Introgression of Submergence1 gene into rice has no impact on seed storability

The results from this study support this null hypothesis.

The severe problem in rice seed production from flooding post-anthesis in the current research was largely limited to pre-harvest sprouting. This issue should be set as a priority in terms of the solutions to prevent sprouting on panicles due to flood events during the ripening phase. The variations amongst genotypes are important in this regard. Some *indica* rice as well as submergence-tolerant cultivars in the group of '*Sub1* Mega varieties" provide great advantages to rice production. Nevertheless, local rice ecosystems (e.g. sub-tropical areas), or customer preferences (i.e. grain shape and cooking quality) may limit the wide adoption of such rices. Therefore, in addition to using less-susceptible *indica* or *japonica* rices where available and suitable, foliar application of molybdenum during the flag leaf phase could also help to alleviate the sprouting problem. Ammonium molybdate at 100 mg L^{-1} provided the best results; less reduction in seed yield per plant (13-21%, Fig. 4.2) and lower sprouted seed percentage (15-21%, Fig.4.3) where flooding occurred at 20 or 30 DAA (Chapter 4). Thus, the results from this study reject the hypothesis below;

3) Molybdenum foliar spray has no effect on pre-harvest sprouting

The reduction in pre-harvest sprouting from molybdenum treatment has been explained by encouraging plant hormone abscisic acid biosynthesis (Tanner, 1978; Cairns and Kritzinger, 1992; Modi and Cairns, 1994; Modi and Kritzinger, 1995; Cairns *et al.*, 1997). Therefore, seed dormancy (i.e. temporary loss of ability to germinate even though favourable conditions are provided) was elevated. In this study, foliar application of molybdenum could moderate the detrimental impact from pre-harvest sprouting after simulated flooding. However, the relation between molybdenum-treated plants and ABA content detected before and after submergence was unclear. A more frequent monitoring of ABA content after spray treatment with molybdenum should be carried out to examine the changing pattern of seed ABA content. In that way it should be possible to determine whether molybdenum provided at the flag leaf stage was contributing to ABA synthesis or not. The present study had a small reduction in pre-harvest sprouting obtained from molybdenum spray, nevertheless it provided promise of an ultimate solution for rice varieties vulnerable to pre-harvest sprouting. An implication, is that rice varieties that have middle or high tolerance (i.e. moderate or high dormant varieties) to wet weather, might become free of sprouting when flooded after such a treatment. Therefore, intensive investigations in physiology may need to identify the mechanism of how molybdenum affects pre-harvest sprouting. If the assumptions above can be confirmed, this would provide a solution to pre-harvest sprouting due to unpredictable floods, erratic rainfall, or lodging.

Selection of an appropriate variety that confers submergence tolerance is the most important practice that can reduce flood damage. The findings in the current research provide the confidence for farmers and rice seed producers that the *Sub1* modified varieties, which are of benefit to early crop growth in flooded conditions (Septiningsih *et al.*, 2009; Mackill *et al.*, 2010; Singh *et al.*, 2011), are of no detriment to subsequent seed storage quality under normal or flooded growing environments. The current thesis found that adverse effects of submergence were mainly through yield loss and pre-harvest sprouting, with a negligible impact on subsequent seed longevity post-harvest. However, it should be noted that the small effect on storability of the former finding was tested with submergence durations up to five days only, and the investigation was a small pot-experiment. Particularly in submergence-tolerant rice varieties, therefore, the effect of prolonged submergence (i.e. more than five days) at ripening phase and maturation on subsequent seed germinability and longevity in field conditions should be investigated, notwithstanding that the visible signs of germination may not be detected at harvest.

The deleterious effect of flooding on rice leading to pre-harvest sprouting is at its greatest close to harvest time. Molecular approaches may help to solve this problem sustainably: identification and fine mapping of major QTL of rice pre-harvest sprouting resistance is suggested. Introgression of such gene(s) into elite rice cultivars could then be made. If the seeds can be prevented from sprouting in panicles in wet weather or flooded conditions before harvest, any effects on postharvest seed storage survival are unlikely to be of concern given the results in this thesis. Ideally, improved varieties would solve the pre-harvest sprouting problem without the need for chemical treatment. However, my study showed that Mo spraying was able to reduce pre-harvest sprouting and at a reasonable cost (Chapter 4). Nevertheless, considerable site- and variety-specific research would be needed to optimise and evaluate the commercial value before this treatment could be recommended to farmers routinely.

In conclusion, there are six main findings from this thesis;

1. Submergence post-anthesis damages rice production, in which the severity of yield loss depending upon rice genotype.
2. In all cases, yield loss was greater the longer the submergence duration and the later in seed development it occurred.
3. Despite detrimental impacts on seed yield due to submergence, the effect on the subsequent air-dry seed longevity of the non-sprouted seed fraction was negligible.

4. Indica rice cvs IR64 and IR64 Sub1 showed superior crop performance than japonica cv. Gleva under both control and flooded environments resulting in greater yield stability, fewer pre-harvest sprouted seeds, and longer seed viability post-harvest.

5. Introgression of *Sub1* gene into the high-yielding rice cultivar IR64 caused no deleterious effect on subsequent seed longevity.

6. Although a significant difference was not detected between foliar sprays, the supply of Mo at 100 mg L^{-1} at the flag leaf stage showed great efficiency in reducing sprouted seed after simulated flooding by up to 20%. This treatment, furthermore, resulted in less yield loss and seed weight reduction

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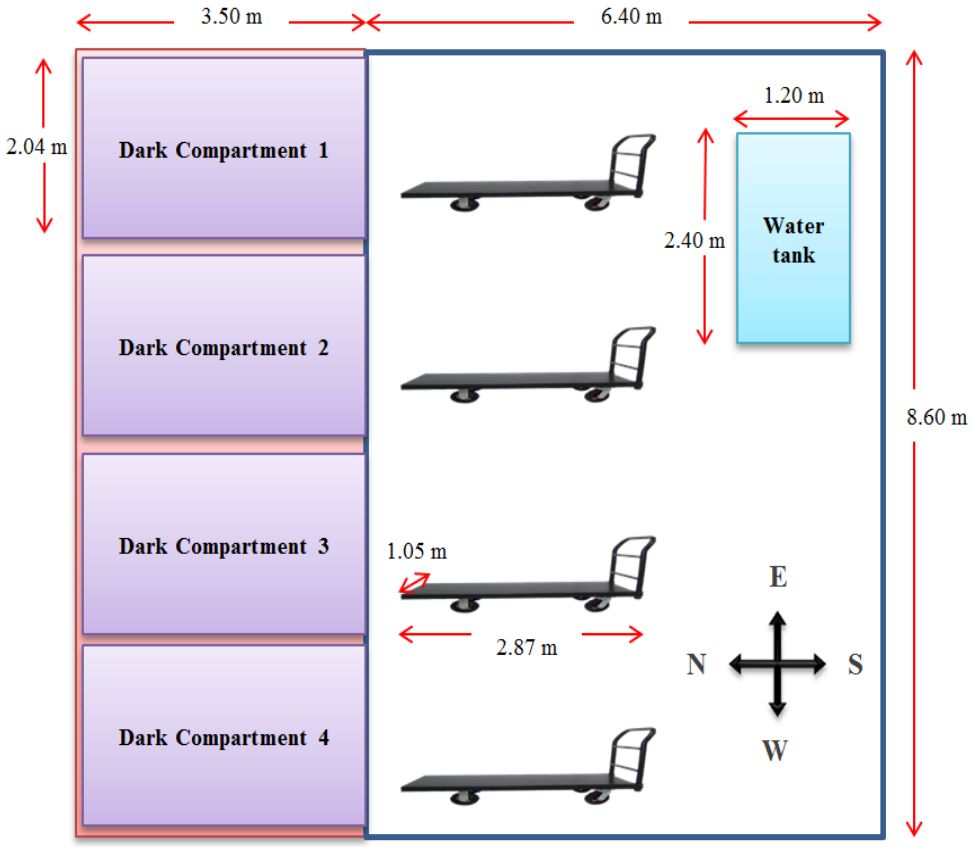
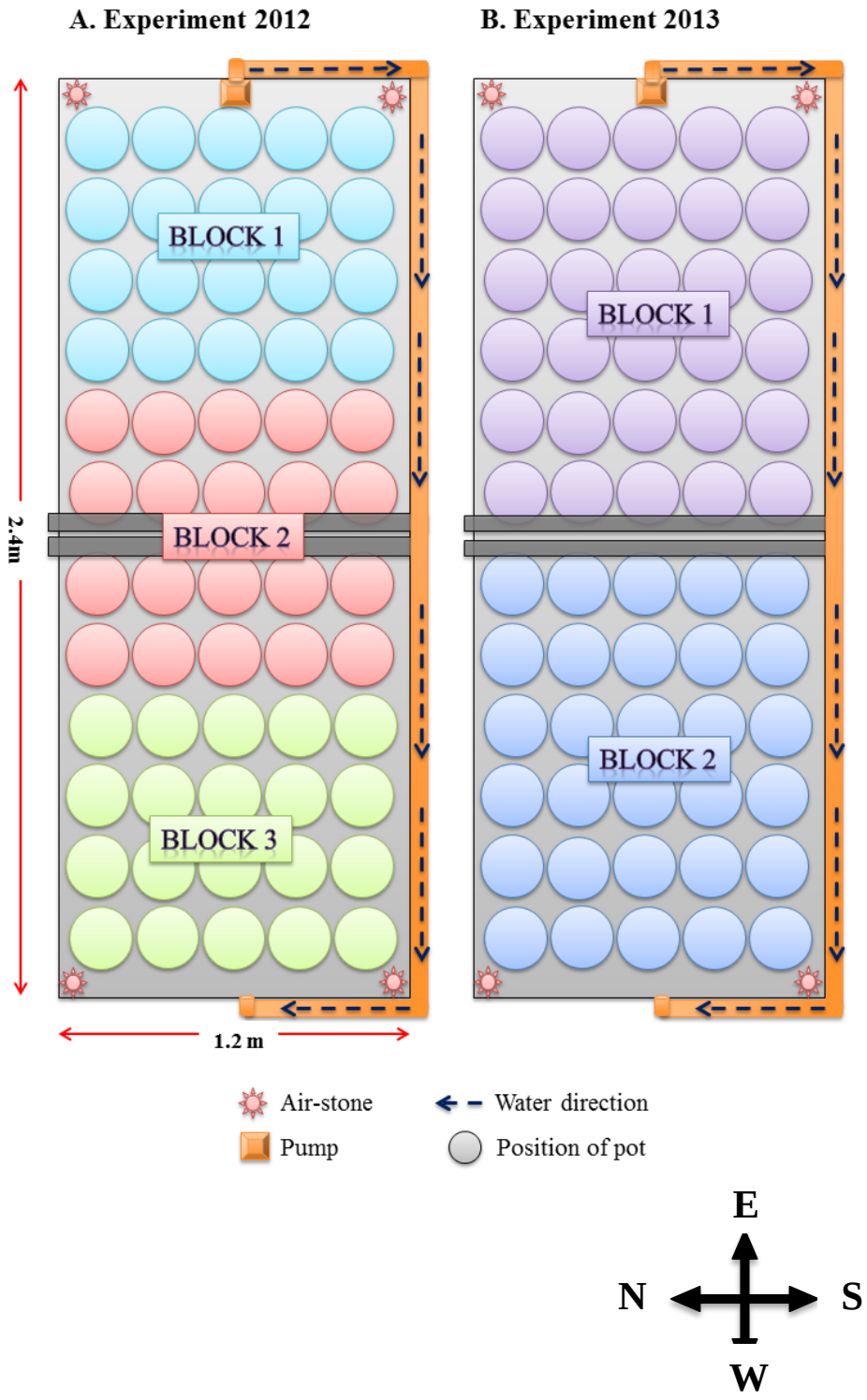
Appendices

Appendix 2.1 Layout inside the glasshouse showing the area of night compartments, trolleys, and the water tank for submergence treatments



Appendix 2.2 Layout inside wooden tank showing pot position including water circulation and aeration system during submergence treatments in 2012 (A) and 2013 (B)



Appendix 2.3 Analysis of variance of yield per pot (dry matter, g) (2012)

Appendix 2.3a One-way ANOVA with 9 treatments (Control included)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	2	14.01	7.00	0.65	
BLOCK.*Units* stratum					
Treatment	8	128.31	16.04	1.49	0.235
Residual	16	171.69	10.73		
Total	26	314.01			

Appendix 2.3b Two-way ANOVA with two factors, i.e. seed development stage and submergence duration (Control excluded)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	2	8.62	4.31	0.36	
BLOCK.*Units* stratum					
Seed development (DAA)	3	70.93	23.64	2	0.160
Submergence duration (d)	1	6.22	6.22	0.53	0.480
DAA . D	3	45.97	15.32	1.3	0.314
Residual	14	165.43	11.82		
Total	23	297.17			

Tables of means

Grand mean 10.42

Seed development (DAA)	9	30	35	40
	7.93	12.59	9.93	11.24

Submergence duration (d)	3	5
	10.93	9.92

	Submergence duration (d)	
Seed development (DAA)	3	5
9	8.0	7.9
30	15.4	9.8
35	9.9	10.0
40	10.4	12.1

Appendix 2.4 Analysis of variance of percentage of pre-harvest sprouting. The data were arcsine transformed (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	2	135.76	67.88	6.49	
BLOCK.*Units* stratum					
Treatment	8	7243.44	905.43	86.57	<.001
Residual	16	167.35	10.46		
Total	26	7546.56			

Least significant differences of means (5% level)

Table	Treatment
rep.	3
d.f.	16
l.s.d.	5.598

Appendix 2.5 Interaction between duration and timing of submergence on pre-harvest sprouting (%) (2012)

Appendix 2.5a Interaction between treatments included Control (9 treatments in total) was performed by restriced maximum likelihood analysis (REML)

Response variate:	PHS_%_Arcsine
Fixed model:	Constant + DAA + d + DAA.d
Random model:	BLOCK
Number of units:	27

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	*P*
Seed developmental stage (DAA)	657.17	4	164.29	16	<0.001
Sumergence duration (d)	22.37	1	22.37	16	<0.001
DAA . d	12.98	3	4.33	16	0.021

Appendix 2.5b Two-way ANOVA with two factors, i.e. seed development stage and submergence duration (Control excluded)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	2	261.21	130.61	6.47	
BLOCK.*Units* stratum					
Seed development (DAA)	3	6528.1	2176.03	107.77	<.001
Submergence duration (d)	1	449.6	449.6	22.27	<.001
DAA . d	3	405.8	135.27	6.7	0.005
Residual	14	282.68	20.19		
Total	23	7927.39			

Least significant differences of means (5% level)

Table	DAA	d	DAA . d
rep.	6	12	3
d.f.	14	14	14
l.s.d.	5.564	3.935	7.869

Tables of means

Grand mean 18.72

Seed development (DAA)	9	30	35	40
	0c	7c	27b	41.2a

Submergence duration (d)	3	5
	14b	23a

	Submergence duration (d)	
Seed development (DAA)	3	5
9	0	0
30	4	10
35	24	30
40	31	53

Appendix 2.6 Analysis of variance of moisture content of freshly harvested seed (%) (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	1.070	0.535	0.38	
Block.*Units* stratum					
Treatment	8	37.478	4.685	3.35	0.019
Residual	16	22.395	1.400		
Total	26	60.942			

Least significant differences of means (5% level)

Table	Treatment
rep.	6
d.f.	16
l.s.d.	2.048

Appendix 2.7 Interaction between duration and time of submergence treatment on moisture content of fresh seed at harvest (%) (2012)

Appendix 2.7a Interaction analysis was included Control and carried out using REML

REML variance components analysis
Response variate: %MC at Harvest
Fixed model: Constant + DAA+ d + DAA . d
Random model: Block + Block.Rep
Number of units: 54

Tests for fixed effects

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	*P*
Developmental stage (DAA)	25.28	4	6.32	45	<0.001
Submergence duration (d)	2.15	1	2.15	45	0.150
DAA .D	5.89	3	1.96	45	0.133

Appendix 2.7b Interaction analysis was excluded Control and carried out using 2-ways-ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	2	1.501	0.75	0.48	
BLOCK.*Units* stratum					
Seed development (DAA)	3	14.797	4.932	3.17	0.058
Submergence duration (d)	1	2.414	2.414	1.55	0.233
DAA . D	3	6.628	2.209	1.42	0.279
Residual	14	21.786	1.556		
Total	23	47.125			

Tables of means

Grand mean 19.40

Seed development (DAA)	9	30	35	40
	20.2	20.1	19.0	18.3

Submergence duration (d)	3	5
	19.7	19.1

	Submergence duration (d)	
Seed development (DAA)	3	5
9	19.7	20.7
30	20.5	19.6
35	20.0	18.1
40	18.7	17.9

Appendix 2.8 Analysis of variance of dry seed weight (g) at 0% moisture content (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	2	0.82976	0.41488	4.16	
BLOCK.*Units* stratum					
Treatments	8	10.77276	1.34659	13.50	<.001
Residual	16	1.59598	0.09975		
Total	26	13.19850			

Least significant differences of means (5% level)

Table	Treatments
rep.	3
d.f.	16
l.s.d.	0.547

Appendix 2.9 Interaction between duration and timing of submergence on dry seed weight (0% moisture content, g) (2012)

Appendix 2.9a Interaction analysis was included Control and carried out using REML

Response variate: Wg_of_1000_Dry_seed_g
Fixed model: Constant + DAA + d + DAA . d
Random model: BLOCK
Number of units: 27

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	*P*
Seed developmental stage (DAA)	95.74	4	23.93	16	<0.001
Submergence duration (d)	0.26	1	0.26	16	0.618
DAA . d	0.01	3	0	16	1.000

Appendix 2.9b Interaction analysis was excluded Control and carried out using 2-ways-ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	2	0.899	0.4495	3.22	
BLOCK.*Units* stratum					
Seed development (DAA)	3	6.8453	2.2818	16.37	<.001
Submergence duration (d)	1	3.921	3.921	28.13	<.001
DAA . D	3	0.2008	0.0669	0.48	0.701
Residual	14	1.9515	0.1394		
Total	23	13.8176			

Least significant differences of means (5% level)

Table	DAA	d	DAA . D
rep.	6	12	3
d.f.	14	14	14
l.s.d.	0.4623	0.3269	0.6538

Tables of means

Grand mean 32.211

Seed development (DAA)	9	30	35	40
	31.5b	31.9b	32.8a	32.6a

Submergence duration (d)	3	5
	32.6a	31.8b

	Submergence duration (d)	
Seed development (DAA)	3	5
9	32.0	30.9
30	32.3	31.5
35	33.2	32.5
40	32.9	32.3

Appendix 2.10 Analysis of variance of germinability of freshly- harvested seed (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	4.741	2.37	0.34	
Block.*Units* stratum					
Treatment	8	199.852	24.981	3.57	0.014
Residual	16	111.926	6.995		
Total	26	316.519			

Least significant differences of means (5% level)

Table	Treatment
rep.	3
d.f.	16
l.s.d.	4.578

Appendix 2.11 Analysis of variance of germinability of dry seed (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	5.95	2.975	0.49	
Block.*Units* stratum					
Treatment	8	113.155	14.144	2.31	0.073
Residual	16	97.906	6.119		
Total	26	217.012			

Least significant differences of means (5% level)

Table	Treatment
rep.	3
d.f.	16
l.s.d.	4.282

Appendix 2.12 Interaction of ability to germinate normally by using freshly-harvested and dried seeds (at 15±0.2% moisture content) (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	1.917	0.958	0.15	
Block.*Units* stratum					
Treatment	8	234.927	29.366	4.57	<.001
Type of seed	1	13.226	13.226	2.06	0.161
Treatment x Type of seed	8	78.073	9.759	1.52	0.188
Residual	34	218.642	6.431		
Total	53	546.785			

Tables of means

Grand mean : 95.10

Treatments	30-3d	30-5d	35-3d	35-5d	40-3d	40-5d
	95.37	95.29	96.69	97.23	94.38	92.01

9-3d	9-5d	Control
95.61	91.39	97.90

Types of seed	Dry seed	Fresh seed
	94.60	95.59

Least significant differences of means (5% level)

Table	Treatment	Types of seed	Treatment Types of seed
rep.	6	27	3
d.f.	34	34	34
l.s.d.	2.975	1.403	4.208

Tukey's 95% confidence intervals

Treatment

	Mean	
Control	97.90	a
35-5d	97.23	a
35-3d	96.69	ab
9-3d	95.61	abc
30-3d	95.37	abc
30-5d	95.29	abc
40-3d	94.38	abc
40-5d	92.01	bc
9-5d	91.39	c

Appendix 2.13 Residuals deviance analysis table for comparing models of loss of viability in hermetic storage at 40 °C with 15.0 ± 0.2% moisture content (2012)

F-test	Res dev	Res d.f.	Res Mean dev
Common slope	4530	733	6.181
Best model	4372	725	6.031
	158	8	
		19.750	
		3.275	
F (0.05, 8, 733)		=	1.951
	P	=	**0.001**
F-test	Res dev	Res d.f.	Res Mean dev
Common line	5731	741	7.734
Best model	4372	725	6.031
	1359	16	
		84.938	
		14.083	
F (0.05, 16, 725)		=	1.657
	P	=	**0.000**
F-test	Res dev	Res d.f.	Res Mean dev
Common Ki	5165	733	7.046
Best model	4372	725	6.031
	793	8	
		99.125	
		16.436	
F (0.05, 8, 725)		=	1.951
	P	=	**0.000**

Appendix 2.14 Analysis of variance of the estimate of constant K_i (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	0.70104	0.35052	9.98	
Block.*Units* stratum					
Treatment	8	2.3672	0.2959	8.43	<.001
Residual	16	0.56192	0.03512		
Total	26	3.63016			

Least significant differences of means (5% level)

Table	Treatment
rep.	3
d.f.	16
l.s.d.	0.3244

Appendix 2.15 Analysis of variance of estimate of standard deviation (σ) (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	43.514	21.757	14.55	
Block.*Units* stratum					
Treatment	8	23.323	2.915	1.95	0.122
Residual	16	23.927	1.495		
Total	26	90.764			

Least significant differences of means (5% level)

Table	Treatment
rep.	3
d.f.	16
l.s.d.	2.117

Appendix 2.16 Analysis of variance of estimate of p_{50} (2012)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	26.636	13.318	3.58	
Block.*Units* stratum					
Treatment	8	70.294	8.787	2.36	0.068
Residual	16	59.499	3.719		
Total	26	156.429			

Least significant differences of means (5% level)

Table	Treatment
rep.	3
d.f.	16
l.s.d.	3.338

Least significant differences of means (20% level)

Table	Treatment
rep.	3
d.f.	16
l.s.d.	2.105

Appendix 2.17 Interaction of grouping between duration of submergence and time of submergence treatment on seed viability (2012)

Appendix 2.17.1 The effect of submergence duration (3, 5 days or non- submergence) on differences of seed viability.

	Res dev	Res d.f.	Res Mean dev
Duration of submergence	4395	729	6.029
Individual treatments ($9K_i$, 9σ)	4372	725	6.031
	23	4	
		5.750	
		0.953	
F (0.05, 4, 725)		=	2.384
	P	=	**0.432**

Appendix 2.17.2 The effect of seed age when submergence occur (from 9, 30, 35, 40DAA and non-submergence) on differences of seed viability.

	Res dev	Res d.f.	Res Mean dev
Times of submergence treatment	4461	731	6.102
Individual treatments ($9K_i$, 9σ)	4372	725	6.031
	89	6	
		14.833	
		2.460	
F (0.05, 6, 725)		=	2.111
	P	=	**0.023**

Appendix 2.18 Comparison of fitted-models for seed survival curves of cv. Gleva showing the effect of seed age when submergence occur (from 9, 30, 35, 40 DAA and non-submergence) on differences of seed viability (Appendix 2.18.2) using paired F-test of residual deviance (2012)

	Res dev	Res d.f.	Res Mean dev	Critical F-value	*P*
Control - 9 DAA					
Common line	2188	262	8.350		
Best model	1725	260	6.634		
	463	2			
		231.5			
		34.90		F(0.05, 2, 260) = 3.031	0.000
Control - 30 DAA					
Common line	1946	264	7.373		
Best model	1908	262	7.284		
	38	2			
		19.0			
		2.61		F(0.05, 2, 262) = 3.030	0.076
Control - 35 DAA					
Common line	1602	247	6.486		
Best model	1576	245	6.431		
	26	2			
		13.0			
		2.02		F(0.05, 2, 245) = 3.033	0.135
Control - 40 DAA					
Common line	1467	223	6.581		
Best model	1173	221	5.307		
	294	2			
		147.0			
		27.70		F(0.05, 2, 221) = 3.037	0.000
9 DAA - 30 DAA					
Common line	2946	354	8.323		
Best model	2500	352	7.101		
	446	2			
		223.0			
		31.40		F(0.05, 2, 352) = 3.021	0.000

Appendix 2.18 (continued)

	Res dev	Res d.f.	Res Mean dev	Critical F-value	*P*
9 DAA - 35 DAA					
Common line	2781	337	8.252		
Best model	2167	335	6.468		
	614	2			
		307.0			
		47.46		F(0.05, 2, 335) = 3.023	0.000
9 DAA - 40 DAA					
Common line	1791	312	5.741		
Best model	1764	310	5.689		
	27	2			
		13.5			
		2.37		F(0.05, 2, 310) = 3.025	0.095
30 DAA - 35 DAA					
Common line	2370	339	6.991		
Best model	2350	337	6.975		
	20	2			
		10.0			
		1.43		F(0.05, 2, 337) = 3.023	0.240
30 DAA - 40 DAA					
Common line	2176	315	6.907		
Best model	1948	313	6.223		
	228	2			
		114.0			
		18.32		F(0.05, 2, 313) = 3.025	0.000
35 DAA - 40 DAA					
Common line	1965	298	6.595		
Best model	1615	296	5.456		
	350	2			
		175.0			
		32.07		F(0.05, 2, 296) = 3.026	0.000

Appendix 2.19 The broken-stick model of grain filling to estimate mass maturity and coefficient of determination, R^2.

cv. Gleva

Source	d.f.	s.s.	m.s.	v.r.
Regression	3	5871.2019	57.068	718.73
Residual	7	19.06	2.723	
Total	10	5890.27	589.027	

Percentage variance accounted for 97.2

Standard error of observations is estimated to be 1.65.

Estimates of parameters

Parameter	estimate	s.e.
Breakpoint_X * Linear	27.570	1.170
Breakpoint_Y	29.467	0.738
Slope 1	1.371	0.118

X value at intersection of lines (Mass maturity)

X value 27.566, approximate s.e. 1.174
95% confidence interval (24.05, 30.89)

Intercepts

Y-axis	-8.332
X-axis (line 1)	6.077
X-axis (line 2)	*

cv. IR64

Source	d.f.	s.s.	m.s.	v.r.
Regression	3	2663.11 8	87.703	612.88
Residual	7	10.14	1.448	
Total	10	2673.25	267.325	

Percentage variance accounted for 97.0

Standard error of observations is estimated to be 1.20.

Estimates of parameters

Parameter	estimate	s.e.
Breakpoint_X	27.480	1.210
* Linear		
Breakpoint_Y	19.756	0.538
Slope_1	0.9316	0.0806

X value at intersection of lines (Mass maturity)

X value 27.478, approximate s.e. 1.208
95% confidence interval (24.66, 30.88)

Intercepts

Y-axis -5.844
X-axis (line 1) 6.272
X-axis (line 2) *

cv. IR64 Sub1

Source	d.f.	s.s.	m.s.	v.r.
Regression	3	2552.26	850.753	128.28
Residual	5	33.16	6.632	
Total	8	2585.42	323.178	

Percentage variance accounted for 78.9

Standard error of observations is estimated to be 2.58.

Estimates of parameters

Parameter	estimate	s.e.
Breakpoint_X	23.98	3.09
* Linear		
Breakpoint_Y	20.29	1.15
Slope_1	1.080	0.361

X value at intersection of lines (Mass maturity)

X value 23.983, approximate s.e. 3.093
95% confidence interval (18.76, 37.25)

Intercepts

Y-axis -5.610
X-axis (line 1) 5.195
X-axis (line 2) *

Appendix 2.20 Analysis of variance of yield (g/pot) measured at 0% moisture content (2013)

Appendix 2.20a One-way ANOVA within cultivar

cv. Gleva

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	2.59	2.59	0.22	
Block.*Units* stratum					
Treatment	3	47.21	15.74	1.34	0.407
Residual	3	35.11	11.70		
Total	7	84.91			

cv. IR64

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	8.738	8.783	4.81	
Block.*Units* stratum					
Treatment	3	18.189	6.063	3.32	0.175
Residual	3	5.478	1.826		
Total	7	32.450			

cv. IR64-Sub1

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	18.256	18.259	4.85	
Block.*Units* stratum					
Treatment	3	24.906	8.302	2.20	0.267
Residual	3	11.300	3.767		
Total	7	54.465			

Appendix 2.20b Interaction analysis between seed development stage and cultivar using 2-ways-ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	1	26.084	26.084	5.18	
BLOCK.*Units* stratum					
Seed development (DAA)	3	64.468	21.489	4.26	0.032
Cultivar (cv)	2	55.185	27.592	5.48	0.022
DAA .cv	6	25.833	4.305	0.85	0.556
Residual	11	55.436	5.04		
Total	23	227.005			

Least significant differences of means (5% level)

Table	DAA	cv	DAA . Cv
rep.	6	8	2
d.f.	11	11	11
l.s.d.	2.853	2.471	4.941

Tables of means

Grand mean 12.63

Seed development (DAA)	Control	10	30	40
	14.5a	10.7a	11.4a	14.0a

Cultivar (cv)	Gleva	IR64	IR64 Sub1
	11.2b	14.7a	11.9ab

	Cultivar (cv)		
Seed development (DAA)	Gleva	IR64	IR64 Sub1
Control	26.6	28.8	27.0
10	28.6	30.4	28.9
30	27.6	23.6	21.9
40	25.9	26.9	20.1

Appendix 2.21 Analysis of variance of pre-harvest sprouting seed percentage (2013)

cv. Gleva

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.141	0.141	0.02	
Block.*Units* stratum					
Treatment	3	6216.956	2072.319	243.51	<.001
Residual	3	25.53	8.51		
Total	7	6242.628			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	9.28

cv. IR64

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.02226	0.02226	1	
Block.*Units* stratum					
Treatment	3	0.34801	0.116	5.21	0.104
Residual	3	0.06679	0.02226		
Total	7	0.43706			

cv. IR64 Sub1

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.007262	0.007262	1	
Block.*Units* stratum					
Treatment	3	0.18556	0.061853	8.52	0.056
Residual	3	0.021786	0.007262		
Total	7	0.214608			

Appendix 2.22 Interaction between submergence treatment and cultivars on pre-harvest sprouting percentage (2013)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.124	0.124	0.05	
Block.*Units* stratum					
CV	2	1809.513	904.756	387.77	<.001
Treatment	3	2125.987	708.662	303.73	<.001
CV . Treatment	6	4091.503	681.917	292.26	<.001
Residual	11	25.666	2.333		
Total	23	8052.792			

Least significant differences of means (5% level)

Table	CV	Treatment	CV Treatment		Mean (ASIN)	
rep.	8	6	2	Gleva	18.524	a
d.f.	11	11	11	IR64	0.12	b
l.s.d.	1.681	1.941	3.362	IR64-Sub1	0.088	b

Least significant differences of means (5% level)

Table	Treatment	CV	Treatment CV		Mean (ASIN)	
				1 Control	0	b
rep.	6	8	2	2 at 10 DAA	0	b
d.f.	11	11	11	3 at 30 DAA	2.529	b
l.s.d.	1.941	1.681	3.362	4 at 40 DAA	22.447	a

Appendix 2.23 Analysis of variance of moisture content (%) of freshly harvested seed of cvs Gleva, IR64 and IR64 Sub1 (2013)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.0007	0.0007	0	
Block.*Units* stratum					
Treatment	11	415.3041	37.7549	43.25	<.001
Residual	35	30.5536	0.873		
Total	47	445.8584			

Least significant differences of means (5% level)

Table	Treatment
rep.	4
d.f.	35
l.s.d.	1.341

Appendix 2.24 Analysis of variance of moisture content (%) of freshly harvested seed among genotype (2013)

cv. Gleva

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.3257	0.3257	1.44	
Block.*Units* stratum					
Treatment	3	16.6406	5.5469	24.54	<.001
Residual	11	2.4861	0.2260		
Total	15	19.4524			

Least significant differences of means (5% level)

Table	Treatment
rep.	4
d.f.	11
l.s.d.	0.74

cv. IR64

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.453	0.453	0.31	
Block.*Units* stratum					
Treatment	3	104.273	34.758	24.1	<.001
Residual	11	15.864	1.442		
Total	15	120.591			

Least significant differences of means (5% level)

Table	Treatment
rep.	4
d.f.	11
l.s.d.	1.869

cv. IR64 Sub1

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.022	0.022	0.02	
Block.*Units* stratum					
Treatment	3	209.333	69.778	67.31	<.001
Residual	11	11.402	1.037		
Total	15	220.757			

Least significant differences of means (5% level)

Table	Treatment
rep.	4
d.f.	11
l.s.d.	1.585

Appendix 2.25 Interaction between submergence treatment and cultivars on harvested moisture content (%) (2013)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0	0.0007	0	
Block.*Units* stratum					
Treatment[1]	3	220	73.3897	84.07	<.001
CV	2	85.1	42.529	48.72	<.001
Treatment[1] . CV	6	110	18.3461	21.02	<.001
Residual	35	30.6	0.873		
Total	47	446			

[1] Seed developmental stage

Least significant differences of means (5% level)

Table	Treatment	CV	Treatment CV	Treatment Control	Mean 27.45b
rep.	12	16	4	10DAA /4d	29.31a
d.f.	35	35	35	30DAA /4d	24.32c
l.s.d.	0.774	0.67	1.341	40DAA /4d	24.28c

Least significant differences of means (5% level)

Table	CV	Treatment	CV Treatment	CV	Mean
rep.	16	12	4	Gleva	27.17a
d.f.	35	35	35	IR64	27.39a
l.s.d.	0.671	0.77	1.341	IR64 Sub1	24.46b

Appendix 2.26 Analysis of variance of seed dry weight at 0 % moisture content (g) (2013)

cv. Gleva

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.0076	0.0076	0.05	
Block.*Units* stratum					
Treatment	3	11.6067	3.8689	23.39	0.014
Residual	3	0.4961	0.1654		
Total	7	12.1105			

Least significant differences of means(5% level)

Table	Treatments
rep.	2
d.f.	3
l.s.d.	1.294

cv. IR64

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.0008	0.0008	0	
Block.*Units* stratum					
Treatment	3	1.6004	0.5335	3.06	0.191
Residual	3	0.5226	0.1742		
Total	7	2.1238			

Least significant differences of means (5% level)

Table	Treatments
rep.	2
d.f.	3
l.s.d.	1.328

cv. IR64-Sub1

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.00006	0.00006	0	
Block.*Units* stratum					
Treatment	3	1.34282	0.44761	19.14	0.019
Residual	3	0.07017	0.02339		
Total	7	1.41305			

Least significant differences of means(5% level)

Table	Treatments
rep.	2
d.f.	3
l.s.d.	0.4867

Appendix 2.27 Interaction between submergence treatment and cultivars on seed dry weight (g) (2013)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.00509	0.00509	0.05	
Block.*Units* stratum					
Treatment[1]	3	8.16488	2.72163	27.41	<.001
CV	2	438.14208	219.07104	2206.13	<.001
Treatment[1].CV	6	6.38501	1.06417	10.72	<.001
Residual	11	1.09231	0.0993		
Total	23	453.78937			

[1] Seed developmental stage

Least significant differences of means (5% level)

Table	CV	Treatment	CV Treatment	CV GLEVA	Mean 28.36	a
rep.	8	6	2	IR64	19.57	b
d.f.	11	11	11	IR64-Sub1	19.04	c
l.s.d.	0.3468	0.4004	0.6936			

Least significant differences of means (5% level)

Table	Treatment	CV	Treatment	Treatment	Mean	
			CV	Control	23.29	a
rep.	6	8	2	10DAA	21.86	b
d.f.	11	11	11	30DAA	22.29	b
l.s.d.	0.4004	0.3468	0.6936	40DAA	21.86	b

Appendix 2.28 Regression analysis for genotype-environment analysis for productivity of cvs Gleva, IR64, and IR64 Sub1 in response to submergence (2013)

Appendix 2.28.1 Seed yield (g pot^{-1})

a.) Response variate: Seed yield (g pot^{-1})
Fitted terms: Constant + Environment

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	161.4	161.388	25.73	<.001
Residual	22	138.0	6.272		
Total	23	299.4	13.016		

Residual variance exceeds variance of response variate.
Standard error of observations is estimated to be 4.32.

b.) Response variate: Seed yield (g pot^{-1})
Fitted terms: Constant + Environment + Cultivar

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	3	408.757	136.2522	310.76	<.001
Residual	20	8.769	0.4384		
Total	23	417.525	18.1533		
Change	-2	-401.573	200.7863	457.95	<.001

Percentage variance accounted for 97.6
Standard error of observations is estimated to be 0.662.

c.) Response variate: Seed yield (g pot^{-1})
Fitted terms: Constant + Environment + Cultivar + Environment x Cultivar

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	5	412.269	82.4537	282.33	<.001
Residual	18	5.257	0.2920		
Total	23	417.525	18.1533		
Change	-2	-3.512	1.7560	6.01	0.010

Percentage variance accounted for 98.4
Standard error of observations is estimated to be 0.540.

Appendix 2.28.2 1000 seed dry weight (g)

a.) Response variate: 1000 Seed dry weight
Fitted terms: Constant + Environment

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	7.2	7.18	0.39	0.541
Residual	22	410.3	18.65		
Total	23	417.5	18.15		

Residual variance exceeds variance of response variate.
Standard error of observations is estimated to be 4.32.

b.) Response variate: 1000 Seed dry weight
Fitted terms: Constant + Environment + Cultivar

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	3	408.757	136.2522	310.76	<.001
Residual	20	8.769	0.4384		
Total	23	417.525	18.1533		
Change	-2	-401.573	200.7863	457.95	<.001

Percentage variance accounted for 97.6
Standard error of observations is estimated to be 0.662.

c.) Response variate: 1000 Seed dry weight
Fitted terms: Constant + Environment + Cultivar + Environment x Cultivar

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	5	412.269	82.4537	282.33	<.001
Residual	18	5.257	0.2920		
Total	23	417.525	18.1533		
Change	-2	-3.512	1.7560	6.01	0.010

Percentage variance accounted for 98.4
Standard error of observations is estimated to be 0.540.

Appendix 2.29 Analysis of variance of longevity parameter of cv. Gleva (2013)

Appendix 2.29.1 Variate: K_i

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.00207	0.00207	0.08	
Block.*Units* stratum					
Treatment	3	0.16706	0.05569	2.19	0.268
Residual	3	0.07626	0.02542		
Total	7	0.24538			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	0.5074

Appendix 2.29.2 Variate:σ

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.973	0.973	2.56	
Block.*Units* stratum					
Treatment	3	6.1831	2.061	5.43	0.099
Residual	3	1.1384	0.3795		
Total	7	8.2946			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	1.96

Appendix 2.29.2 Variate: p_{50}

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	3.302	3.302	1.61	
Block.*Units* stratum					
Treatment	3	20.538	6.846	3.34	0.174
Residual	3	6.156	2.052		
Total	7	29.996			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	4.559

Appendix 2.30 Analysis of variance of longevity parameter of cv. IR64 (2013)

Appendix 2.30.1 Variate: K_i

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.0101	0.0101	0.07	
Block.*Units* stratum					
Treatment	3	0.0398	0.0133	0.09	0.961
Residual	3	0.4428	0.1476		
Total	7	0.4928			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	1.223

Appendix 2.30.2 Variate:σ

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	4.76	4.76	0.31	
Block.*Units* stratum					
Treatment	3	1.17	0.39	0.03	0.993
Residual	3	45.66	15.22		
Total	7	51.58			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	12.42

Appendix 2.30.3 Variate: p_{50}

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	4.79	4.79	0.72	
Block.*Units* stratum					
Treatment	3	24.012	8.004	1.21	0.44
Residual	3	19.849	6.616		
Total	7	48.65			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	8.186

Appendix 2.31 Analysis of variance of longevity parameter of cv. IR64 Sub1 (2013)

Appendix 2.31.1 Variate: K_i

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.0415	0.0415	0.28	
Block.*Units* stratum					
Treatment	3	0.2231	0.0744	0.51	0.705
Residual	3	0.4406	0.1469		
Total	7	0.7052			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	1.22

Appendix 2.31.2 Variate:σ

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	1.702	1.702	0.64	
Block.*Units* stratum					
Treatment	3	6.94	2.313	0.87	0.546
Residual	3	8.02	2.673		
Total	7	16.662			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	5.204

Appendix 2.31.3 Variate: p_{50}

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.644	0.644	0.27	
Block.*Units* stratum					
Treatment	3	13.564	4.521	1.9	0.306
Residual	3	7.151	2.384		
Total	7	21.358			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	3
l.s.d.	4.913

Appendix 2.32 Analysis of variance of longevity parameter between genotypes of cvs Gleva, IR64 and IR64 Sub1 (2013)

Appendix 2.32.1 Variate: K_i

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	0.0223	0.0223	0.25	
Block.*Units* stratum					
Treatment	11	2.12309	0.19301	2.14	0.111
Residual	11	0.99105	0.0901		
Total	23	3.13644			

Least significant differences of means (5% level)

Table	Ttreatment
rep.	2
d.f.	11
l.s.d.	0.6606

Appendix 2.32.2 Variate: σ

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	6.668	6.668	1.32	
Block.*Units* stratum					
Treatment	11	273.332	24.848	4.92	0.007
Residual	11	55.583	5.053		
Total	23	335.582			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	11
l.s.d.	4.948

Least significant differences of means (10% level)

Table	Treatment
rep.	2
d.f.	11
l.s.d.	4.037

Appendix 2.32.3a Variate: p_{50}

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	7.707	7.707	2.48	
Block.*Units* stratum					
Treatment	11	1176.952	106.996	34.43	<.001
Residual	11	34.185	3.108		
Total	23	1218.844			

Least significant differences of means (5% level)

Table	Treatment
rep.	2
d.f.	11
l.s.d.	3.88

IR64 Sub1_30 DAA	34.72	a
IR64 Sub1_Control	33.77	a
IR64 Sub1_40 DAA	33.31	a
IR64 Sub1_10 DAA	31.16	ab
IR64_Control	28.95	ab
IR64_40 DAA	25.74	bc
IR64_30 DAA	25.33	bc
IR64_10 DAA	24.33	bc
Gleva_30 DAA	19.16	cd
Gleva_40 DAA	16.64	d
Gleva_Control	15.29	d
Gleva_10 DAA	15.19	d

Appendix 2.32..3b Variate: p_{50} Interaction analysis between seed development stage and cultivar

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
BLOCK stratum	1	7.707	7.707	2.48	
BLOCK.*Units* stratum					
Seed development (DAA)	3	28.483	9.494	3.06	0.074
Cultivar (cv)	2	1118.839	559.419	180.01	<.001
DAA .cv					
	6	29.631	4.938	1.59	0.239
Residual	11	34.185	3.108		
Total	23	1218.844			

Least significant differences of means (5% level)

Table	DAA	cv	DAA . Cv
rep.	6	8	2
d.f.	11	11	11
l.s.d.	2.24	1.94	3.88

Tables of means

Grand mean 25.30

Seed development (DAA)	Control	10	30	40
	26.0	23.6	26.4	25.2

Cultivar (cv)	Gleva	IR64	IR64 Sub1
	16.6c	26.1b	33.2a

Seed development (DAA)	Cultivar (cv) Gleva	IR64	IR64 Sub1
Control	15.3	29.0	33.8
10	15.2	24.3	31.2
30	19.2	25.3	34.7
40	16.6	25.7	33.3

Appendix 2.33 Residuals deviance analysis table for comparing models of loss of viability in hermetic storage (at 40 °C with 14.7 ± 0.2% moisture content) among cultivar (2013)

cv. Gleva

F-test	Res dev	Res d.f.	Res Mean dev
Common slope	800.8	199	4.024
Best model	733.6	196	3.743
	67.2	3	
		22.400	
		5.985	
F(0.05, 3, 199)		=	2.650
	P	=	**0.001**

F-test	Res dev	Res d.f.	Res Mean dev
Common line	1118.0	202	5.534
Best model	733.6	196	3.743
	384.4	6	
		64.067	
		17.116	
F(0.005, 6, 196)		=	2.145
	P	**=**	**0.000**

F-test	Res dev	Res d.f.	Res Mean dev
Common Ki	753.4	199	3.786
Best model	733.6	196	3.743
	19.8	3	
		6.600	
		1.763	
F(0.05, 3, 196)		=	2.651
	P	**=**	**0.156**

cv. IR64

F-test	Res dev	Res d.f.	Res Mean dev
Common slope	2101	251	8.370
Best model	2096	248	8.450
	5	3	
		1.667	
		0.197	
F(0.05, 3, 251)		=	2.641
	P	**=**	**0.898**

F-test	Res dev	Res d.f.	Res Mean dev
Common line	2240	254	8.819
Best model	2096	248	8.450
	144	6	
		24.000	
		2.840	
F(0.05, 6, 248)		=	2.135
	P	**=**	**0.011**

F-test	Res dev	Res d.f.	Res Mean dev
Common Ki	2118	251	8.439
Best model	2096	248	8.45
	22	3	
		7.333	
		0.868	
F(0.05, 3, 248)		=	2.641
	P	**=**	**0.458**

cv. IR64 Sub1

F-test	Res dev	Res d.f.	Res Mean dev
Common slope	3644	251	14.520
Best model	3615	248	14.580
	29	3	
		9.667	
		0.663	
F(0.05, 3, 251)		=	2.641
	P	=	**0.576**

F-test	Res dev	Res d.f.	Res Mean dev
Common line	3719	254	14.640
Best model	3615	248	14.580
	104	6	
		17.333	
		1.189	
F(0.05, 6, 248)		=	2.135
	P	=	**0.313**

F-test	Res dev	Res d.f.	Res Mean dev
Common Ki	3654	251	14.560
Best model	3615	248	14.580
	39	3	
		13.000	
		0.892	
F(0.05, 3, 248)		=	2.641
	P	=	**0.446**

Appendix 2.34 Interaction of grouping between genotypes and time of submergence treatment on seed viability (2013)

Appendix 2.34.1 The effect of seed age when submergence occur (from 10, 30, 40DAA and non-submergence) on differences of seed viability

	Res dev	Res d.f.	Res Mean dev
Times of submergence treatment	6545	731	9.337
Individual treatments ($12K_i$, 12σ)	6445	701	9.313
	100	30	
		3.333	
		0.358	
F(0.05, 30, 701)		=	1.475
	P	=	**0.999**

Appendix 2.34.2 The effect of submergence tolerance according to genetic background on differences of seed viability

	Res dev	Res d.f.	Res Mean dev
Genotypes	8063	700	11.520
Individual treatments ($12K_i$, 12σ)	6445	692	9.313
	1618	8	
		202.250	
		21.717	
F(0.05, 8, 692)		=	1.952
	P	=	**0.000**

Appendix 2.35 Interaction between submergence treatment and cultivars on seed viability (p50) (2013)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	1	7.707	7.707	2.48	
Block.*Units* stratum					
Treatment[1]	3	28.483	9.494	3.06	0.074
CV	2	1118.839	559.419	180.01	<.001
Treatment[1].CV	6	29.631	4.938	1.59	0.239
Residual	11	34.185	3.108		
Total	23	1218.844			

[1] Seed developmental stage

Least significant differences of means (5% level)

Table	Treatment	CV	Treatment CV	Treatment	Mean	
				Control	26.01	a
rep.	6	8	2	10 DAA	23.56	a
d.f.	11	11	11	30 DAA	26.41	a
l.s.d.	2.24	1.94	3.88	40 DAA	25.23	a

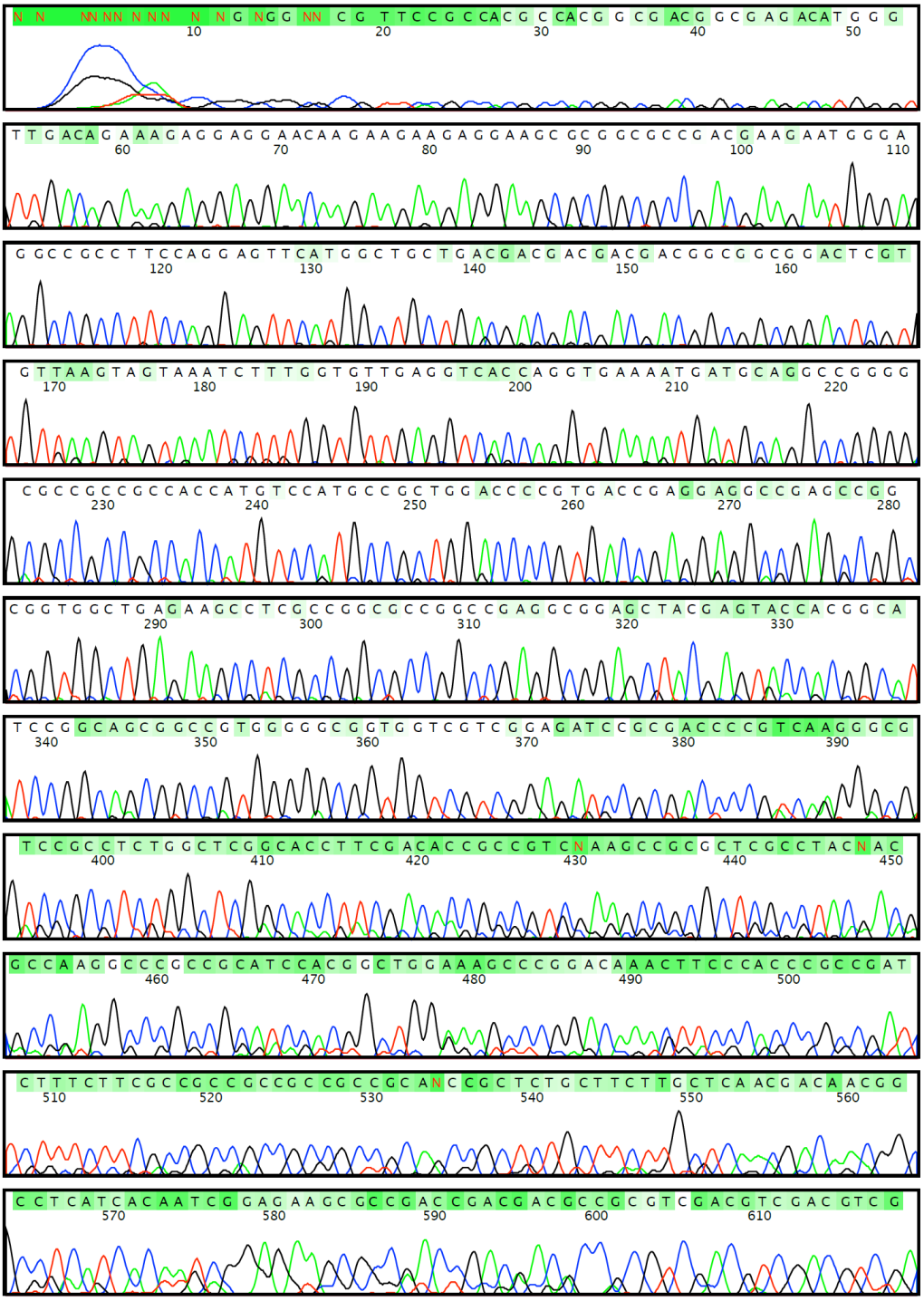
Least significant differences of means (5% level)

Table	CV	Treatment	CV Treatment	CV	Mean	
				Gleva	16.57	c
rep.	8	6	2	IR64	26.09	b
d.f.	11	11	11	IR64 Sub1	33.24	a
l.s.d.	1.94	2.24	3.88			

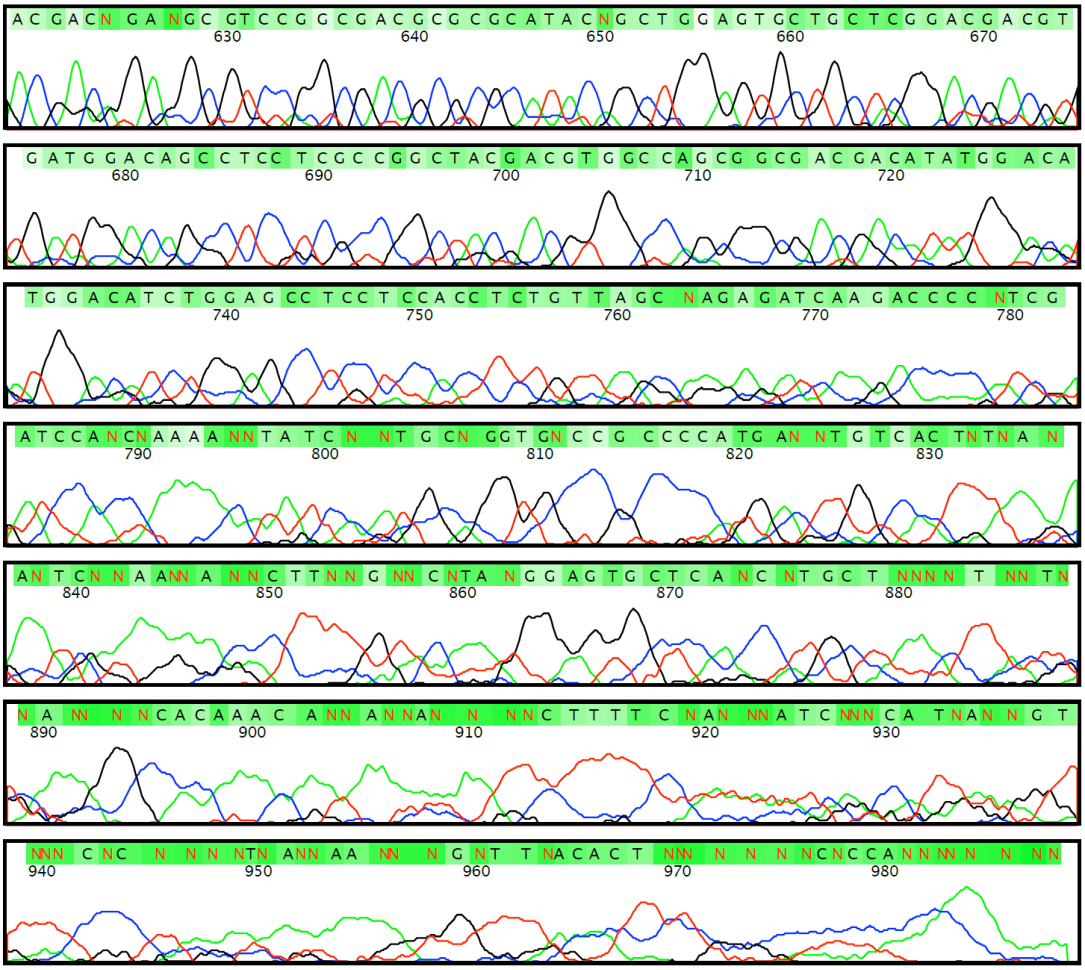
Appendix 3.1 Chromatograms of nucleotide sequence analysis of the pair of near-isogenic cultivars IR64 and IR64 Sub1, using the seeds provided by IRRI and the seeds that had been reproduced at PEL (2014)

Appendix 3.1.1 IR64

a.) PCR products of seed obtained from IRRI, amplified by using primers for forward reaction (*Sub1A_1_fw*)

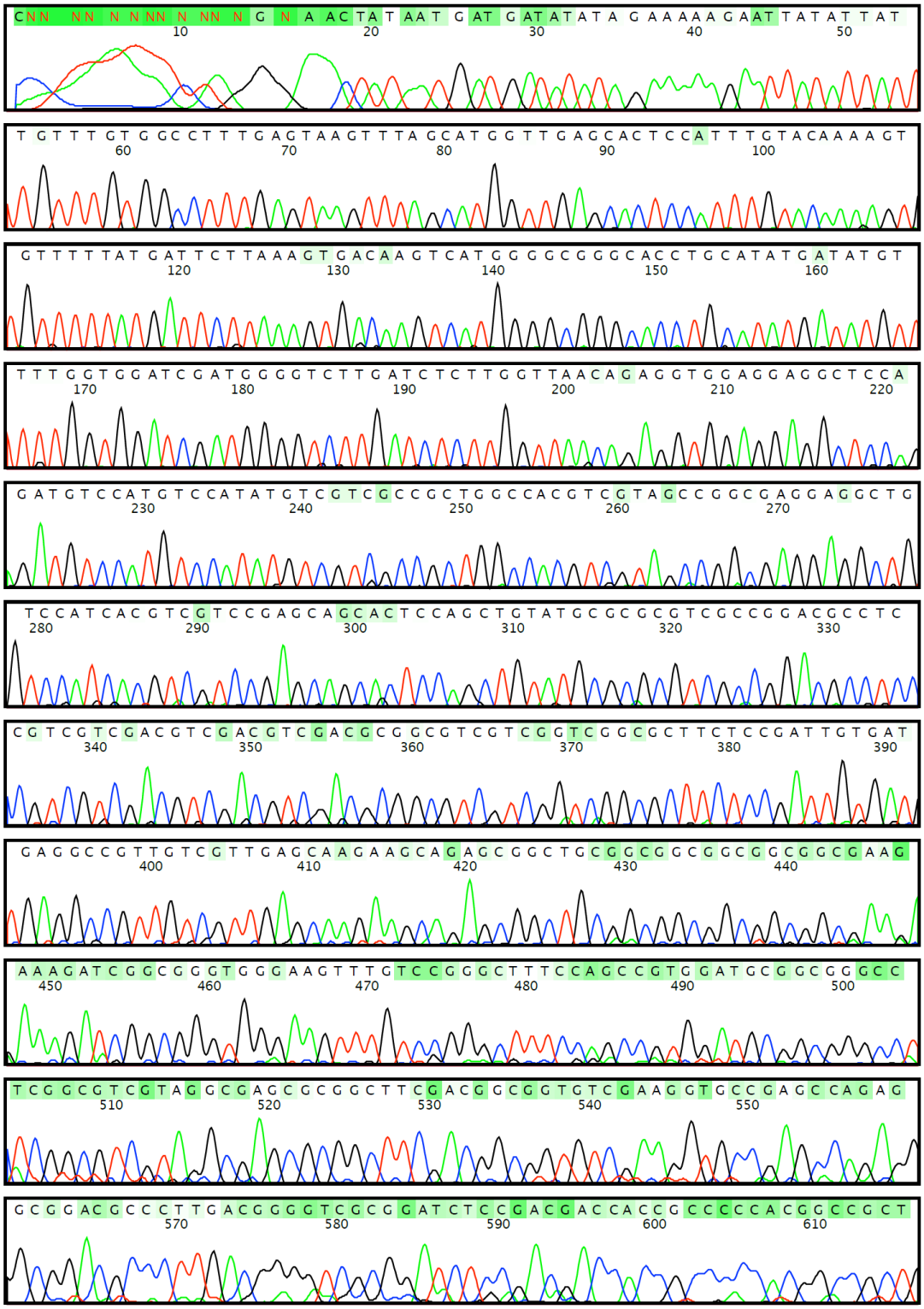


Appendix 3.1.1a (continued)

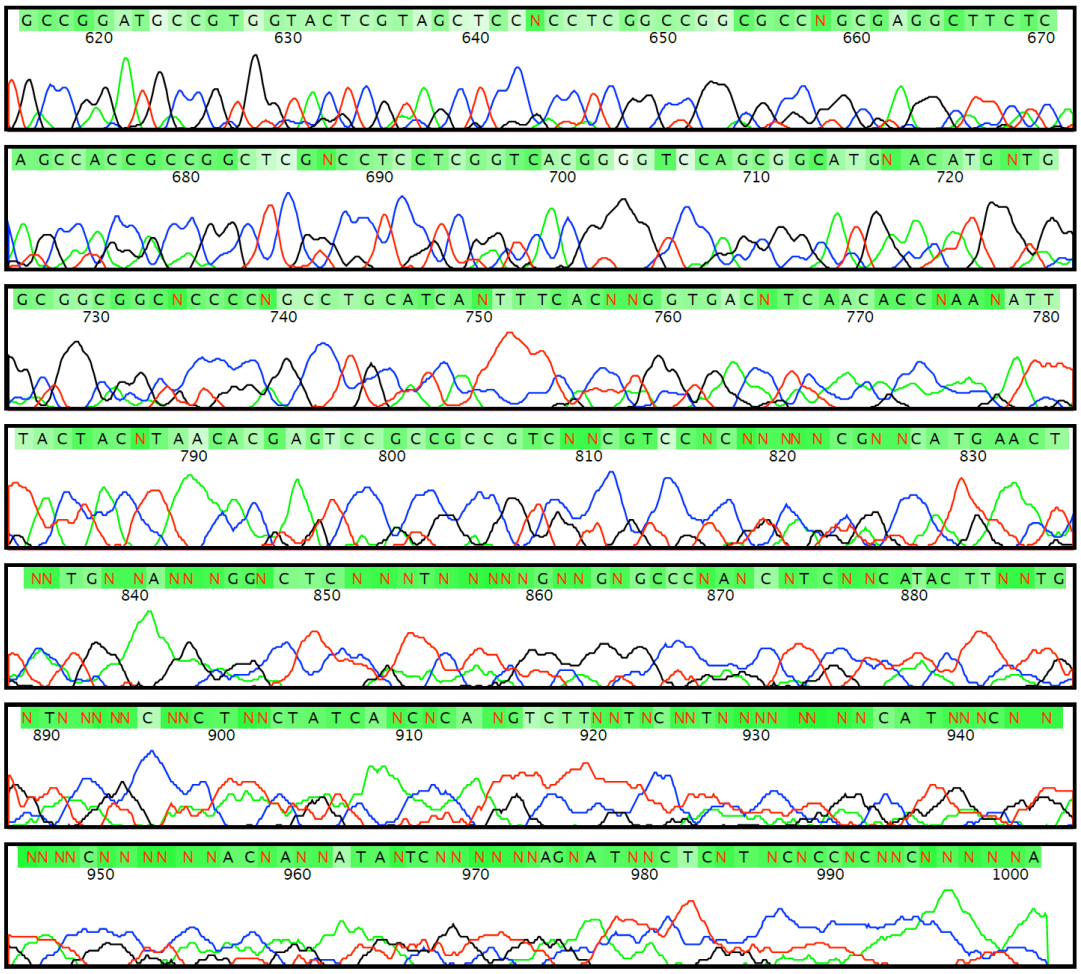


Appendix 3.1.1 IR64

b.) PCR products of seed obtained from IRRI, amplified by using primers for forward reaction (*Sub1A_1_rev*)

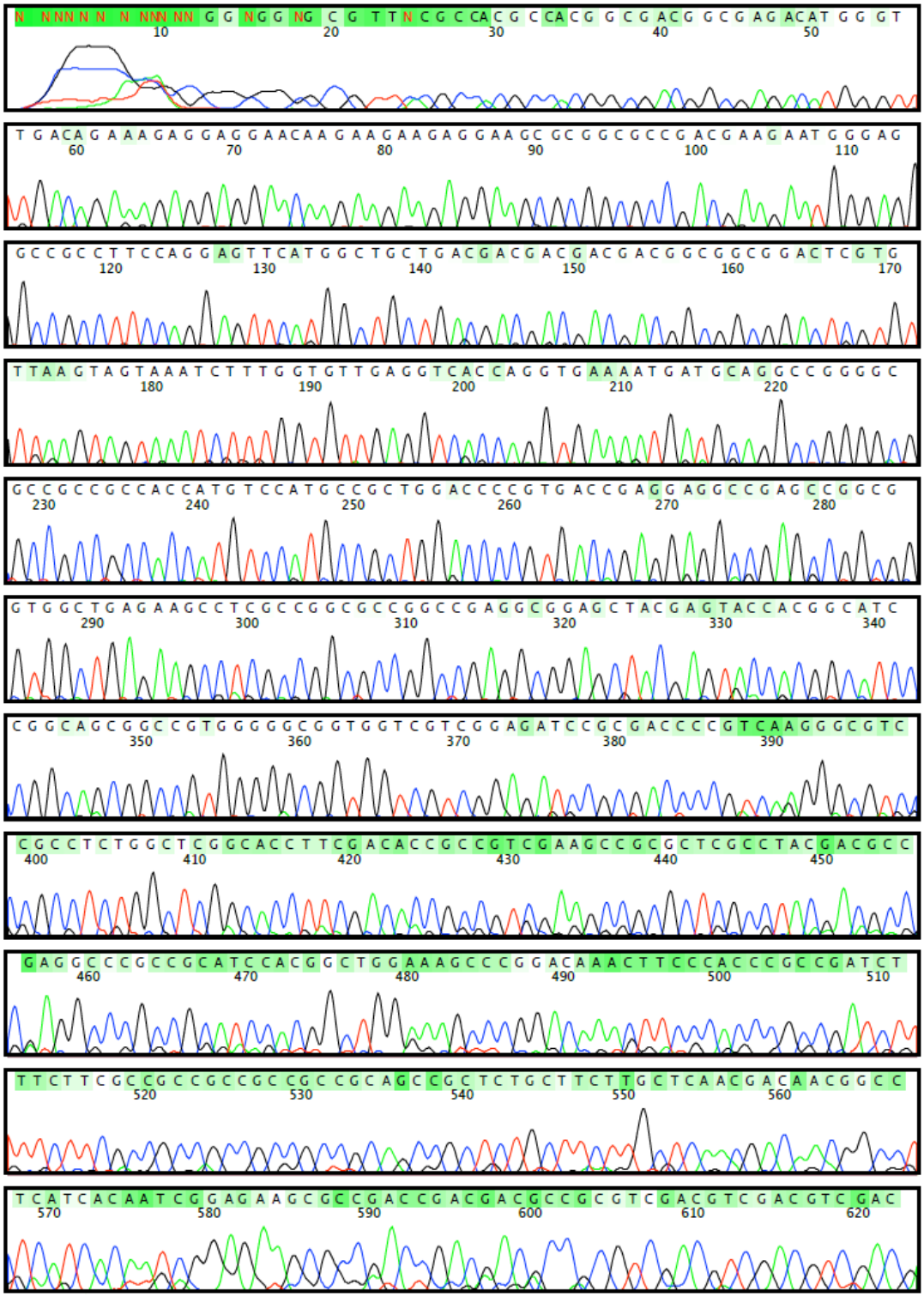


Appendix 3.1.1b (continued)

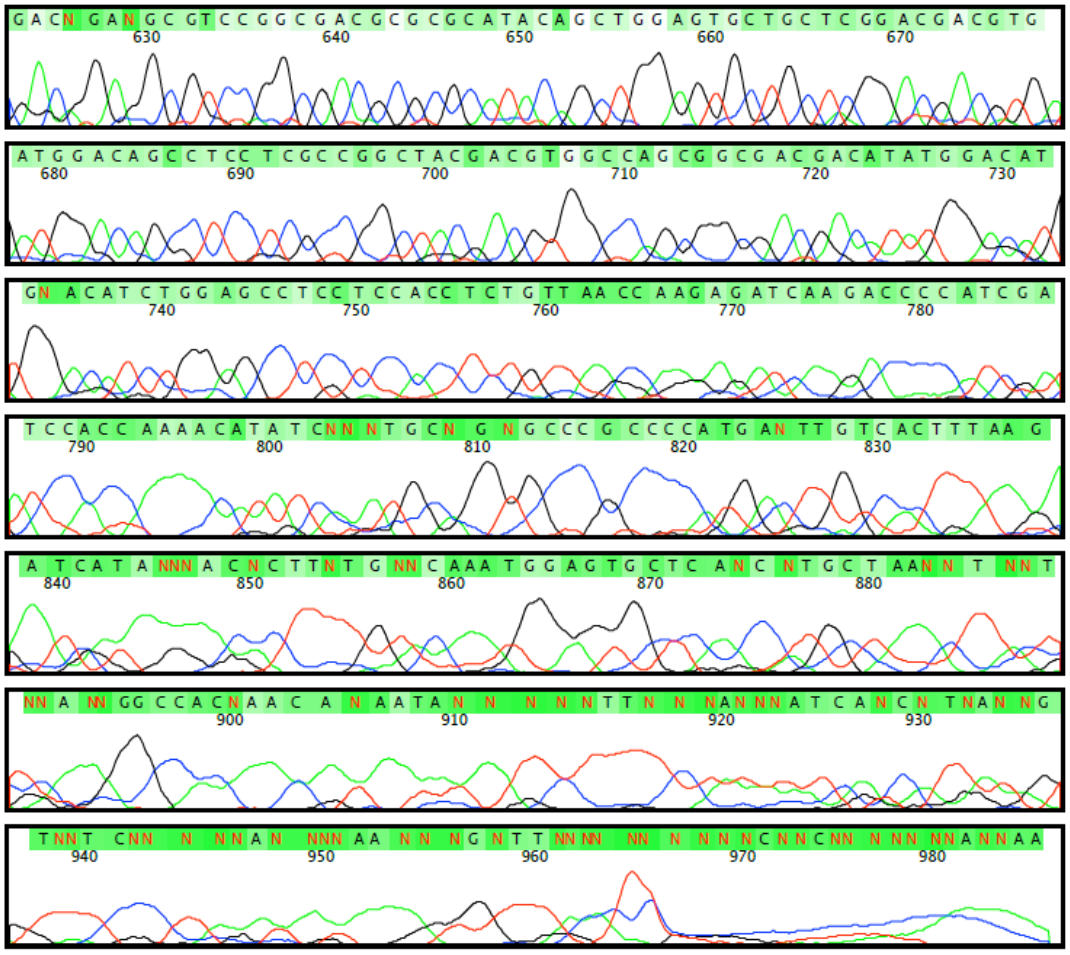


Appendix 3.1.1 IR64

c.) PCR products of seed reproduced at PEL in 2014, amplified by using primers for forward reaction (*Sub1A_1_fw*)

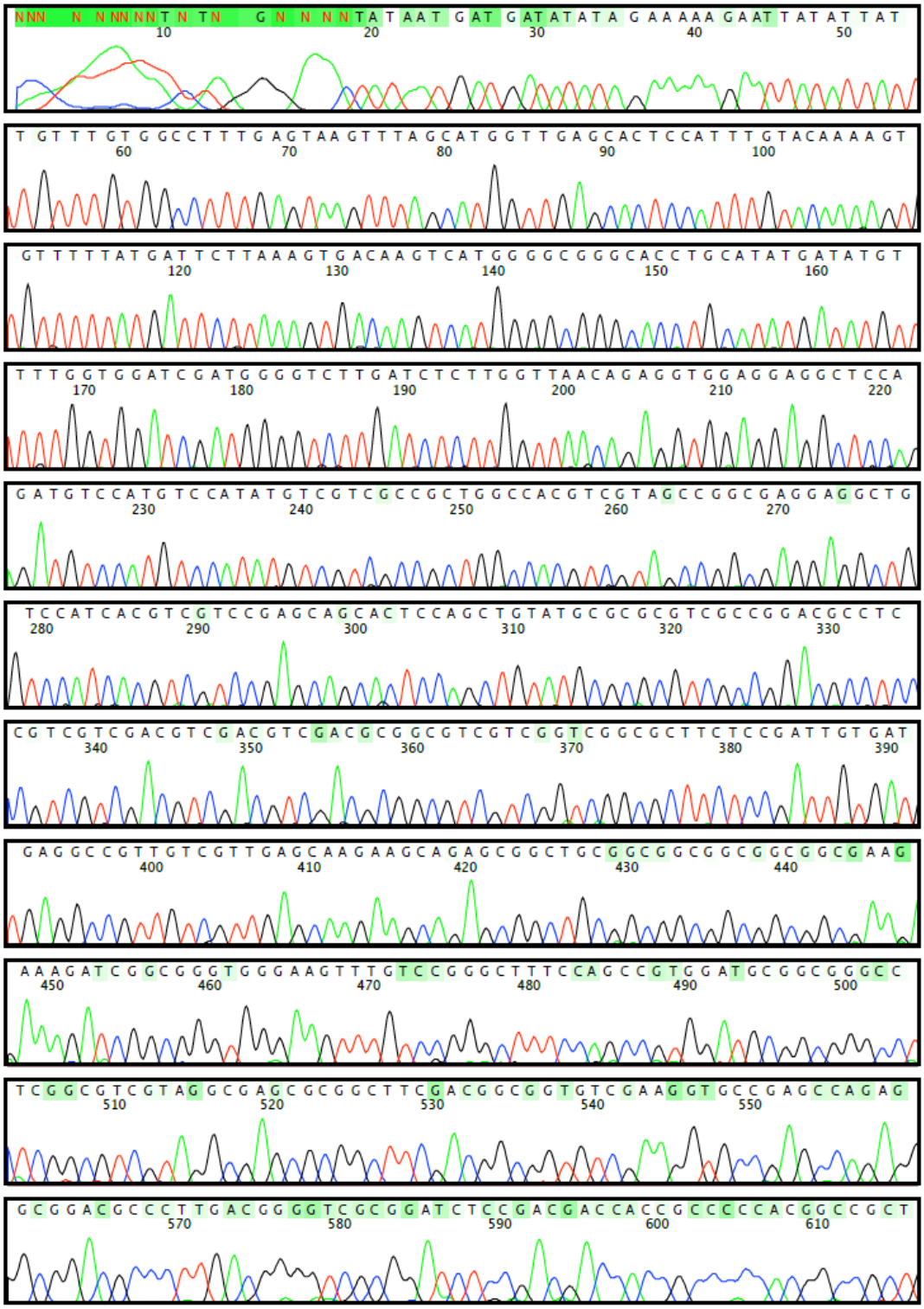


Appendix 3.1.1c (continued)

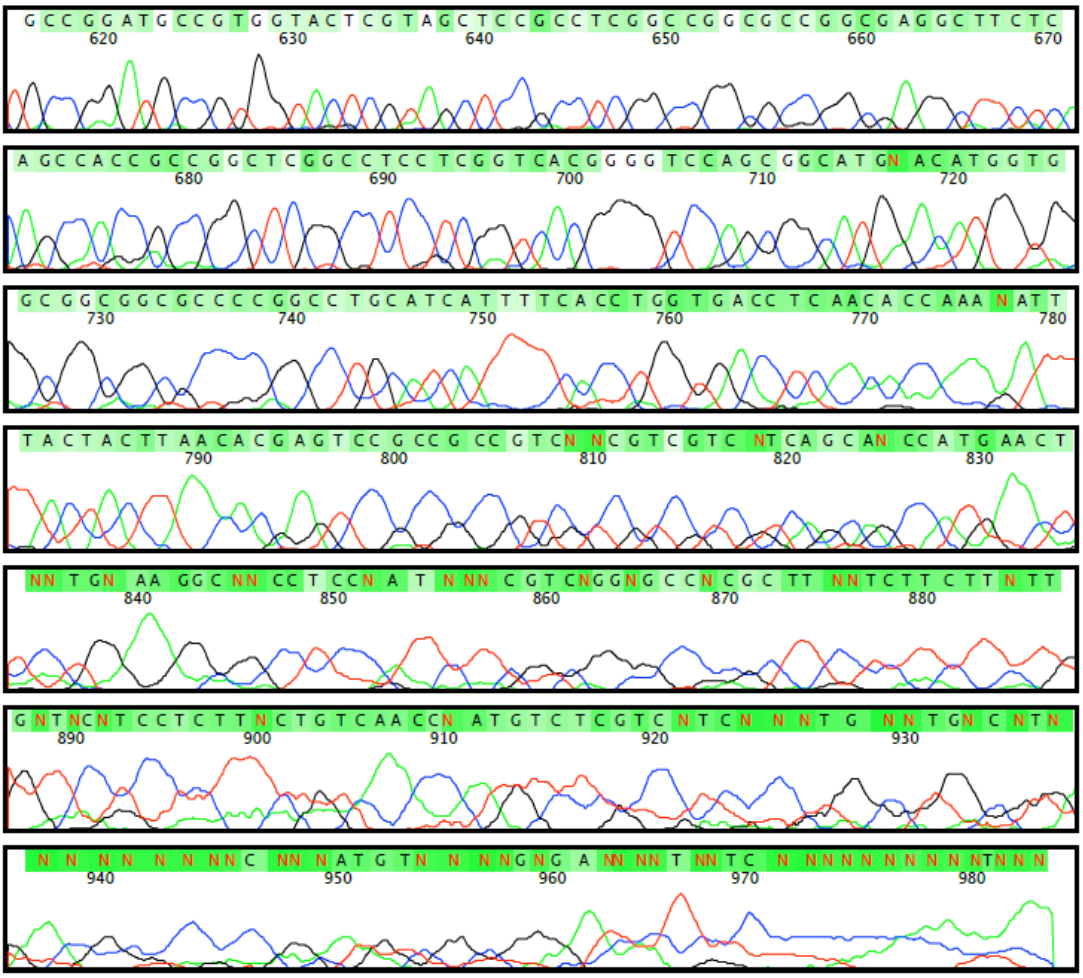


Appendix 3.1.1 IR64

d.) PCR products of seed reproduced at PEL in 2014, amplified by using primers for forward reaction (*Sub1A_1_rev*)

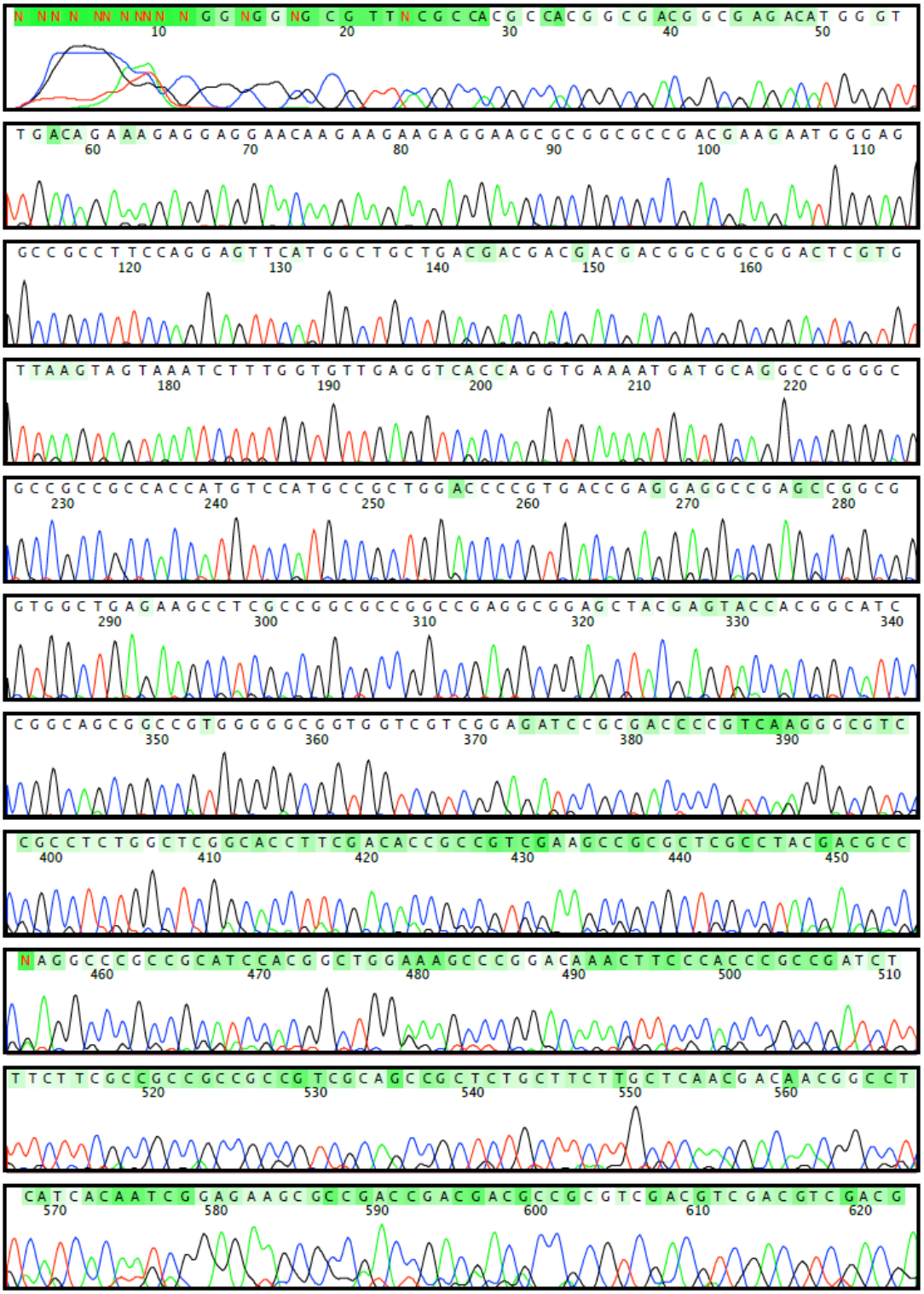


Appendix 3.1.1d (continued)

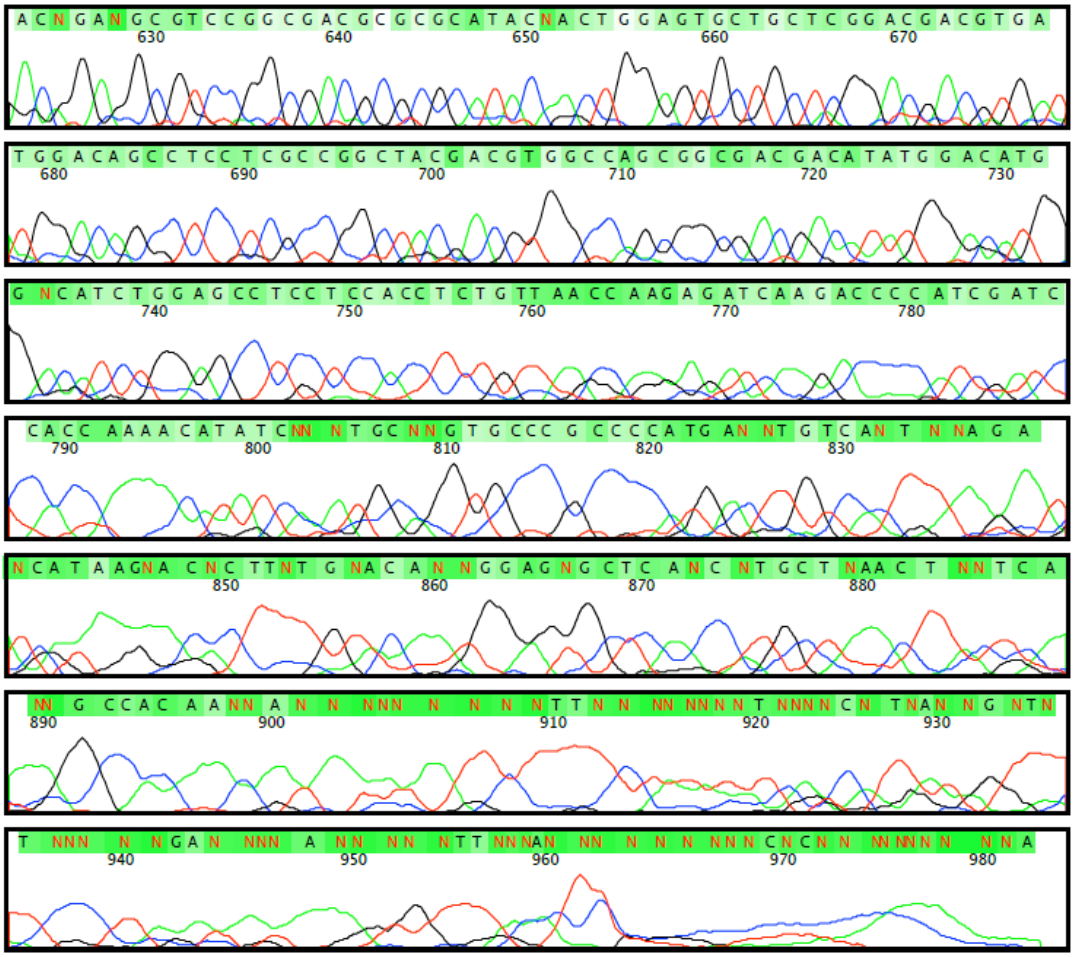


Appendix 3.1.2 IR64 Sub1

a.) PCR products of seed obtained from IRRI, amplified by using primers for forward reaction (*Sub1A_1_fw*)

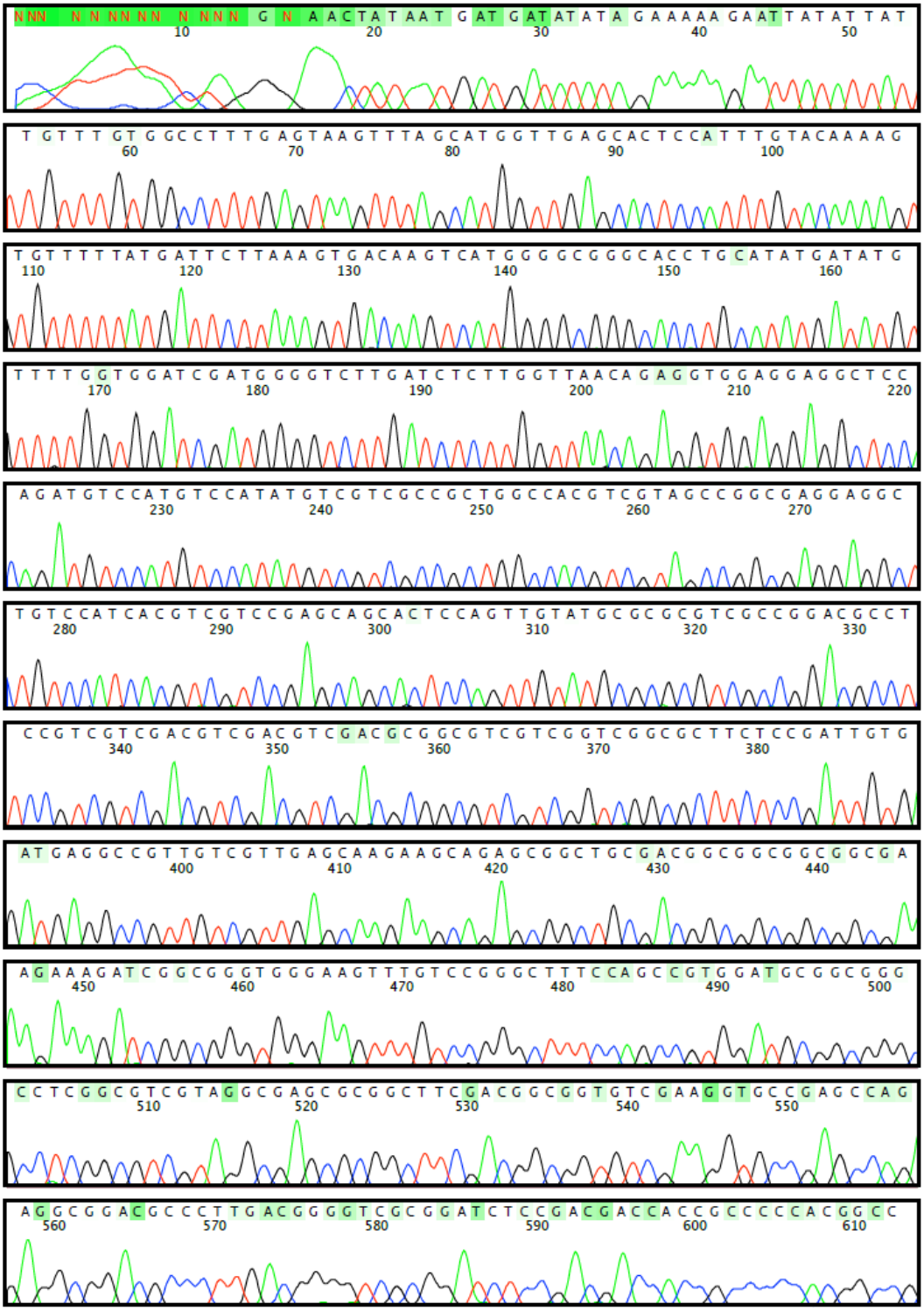


Appendix 3.1.2a (continued)

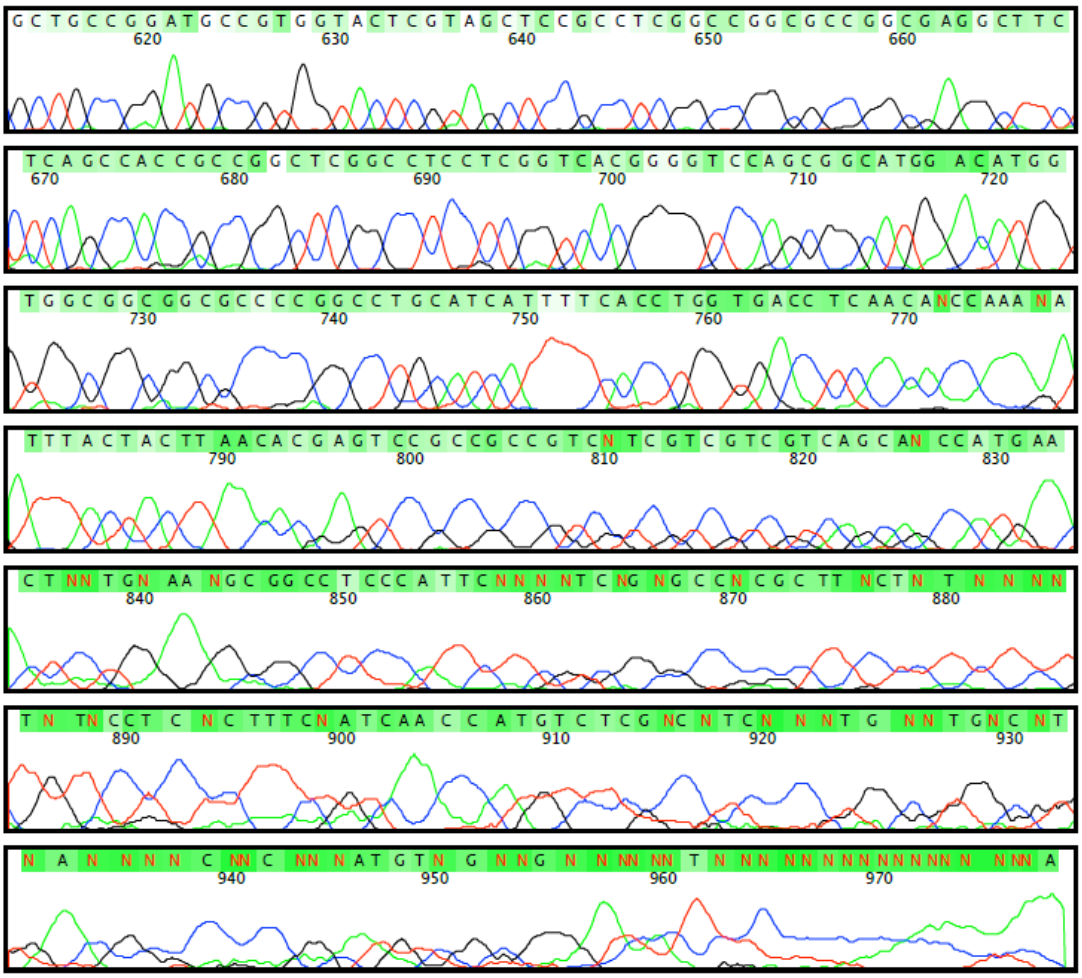


Appendix 3.1.2 IR64 Sub1

b.) PCR products of seed obtained from IRRI, amplified by using primers for forward reaction (*Sub1A_1_rev*)

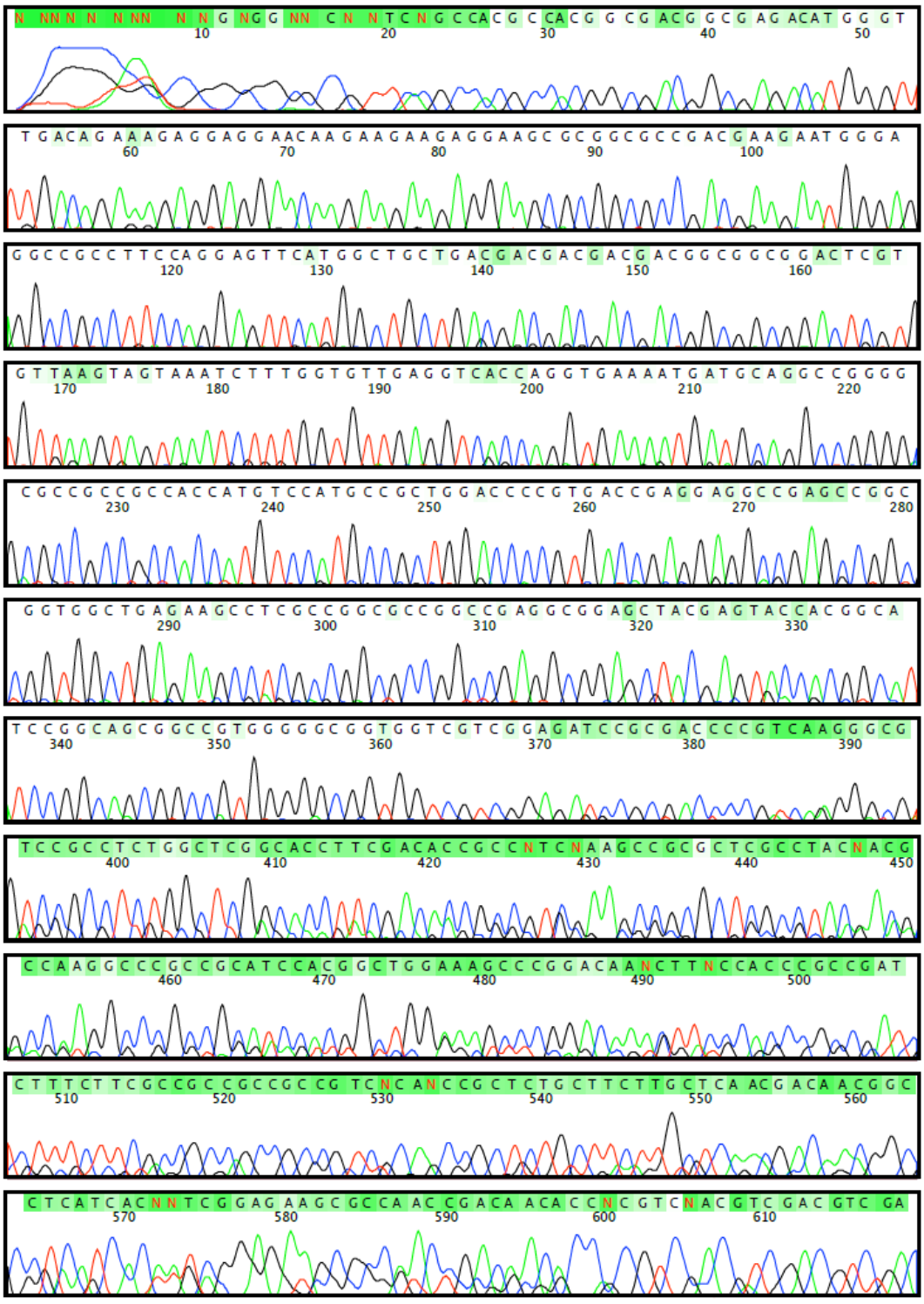


Appendix 3.1.2b (continued)

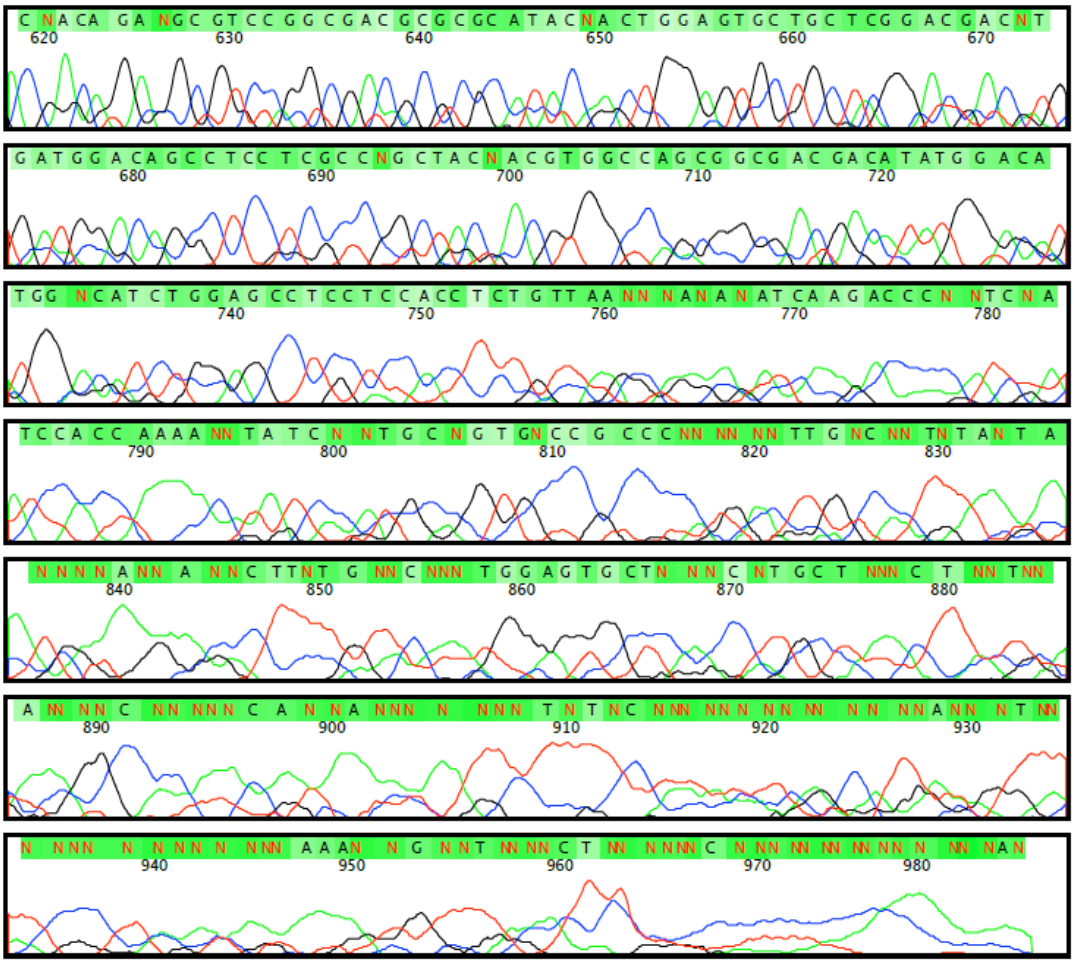


Appendix 3.1.2 IR64 Sub1

c.) PCR products of seed reproduced at PEL in 2014, amplified by using primers for forward reaction (*Sub1A_1_fw*)

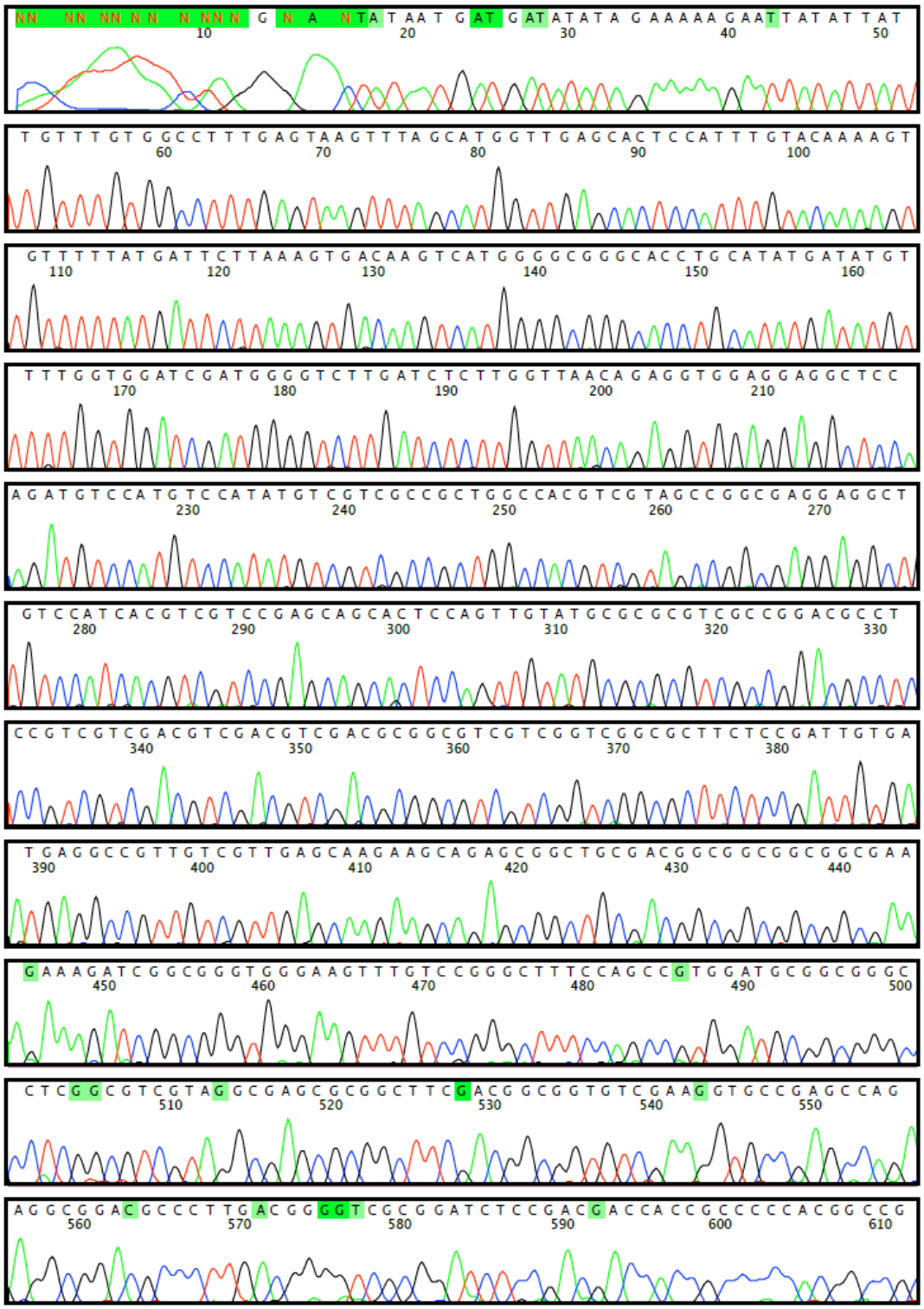


Appendix 3.1.2c (continued)

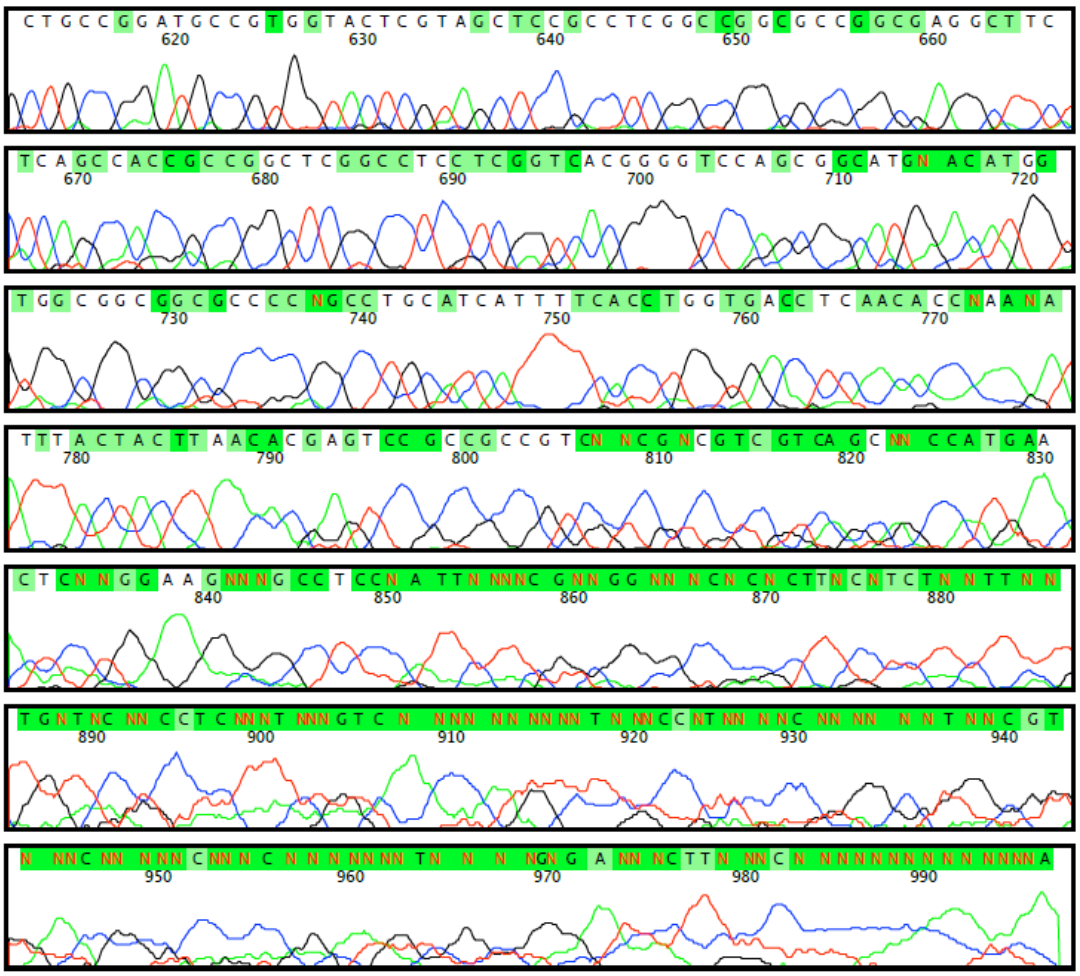


Appendix 3.1.2 IR64 Sub1

d.) PCR products of seed reproduced at PEL in 2014, amplified by using primers for forward reaction (*Sub1A_1_rev*)



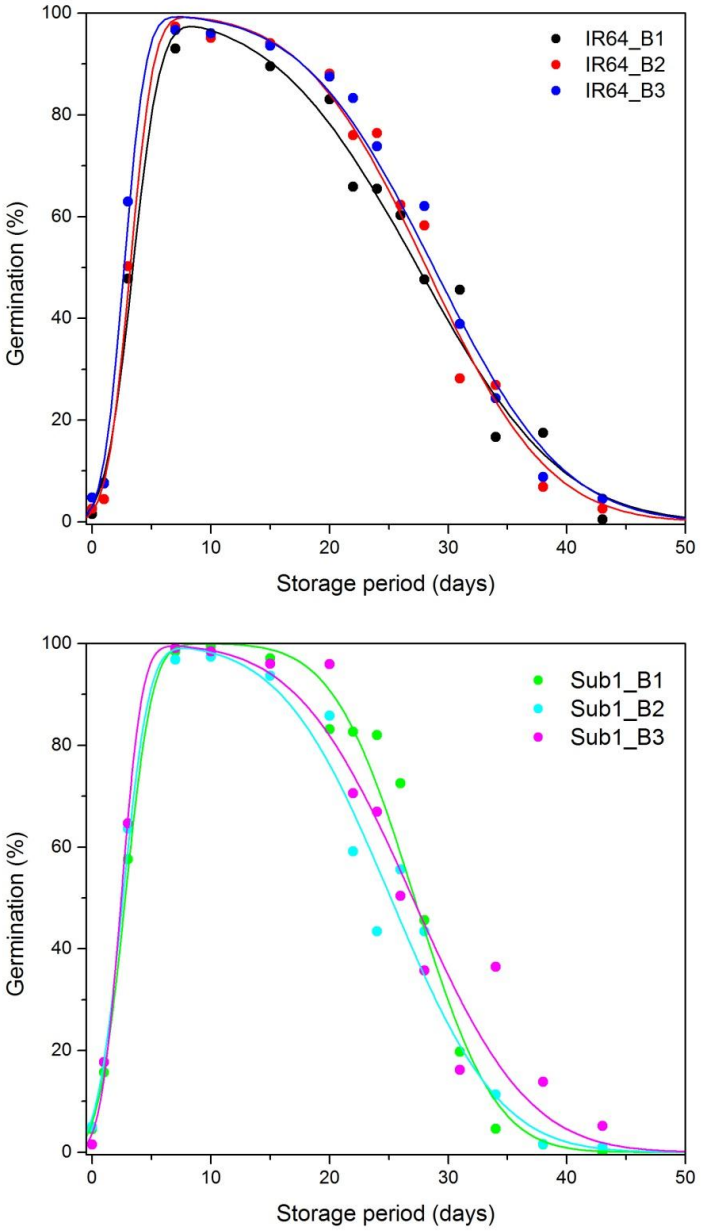
Appendix 3.1.2d (continued)



Appendix 3.2 Two samples t-test of yield per pot, moisture content at harvest, and 1000 seed fresh and dry weight of cvs IR64 and IR64 Sub1 (2014)

Parameter	CVS	Size	Mean	Variance	s.d.	s.e.m.	Difference of means	s.e.	95% CI	t-test value	d.f.	*P*
Yield (g pot^{-1})												
	IR64	3	102.2	256.1	16	9.24	-44.19	9.90	(-71.67, -16.72)	-4.47	4	0.011
	IR64 Sub1	3	146.4	37.8	6.15	3.546						
Moisture content at harvest												
	IR64	6	30.9	0.05915	0.2432	0.09929	1.322	0.14	(1.010, 1.634)	9.43	10	<0.001
	IR64 Sub1	6	29.6	0.05866	0.2422	0.09887						
1000 seed fresh weight												
	IR64	3	30.5	0.19379	0.4402	0.2542	2.56	0.26	(1.485, 3.625)	9.97	2.06	0.009
	IR64 Sub1	3	27.9	0.00308	0.0555	0.0320						
1000 seed dry weight												
	IR64	3	21.0	0.10897	0.3301	0.1906	1.386	0.20	(0.8300, 1.942)	6.92	4	0.002
	IR64 Sub1	3	19.7	0.01123	0.1060	0.0612						

Appendix 3.3 Seed survival curves (% normal germination plotted against period in hermetic storage at 40 °C with 13.7 ± 0.2% moisture content) fitted with the combined model of loss in dormancy and viability suggested by Whitehouse et al., (2015) for seeds harvested at harvest maturity of indica rice cv. IR64 (a) and IR64 Sub1 (b) (2014). The different lines represent the results for seeds from Blocks 1, 2, or 3. The parameters of the fitted curves are provided in Appendix 3.4.



Appendix 3.4 Parameters of the combined model of loss in dormancy and viability suggested by Whitehouse *et al.*, (2015) in hermetic storage at 40° C with 13.7 ± 0.2% moisture content for seeds harvested at harvest maturity of of *indica* rice cv. IR64 and IR64 Sub1 (2014)

Cultivar	Block	K_d		β1		K_i		Slope (1/σ)		σ (days)	p_{50} (days)
		Estimate	s.e.	Estimate	s.e.	Estimate	s.e.	Estimate	s.e.	Estimate	
IR64	1	-1.87	0.246	0.548	0.0818	2.88	0.254	-0.105	0.0093	9.5	27.4
	2	-2.05	0.256	0.644	0.0933	3.45	0.259	-0.122	0.0093	8.2	28.3
	3	-1.86	0.248	0.697	0.1070	3.33	0.245	-0.116	0.0087	8.6	28.6
IR64 Sub1	1	-1.57	0.222	0.565	0.0825	5.07	0.450	-0.187	0.0169	5.4	27.4
	2	-1.50	0.235	0.583	0.1010	3.48	0.323	-0.138	0.0124	7.2	25.1
	3	-1.81	0.376	0.740	0.1690	3.52	0.405	-0.130	0.0149	7.7	27.1

Appendix 3.5 Comparison of fitted models for combining loss in dormancy with loss in viability using paired F-test of residual deviance (2014)

F-test	Res dev	Res d.f.	Res Mean dev	F value	*P*
Common line	322.1	84	3.834		
Best fit	246.3	80	3.079		
	75.8	4			
		18.95		F(4 , 84) = 6.155	<0.001
Common slope for loss in dormancy, best fit for loss in viability	246.3	81	3.041		
Best fit	246.3	80	3.079		
	0	1			
		0.00		F(1 , 80) = 0.000	1.000
Best fit for loss in dormancy, common slope of loss in seed viability	277.9	82	3.389		
Best fit	246.3	80	3.079		
	31.6	2			
		15.80		F(2 , 80) = 5.132	0.008

Appendix 4.1 Analysis of variance of yield in grams per pot and interaction between foliar applications and conducting time of submergence (DAA) of dry seed (0% moisture content) (2014).

Appendix 4.1.1 Submergence treatments at 20 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	8.405	4.202	3.76	
Block.*Units* stratum					
Foliar treatment	4	10.259	2.565	2.29	0.148
Residual	8	8.947	1.118		
Total	14	27.611			

Appendix 4.1.2 Submergence treatment at 30 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	21.353	10.676	1.75	
Block.*Units* stratum					
Foliar treatment	4	27.849	6.962	1.14	0.402
Residual	8	48.727	6.091		
Total	14	97.928			

Appendix 4.1.3 Interaction analysis between seed development stage and foliar treatment

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	28.060	14.030	4.25	
Block.*Units* stratum					
Seed development (DAA)	1	5.699	5.699	1.73	0.205
Foliar treatment	4	35.388	8.847	2.68	0.065
DAA.Foliar treatment	4	2.720	0.680	0.21	0.932
Residual	18	59.372	3.298		
Total	29	131.239			

Appendix 4.1.4 Interaction analysis between seed development stage and molybdenum treatment (ABA spray was excluded)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	25.655	12.827	3.25	
Block.*Units* stratum					
Molybdenum (Mo)	3	35.214	11.738	2.98	0.068
Seed development (DAA)	1	4	4	1.01	0.331
Mo . DAA					
	3	2.629	0.876	0.22	0.879
Residual	14	55.199	3.943		
Total	23	122.697			

Tables of means

Grand mean 5.83

Molybdenum (Mo)	0 mg L^{-1}	100 mg L^{-1}	600 mg L^{-1}	3000 mg L^{-1}
	4.5	7.2	4.8	6.9

Seed development (DAA)	20	30
	5.4	6.2

	Seed development (DAA)	
Molybdenum (Mo)	20	30
0 mg L^{-1}	4.6	4.4
100 mg L^{-1}	6.6	7.8
600 mg L^{-1}	4.5	5.1
3000 mg L^{-1}	6.1	7.7

Appendix 4.2 Analysis of variance of pre-harvest sprouting percentage and interaction between foliar applications and conducting time of submergence (DAA) (2014)

Appendix 4.2.1 Submergence at 20 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	365.05	182.52	4.07	
Block.*Units* stratum					
Foliar treatment	4	468.04	117.01	2.61	0.116
Residual	8	358.84	44.85		
Total	14	1191.92			

Appendix 4.2.2 Submergence at 30 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	561.37	280.69	3.76	
Block.*Units* stratum					
Foliar treatment	4	1103.20	275.80	3.70	0.055
Residual	8	597.03	74.63		
Total	14	2261.60			

Appendix 4.2.3 Interaction analysis between seed development stage and foliar treatment

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	891.27	445.63	8.09	
Block.*Units* stratum					
Foliar treatment	4	1271.29	317.82	5.77	0.004
DAA	1	16796.31	16796.31	305.07	<.001
Foliar treatment . DAA	4	299.94	74.99	1.36	0.286
Residual	18	991.02	55.06		
Total	29	20249.84			

Least significant differences of means (5% level)

Table	DAA	Foliar treatment	DAA Foliar treatment
rep.	15	6	3
d.f.	18	18	18
l.s.d.	5.69	9.00	12.73

Tukey's 95% confidence intervals

	Mean	
Mo 0 mg L^{-1}	51.17	a
Mo 3000 mg L^{-1}	40.68	ab
ABA 50 µM	40.44	ab
Mo 600 mg L^{-1}	34.31	b
Mo 100 mg L^{-1}	32.64	b

DAA	Mean	
30 DAA	63.5	a
20 DAA	16.2	b

Appendix 4.2.4 Interaction analysis between seed development stage and molybdenum treatment (ABA spray was excluded)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	662.52	331.26	5.07	
Block.*Units* stratum					
Molybdenum (Mo)	3	1268.68	422.89	6.47	0.006
Seed development (DAA)	1	12380.65	12380.65	189.32	<.001
Mo . DAA	3	191.84	63.95	0.98	0.431
Residual	14	915.53	65.4		
Total	23	15419.22			

Least significant differences of means (5% level)

Table	Mo	DAA	Mo . DAA
rep.	6	12	3
d.f.	14	14	14
l.s.d.	10.01	7.08	14.16

Tables of means

Grand mean 39.7

Molybdenum (Mo)	0 mg L^{-1}	100 mg L^{-1}	600 mg L^{-1}	3000 mg L^{-1}
	51a	323b	41ab	34b

Seed development (DAA)	20	30
	17b	62a

	Seed development (DAA)	
Molybdenum (Mo)	20	30
0 mg L^{-1}	27.1	75.2
100 mg L^{-1}	11.6	53.6
600 mg L^{-1}	14.1	67.3
3000 mg L^{-1}	15.1	53.5

Appendix 4.3 Analysis of variance of a thousand non-sprouted seed dry weight in grams and interaction between foliar applications and time of submergence (DAA) (2014)

Appendix 4.3.1 Submergence treatments at 20 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	4.1495	2.0747	3.10	
Block.*Units* stratum					
Foliar treatment	4	0.8257	0.2064	0.31	0.864
Residual	8	5.3501	0.6688		
Total	14	10.3252			

Appendix 4.3.2 Submergence treatments at 30 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	3.3389	1.6694	2.89	
Block.*Units* stratum					
Foliar treatment	4	3.3522	0.8380	1.45	0.302
Residual	8	4.6187	0.5773		
Total	14	11.3097			

Appendix 4.3.3 Interaction analysis between seed development stage and foliar treatment

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	6.2870	3.1435	5.07	
Block.*Units* stratum					
Foliar treatment	4	0.7776	0.1944	0.31	0.865
DAA	1	0.5333	0.5333	0.86	0.366
Foliar treatment . DAA	4	3.4002	0.8501	1.37	0.284
Residual	18	11.1701	0.6206		
Total	29	22.1683			

Appendix 4.3.4 Interaction analysis between seed development stage and molybdenum treatment (ABA spray was excluded)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	5.3632	2.6816	4.69	
Block.*Units* stratum					
Molybdenum (Mo)	3	0.6525	0.2175	0.38	0.769
Seed development (DAA)	1	1.5492	1.5492	2.71	0.122
Mo . DAA	3	1.651	0.5503	0.96	0.437
Residual	14	8.0004	0.5715		
Total	23	17.2164			

Tables of means

Grand mean 26.97

Molybdenum (Mo)	0 mg L^{-1}	100 mg L^{-1}	600 mg L^{-1}	3000 mg L^{-1}
	26.8	26.9	26.9	27.2

Seed development (DAA)	20	30
	27.2	26.7

	Seed development (DAA)	
Molybdenum (Mo)	20	30
0 mg L^{-1}	27.5	26.14
100 mg L^{-1}	27.07	26.67
600 mg L^{-1}	27.1	26.76
3000 mg L^{-1}	27.21	27.27

Appendix 4.4 Analysis of variance of a thousand sprouted seed dry weight in grams and interaction between foliar applications and time of submergence (DAA) (2014)

Appendix 4.4.1 Submergence treatments at 20 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	7.3125	3.6562	7.05	
Block.*Units* stratum					
Foliar treatment	4	5.0901	1.2725	2.45	0.130
Residual	8	4.1467	0.5183		
Total	14	16.5493			

Appendix 4.4.2 Submergence treatments at 30 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	1.3870	0.6935	1.05	
Block.*Units* stratum					
Foliar treatment	4	1.7587	0.4397	0.67	0.632
Residual	8	5.2694	0.6587		
Total	14	8.4151			

Appendix 4.4.3 Interaction analysis between seed development stage and foliar treatment

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	7.3502	3.6751	6.14	
Block.*Units* stratum					
Foliar treatment	4	4.7048	1.1762	1.97	0.143
DAA	1	2.9611	2.9611	4.95	0.039
Foliar treatment . DAA	4	2.1440	0.5360	0.90	0.487
Residual	18	10.7654	0.5981		
Total	29	27.9255			

Appendix 4.4.4 Interaction analysis between seed development stage and molybdenum treatment (ABA spray was excluded)

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	5.6015	2.8008	4.72	
Block.*Units* stratum					
Molybdenum (Mo)	3	1.9351	0.645	1.09	0.387
Seed development (DAA)	1	2.9218	2.9218	4.92	0.044
Mo . DAA	3	1.5421	0.514	0.87	0.482
Residual	14	8.3116	0.5937		
Total	23	20.3121			

Least significant differences of means (5% level)

Table	Mo	DAA	Mo . DAA
rep.	6	12	3
d.f.	14	14	14
l.s.d.	0.954	0.675	1.349

Tables of means

Grand mean
30.06

Molybdenum (Mo)	0 mg L^{-1}	100 mg L^{-1}	600 mg L^{-1}	3000 mg L^{-1}
	29.6	30.4	30.0	30.2

Seed development (DAA)	20	30
	30.4a	29.7b

	Seed development (DAA)	
Molybdenum (Mo)	20	30
0 mg L^{-1}	29.7	29.6
100 mg L^{-1}	31.0	29.7
600 mg L^{-1}	30.2	29.9
3000 mg L^{-1}	30.8	29.7

Appendix 4.5 Regression analysis for responses of exogenous spraying of molybdenum (at 0, 100, 600, or 3000 mg L^{-1}) on Mo content of seed (2014)

a.) Non-sprouted seed

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	1.716	1.7159	14.82	<.001
Residual	22	2.546	0.1157		
Total	23	4.262	0.1853		

Percentage variance accounted for 37.5

Standard error of observations is estimated to be 0.340.

b.) Sprouted seed

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	5.355	5.3552	33.40	<.001
Residual	22	3.528	0.1604		
Total	23	8.883	0.3862		

Percentage variance accounted for 58.5

Standard error of observations is estimated to be 0.400.

c.) Linear regression analysis of applied Mo and measured Mo in sprouted and non-sprouted seed

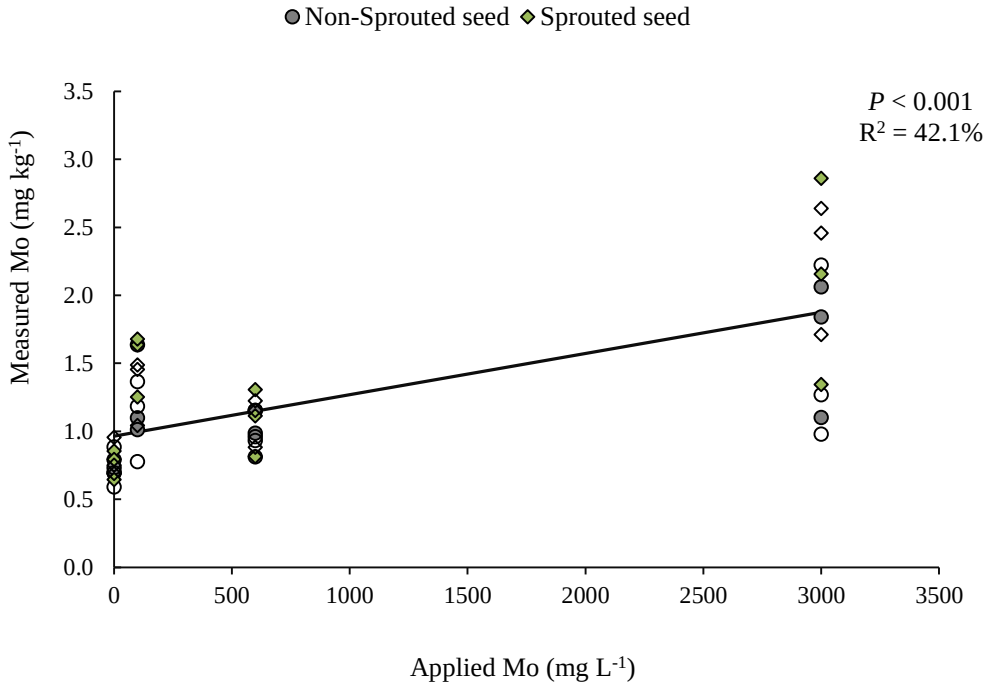
Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	6.567	6.5669	41.26	<.001
Residual	46	7.321	0.1592		
Total	47	13.888	0.2955		

Percentage variance accounted for 46.1

Standard error of observations is estimated to be 0.399.

Estimates of parameters

Parameter	estimate	s.e.	t(46)	t pr.
Constant	0.9652	0.0723	13.35	<.001
Applied_Mo	0.0003033	0.0000472	6.42	<.001



Linear regression line of applied Mo and measured Mo in sprouted (♦, ◊,) and non-sprouted seed (●, ○). The solid- and open- symbols show results of seed samples obtained from 4 days' submergence at 20 and 30 DAA, respectively.

Appendix 4.6 Analysis of variance of Mo content and interaction between foliar application and types of seed i.e. sprouted and non-sprouted seed samples when simulated submergence applied at 20 DAA (2014)

Appendix 4.6.1 Non-sprouted seed

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	0.43517	0.21759	4.64	
Block.*Units* stratum					
Foliar treatment	4	1.91743	0.47936	10.23	0.003
Residual	8	0.37492	0.04686		
Total	14	2.72752			

Least significant differences of means (5% level)

Table	Foliar treatment
rep.	3
d.f.	8
l.s.d.	0.4076

Appendix 4.6.2 Sprouted seed

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	0.79519	0.39760	5.08	
Block.*Units* stratum					
Foliar treatment	4	4.03857	1.00964	12.89	0.001
Residual	8	0.62648	0.07831		
Total	14	5.46025			

Least significant differences of means (5% level)

Table	Foliar treatment
rep.	3
d.f.	8
l.s.d.	0.5269

Appendix 4.6.3 Sprouted-and non-sprouted seed samples

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	1.18419	0.59209	10.17	
Block.*Units* stratum					
Foliar treatment	9	6.19097	0.68789	11.82	<.001
Residual	18	1.04758	0.05820		
Total	29	8.42274			

Least significant differences of means (5% level)

Table	Treatments
rep.	3
d.f.	18
l.s.d.	0.4138

Appendix 4.6.4 Interaction

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	1.18419	0.59209	10.17	
Block.*Units* stratum					
Foliar treatment	4	5.75762	1.43941	24.73	<.001
Type of seed	1	0.23497	0.23497	4.04	0.060
Foliar treatment . Type of seed	4	0.19838	0.04959	0.85	0.511
Residual	18	1.04758	0.05820		
Total	29	8.42274			

Least significant differences of means (5% level)

Table	Concentration	Type of seed	Foliar treatment Type of seed
rep.	6	15	3
d.f.	18	18	18
l.s.d.	0.2926	0.1851	0.4138

Tukey's 95% confidence intervals

	Mean	
Mo 3000 mg L^{-1}	1.893	a
Mo 600 mg L^{-1}	1.385	b
Mo 100 mg L^{-1}	1.030	bc
Mo 0 mg L^{-1}	0.751	c
ABA 50 µM	0.727	c

Appendix 4.7 Analysis of variance of Mo content and interaction between foliar application and types of seed i.e. sprouted and non-sprouted seed samples when simulated submergence applied at 30 DAA (2014)

Appendix 4.7.1 Non-sprouted seed

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	0.67439	0.33720	5.86	
Block.*Units* stratum					
Foliar treatment	4	1.34504	0.33626	5.84	0.017
Residual	8	0.46028	0.05754		
Total	14	2.47971			

Least significant differences of means (5% level)

Table	Foliar treatment
rep.	3
d.f.	8
l.s.d.	0.4516

Appendix 4.7.2 Sprouted seed

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	0.26191	0.13095	2.21	
Block.*Units* stratum					
Foliar treatment	4	4.41929	1.10482	18.69	<.001
Residual	8	0.47303	0.05913		
Total	14	5.15423			

Least significant differences of means (5% level)

Table	Foliar treatment
rep.	3
d.f.	8
l.s.d.	0.4578

Appendix 4.7.3 Sprouted and non-sprouted seed samples

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.81279	0.40639	6.92	
Block.*Units* stratum					
Treatments	9	6.27166	0.69685	11.87	<.001
Residual	18	1.05683	0.05871		
Total	29	8.14128			

Least significant differences of means (5% level)

Table	Treatments
rep.	3
d.f.	18
l.s.d.	0.4157

Appendix 4.7.4 Interaction

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	0.81279	0.40639	6.92	
Block.*Units* stratum					
Foliar treatment	4	5.23579	1.30895	22.29	<.001
Type of seed[1]	1	0.50734	0.50734	8.64	0.009
Foliar treatment . Type of seed	4	0.52854	0.13213	2.25	0.104
Residual	18	1.05683	0.05871		
Total	29	8.14128			

[1] Sprouted and Non-sprouted seed

Least significant differences of means (5% level)

Table	Foliar treatment	Type of seed	Foliar treatment Type of seed
rep.	6	15	3
d.f.	18	18	18
l.s.d.	0.2939	0.1859	0.4157

Tukey's 95% confidence intervals

	Mean			Mean	
Mo 3000 mg L^{-1}	1.880	a	Sprouted seed	1.257	a
Mo 600 mg L^{-1}	1.217	b	Non-sprouted seed	0.997	b
Mo 100 mg L^{-1}	1.050	bc			
Mo 0 mg L^{-1}	0.760	c			
ABA 50 µM	0.730	c			

Appendix 4.8 Interaction analysis between foliar application (i.e. Mo at 0, 100, 600, 3000 mg L^{-1}, and ABA 50µM), time of submergence (20 or 30 DAA), and type of seed (i.e. sprouted and non-sprouted seed) on Mo content (2014)

Appendix 4.8.1 Interaction analysis between seed development stage and foliar treatment

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	*P*
a.) Experiment factor: Foliar application					
Foliar application	172.27	4	38.34	15.7	<0.001
Time of submergence	0.00	1	0.00	16.4	0.945
Type of seed	14.74	1	14.74	16.4	0.001
Foliar application . Time of submergence	2.05	4	0.46	15.7	0.767
Foliar application .Type of seed	6.45	4	1.44	15.7	0.268
Time of submergence . Type of seed	2.85	1	2.85	16.4	0.110
Foliar application . Time of submergence. Type of seed	1.14	4	0.25	15.7	0.903
b.) Experiment factor: Time of submergence					
Foliar application	195.36	4	48.84	38.0	<0.001
Time of submergence	0.24	1	0.24	38.0	0.625
Type of seed	12.85	1	12.85	38.0	<0.001
Foliar application . Time of submergence	1.30	4	0.32	38.0	0.859
Foliar application .Type of seed	11.81	4	2.95	38.0	**0.032**
Time of submergence .Type of seed	0.46	1	0.46	38.0	0.500
Foliar application . Time of submergence . Type of seed	1.23	4	0.31	38.0	0.872

Appendix 4.8.1 (Continued)

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	*P*
c.) Experimental factor: Type of seed					
Foliar application	186.04	4	46.51	36.9	<0.001
Time of submergence	0.37	1	0.37	36.9	0.548
Type of seed	12.83	1	12.83	37.2	<0.001
Foliar application . Time of submergence	1.22	4	0.30	36.9	0.873
Foliar application .Type of seed	11.79	4	2.95	37.2	**0.033**
Time of submergence .Type of seed	0.46	1	0.46	37.2	0.500
Foliar application . Time of submergence . Type of seed	1.23	4	0.31	37.2	0.872

Appendix 4.8.2 Interaction analysis between seed development stage and molybdenum treatment (ABA spray was excluded)

Term	d.f.	Wilk's lambda	Rao F	n.d.f.	d.d.f.	*P*
Molybdenum (Mo)	3	0.1846	44.16	3	30	0.000
Seed development (DAA)	1	0.9908	0.28	1	30	0.601
Type of seed (Type)	1	0.718	11.78	1	30	0.002
Mo . DAA	3	0.965	0.36	3	30	0.781
Mo . Type	3	0.7582	3.19	3	30	0.038
DAA . Type	1	0.9908	0.28	1	30	0.601
Mo . DAA. Type	3	0.9654	0.36	3	30	0.783

Term	d.f.	Pillai-Bartlett trace	Roy's maximum root test	Lawley-Hotelling trace
Molybdenum (Mo)	3	0.8154	0.8154	4.4162
Seed development (DAA)	1	0.0092	0.0092	0.0093
Type of seed (Type)	1	0.282	0.282	0.3927
Mo . DAA	3	0.035	0.035	0.0362
Mo . Type	3	0.2418	0.2418	0.3189
DAA . Type	1	0.0092	0.0092	0.0093
Mo . DAA. Type	3	0.0346	0.0346	0.0359

Appendix 4.9 Nonlinear regression analysis of exponential decline of seed ABA concentration during grain filling and maturation of japonica rice cv. Gleva (2012)

Response variate: ABA (ng g^{-1} fresh weight)

Explanatory: DAA

Fitted Curve: $A + (B. R^X)$

Constraints: $R < 1$

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	2	251021.	125510.7	1026.12	<.001
Residual	29	3547.	122.3		
Total	31	254569.	8211.9		

Percentage variance accounted for 98.5

Standard error of observations is estimated to be 11.1.

Estimates of parameters

Parameter	estimate	s.e.
R	0.7023	0.0148
B	4531.0	779.0
A	86.49	2.45

Appendix 4.10 Analysis of variance of ABA concentration before (at 20 or 30 DAA) and after (at 24 or 34 DAA) submergence treatments (2014)

Appendix 4.10.1 ABA determination at 20 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	3627.3	1813.6	2.87	
Block.*Units* stratum					
Foliar treatment	4	2867.1	716.8	1.13	0.406
Residual	8	5062.0	632.8		
Total	14	11556.4			

Appendix 4.10.2 ABA determination at 24 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	848.7	424.4	1.41	
Block.*Units* stratum					
Foliar treatment	4	1388.3	347.1	1.15	0.399
Residual	8	2411.0	301.4		
Total	14	4648.0			

Appendix 4.10.3 ABA determination at 30 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	457.5	228.7	0.41	
Block.*Units* stratum					
Foliar treatment	4	611.7	152.9	0.27	0.888
Residual	8	4509.2	563.7		
Total	14	5578.4			

Appendix 4.10.4 ABA determination at 34 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	1490.9	745.5	0.94	
Block.*Units* stratum					
Foliar treatment	4	3442.0	860.5	1.08	0.426
Residual	8	6350.6	793.8		
Total	14	11283.5			

Appendix 4.11 Interactions of ABA concentration between foliar application and submergence treatments (2014)

Appendix 4.11.1 ABA concentration before (20 DAA) and after (24 DAA) 4 days' submergence at 20 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	1104.5	552.2	0.92	
Block.*Units* stratum					
Foliar treatment	4	925.0	231.2	0.38	0.817
ABA determination time[1]	1	91720.6	91720.6	152.24	<.001
Foliar treatment.ABA determination	4	3330.4	832.6	1.38	0.280
Residual	18	10844.6	602.5		
Total	29	107925.0			

[1] Before and after submergence treatment

Least significant differences of means (5% level)

Table	Foliar treatment	ABA determination
		Foliar treatment
		ABA determination time
rep.	6	15	3
d.f.	18	18	18
l.s.d.	29.77	18.83	42.11

Tukey's 98% confidence intervals

ABA determination

	Mean	
20 DAA	137.4	a
24 DAA	26.8	b

Appendix 4.11.2 ABA concentration before (30 DAA) and after (34 DAA) 4 days' submergence at 30 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	1388.7	694.3	1.09	
Block.*Units* stratum					
Foliar treatment	4	3120.5	780.1	1.23	0.333
ABA determination time[1]	1	85675.0	85675.0	135.04	<.001
Foliar treatment.ABA determination	4	933.1	233.3	0.37	0.828
Residual	18	11419.6	634.4		
Total	29	102536.9			

[1] Before and after submergence treatment

Least significant differences of means (5% level)

Table	Foliar treatment	ABA determination	Foliar treatment ABA determination time
rep.	6	15	3
d.f.	18	18	18
l.s.d.	30.55	19.32	43.21

Tukey's 98% confidence intervals

ABA determination

	Mean	
30 DAA	125.2	a
34 DAA	18.3	b

Appendix 4.11.3 ABA concentration at 20, 24, 30, and 34 DAA

Source of variation	d.f.	s.s.	m.s.	v.r.	*P*
Block stratum	2	2310.9	1155.5	1.96	
Block.*Units* stratum					
ABA determination time[1]	3	178989.0	59663.0	101.01	<.001
Foliar treatment	4	2398.0	599.5	1.01	0.412
ABA determination.Foliar treatment	12	5911.1	492.6	0.83	0.616
Residual	38	22446.3	590.7		
Total	59	212055.3			

[1] ABA determination dates of 20, 24, 30 and 34 DAA

Least significant differences of means (5% level)

Table	ABA determination	Foliar treatment	ABA determination time Foliar treatment
rep.	15	12	3
d.f.	38	38	38
l.s.d.	17.97	20.09	40.17

Tukey's 95% confidence intervals

DAA	Mean	
20	137.36	a
30	125.20	a
24	26.77	b
34	18.32	b

Appendix 4.12 Regression analysis of ABA concentration and molybdenum application (2014)

Appendix 4.12.1 at 20 DAA

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	368.	368.5	0.43	0.529
Residual	10	8666.	866.6		
Total	11	9034.	821.3		

Residual variance exceeds variance of response variate.
Standard error of observations is estimated to be 29.4.

Estimates of parameters

Parameter	estimate	s.e.	t(10)	t pr.
Constant	139.0	10.7	13.04	<.001
Foliar treatment	0.00454	0.00697	0.65	0.529

Appendix 4.12.2 at 24 DAA

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	183.0	183.00	7.19	0.025
Residual	9	229.2	25.47		
Total	10	412.2	41.22		

Percentage variance accounted for 38.2
Standard error of observations is estimated to be 5.05.

Estimates of parameters

Parameter	estimate	s.e.	t(9)	t pr.
Constant	23.76	1.95	12.18	<.001
Foliar treatment	-0.00327	0.00122	-2.68	0.025

Appendix 4.12.3 at 30 DAA

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	258.	258.3	0.52	0.489
Residual	10	4997.	499.7		
Total	11	5255.	477.8		

Residual variance exceeds variance of response variate.
Standard error of observations is estimated to be 22.4.

Estimates of parameters

Parameter	estimate	s.e.	t(10)	t pr.
Constant	128.00	8.10	15.80	<.001
Foliar treatment	-0.00380	0.00529	-0.72	0.489

Appendix 4.12.4 at 34 DAA

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	0.47	0.47	0.04	0.840
Residual	9	99.19	11.02		
Total	10	99.67	9.97		

Residual variance exceeds variance of response variate.
Standard error of observations is estimated to be 3.32.

Estimates of parameters

Parameter	estimate	s.e.	t(9)	t pr.
Constant	10.32	1.25	8.24	<.001
Foliar treatment	-0.000163	0.000788	-0.21	0.840

Appendix 4.12.5 Regression analysis of measured ABA and molybdenum at 20 and 30 DAA

Source	d.f.	s.s.	m.s.	v.r.	*P*
Regression	1	889.	888.9	1.26	0.274
Residual	22	15514.	705.2		
Total	23	16403.	713.2		

Percentage variance accounted for 1.1
Standard error of observations is estimated to be 26.6.

Estimates of parameters

Parameter	estimate	s.e.	t(22)	t pr.
Constant	150.1	15.4	9.74	<.001
Measured mo	-14.4	12.9	-1.12	0.274