Waterlogging tolerance at germination in pea

(Pisum sativum L.)

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This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

Centre for Plant Genetics and Breeding
School of Agriculture and Environment

August 2019
I, Md Shahin Uz Zaman, certify that:

This thesis has been substantially accomplished during enrolment in this degree.

Only a small part of research- the evaluation of pea germplasm accessions in relay cropping in Chapter 3 has been conducted in the field in Bangladesh before the enrolment.

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ABSTRACT

Peas (*Pisum sativum* L.) are an important pulse crop, ranks second in global production after beans among the pulse crops. It is a good source of plant–based protein for human diet. The crop can fix substantial quantities of atmospheric nitrogen to soil to improve soil fertility. Therefore, it is grown as rotational crop for sustainable productivity in the cereal based cropping around the world. In South Asia, there is a traditional practice of relay-sowing of pea, where pea seeds are hand-broadcasted onto waterlogged soil into the standing rice 2-3 weeks prior to rice harvest. A short period of WL at this stage leads to delayed germination, germination failure and finally reduced yield.

Following the initial evaluation of 91 germplasm accessions in relay cropping in Bangladesh, three contrasting genotypes were further evaluated in three waterlogged treatments (drained control, 4 and 8 d WL) in controlled environments at the University of Western Australia (UWA). The mechanism of WL tolerance was studied by whole genome RNAseq to capture differentially expressing genes during WL. Finally, a recombinant inbred line (RIL) population developed from a bi-parental cross between WL tolerant genotype Kaspa and sensitive BM-3, and a germplasm diversity panel were studied to identify extreme phenotypes, understand the genetic basis of WL tolerance and identify traits for possible use in indirect selection.

During WL in the field there was significant variation in seedling emergence between genotypes Natore local-2 (NL-2) (13 plants m$^{-2}$) and BARI Motorshuti-3 (BM-3) (6 plants m$^{-2}$). This contrasting response to WL was confirmed under controlled environments at UWA with an additional local cultivar Kaspa. There was 14% emergence in BM-3, 40% in NL-2 and 55% in Kaspa after 8 d of WL. The ability of genotypes to survive in WL was associated with testa colour and testa integrity. Dark testa Kaspa showed visually intact testa, low electrical conductivity (EC) and higher percent of germination. In contrast, light testa BM-3 exhibited solute leakage, higher EC and lower germination.
Among differentially expressed genes, the fat metabolism genes- LOX5 and PNC2 known elsewhere to cause membrane damage and subsequent solute leakage were downregulated in tolerant Kaspa but upregulated in sensitive BM-3.

A wide range of variation in germination % in waterlogged soil was observed in the RIL population (6 - 93%) and the diversity panel (5 - 100%) with a high broad-sense heritability (H² > 85%). The variation was continuously distributed suggesting polygenic control. Most genotypes with a dark coloured testa (90%) were WL tolerant, whereas those with a light testa were all WL sensitive in both the RIL population and the diversity panel. Testa integrity, measured by EC of the leakage solute, was strongly associated with WL tolerance in the RIL population (r_G = -1.00) and the diversity panel (r_G = -0.90). Indirect selection for WL tolerance via EC of the leakage solute was more efficient than direct selection for WL tolerance.

The presence of genotypic variation for WL tolerance in pea at germination raises the prospect of rainfed Mediterranean ecotype pea into relay-sowing on moist soils with rice. Climate change predictions for South Asia suggest alterations in the intensity of rainfall events, an increase in inter-annual precipitation variability and delayed monsoon rains which could significantly change crop productivity. We anticipate that selection for WL tolerance at an early stage of crop growth could significantly reduce 6 – 42% seed yield loss in pea (Belford 1980) and help climate-proof crop yield as part of a strategy to enhance productivity in South Asia.
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## AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

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Chapter 1

General Introduction

Waterlogging (WL) of soil is a widespread problem in crop production in both irrigated and non-irrigated cropping systems around the world. Approximately one-tenth of the irrigated cropland globally suffers occasional or frequent WL (Mancuso & Shabala 2010). In USA, 16% of cropland is affected by WL (Boyer 1982), which is also a major constraint to crop production in India, Pakistan, and China (Crosson & Anderson 1992). Waterlogging is frequently observed in South and South East Asia including Bangladesh, India, Nepal and Pakistan (Samad et al. 2001) in irrigated rice-based cropping systems, where soil is puddled for rice (Cass et al. 1994). Puddling increases soil bulk density and causes a plough-pan in the upper layer of soil that reduces drainage (Prihar et al. 1985), and causes WL in any succeeding dryland crops. By contrast, in non-irrigated cropping systems WL occurs mainly in duplex soil types - widespread in Australia (Chittleborough 1992) - that comprise sandy soil in the A-horizon and impermeable rocks in a layer in the B-horizon where rainwater accumulates on compacted clay subsoil and causes WL (Tennant et al. 1992).

Waterlogging may appear at different growth/developmental stages of crops. In rice-based cropping, WL is a common problem at germination for dryland crops. For instance, the cultivation of pea after the harvest of transplanted monsoonal rice (henceforth called T. aman) (late October/November) is delayed (up to three weeks) due to high soil moisture content. This reduces pulse yield and also delays subsequent irrigated boro rice transplanting (February to early March) with knock-on effects on rice yield. Therefore, peas (Pisum sativum L.) are often sown as relay with monsoonal rice on waterlogged soil (Oryza sativa L.) (Ali & Sarker 2013). A short period of waterlogging can lead to delayed germination (Zaidi et al. 2012, maize (Zea mays L.)), germination failure (Crawford 1977, pea; Sarlistyaningsih et al. 1995, narrow-leaf lupin (Lupinus angustifolius L.); Kumutha et al. 2008,
pigeonpea (*Cajanus cajan* (L.) Millsp.), and the reduced growth of coleoptiles (Biswas & Yamauchi 1997, rice), roots and shoots (Ismail et al. 2009, rice) and seedlings (Rowland & Gusta 1977, fababean (*Vicia faba* L.) and pea).

Genetic variation is a potential way to mitigate WL stress. However, the capacity to tolerate waterlogging varies from species to species (McDonald et al. 2001) and even within species. Moreover, the growth stages of the plant also show different responses in tolerance to WL (Setter & Waters 2003), but most research has focused on variation in WL tolerance at seedling, vegetative and flowering stages of plants. Some studies, like lentil (*Lens culinaris* Medik. ssp. *culinaris*) (Wiraguna et al. 2017), pigeonpea (Sultana et al. 2013), soybean (*Glycine max* (L.) Merr.) (Hou & Thseng 1991), wheat (*Triticum aestivum* L.) (Ueno & Takahashi 1997), maize (Zaidi et al. 2012) and barley (*Hordeum vulgare* L.) (Takeda & Fukuyama 1986) have examined the effect of waterlogging at the stage of germination.

During WL, plants switch from high energy yield respiratory metabolism to low energy yield fermentative metabolism as a survival mechanism (Ismond et al. 2003; Banti et al. 2013). However, metabolic and physiological processes as well as underlying genomic regulation responsible for WL tolerance are poorly understood at germination when root and shoot systems are absent and emergence totally depends on reserved food materials in seeds. The morphological trait, testa colour is associated with WL tolerance at germination in such crops as soybean, wheat and rapeseed (Hou & Thseng 1991; Ueno & Takahashi 1997; Zhang et al. 2008).
Pea- the foundation of modern plant genetics- is an important crop that ranks second in global production among the pulse crops (FAOSTAT 2017). The total world dry pea production was 16.2 million tons, with Canada as the leading producer, followed by USA, India, Russia, France and China in 2017 (FAOSTAT 2017). It is grown worldwide in temperate to elevated tropical environments in a wide range of fine and medium textured well-drained soil (Redden et al. 2005) within different cropping systems, particularly as a rotational crop with cereals for sustainable cropping (Nemecek et al. 2008). In South Asia pea is often sown as relay with rice (Ali & Sarker 2013) that causes WL at germination. Therefore, it is essential to study WL tolerance for survival of pea in South Asia. Dramatic differences between field pea genotypes in waterlogging tolerance were observed in a relay-sown crop in the field in Bangladesh that led to this study and made a prospect for pea in waterlogged soil in South Asia, although pea was domesticated in a rainfed Mediterranean ecosystem (Zohary & Hopf 1973).

The specific aims of this thesis were to:

- Identify the WL tolerance at germination in pea
- Understand the metabolic, physiological and molecular mechanisms for WL tolerance at germination
- Investigate the morpho-physiological traits that could be used in indirect selection for waterlogging tolerance
- Study the inheritance of waterlogging tolerance at germination
References


Chapter 2

Literature review

Authors:
Md Shahin Uz Zaman, Al Imran Malik, Parwinder Kaur and William Erskine

Attribution Statement
Due to the publication-based nature of this thesis, some similarities and repetition may exist in this chapter and thereafter.

The Ph.D. candidate M. S. U. Zaman made the following contributions:

- Reviewed the scientific literature
- Structured and wrote the chapter

Co-authors made the following contributions:

- Provided feedback and edited the chapter
Chapter 2

Literature review

Peas (*Pisum sativum* L.) are important grain legumes grown globally, covering an area of 8.14 million ha with a production of 16.20 million tonnes for dry peas in 2017 (FAOSTAT 2017). In South Asia dry pea is cultivated in the area of 0.89 million ha with the production of 0.78 million tonnes. However, soil waterlogging (WL) is a major constraint to production in some instances. Approximately one-tenth of the irrigated cropland globally suffers WL (Mancuso & Shabala 2010). Waterlogging is frequently observed in irrigated rice-based cropping systems, particularly when peas are grown as relay cropping with rice. This literature review first outlines first pea domestication, distribution, production in different cropping systems and production constraints. Next, it particularly focuses on soil WL problem and its effects on pea production at germination in relay cropping. This is followed by a review of the genetic control of WL tolerance at germination as a strategy to mitigate WL stress. It surveys information on genotypic variation in WL tolerance, morphological, physiological and molecular mechanisms of WL tolerance and finally the genetic basis of WL tolerance. The review concludes with a discussion of gaps in knowledge on genotypic variation and the potential mechanisms of WL tolerance in pea at germination.

2.1 Background of pea

2.1.1 Domestication of cultivated pea

The genus *Pisum* belongs to the Leguminosae or Fabaceae family (subfamily- Faboideae) within tribe Fabae which contains three species- i) Wild species *Pisum fulvum* var. *amphicarpum* which is observed in Jordan, Syria, Lebanon and Israel; ii) Domesticated species *Pisum abyssinicum* (syn. *Pisum sativum* subsp. *abyssinicum*) which is restricted in Yemen and Ethiopia and iii) The other dominated domesticated species *Pisum sativum*, which has two sub-species,
domesticated sub-species *Pisum sativum* subsp. *sativum* and a wild sub-species *Pisum sativum* subsp. *elatius* (syn. *Pisum elatius, Pisum syriacum*). *Pisum sativum* L. is one of the world’s oldest domesticated crops. Archaeological evidence shows the existence of pea back to 7,000-6,000 B.C. in early Neolithic farming villages of the Near East (Turkey to Iran) (Zohary & Hopf 1973). The earliest carbonized peas with smooth testa were discovered in aceramic Khirokitia, Cyprus (5,500 B.C.) which suggest domesticated peas were found at Cayoni, southeast Turkey (Zeist 1972). During the Neolithic and Bronze ages they spread to Europe, the Mediterranean, Africa and then to India. Domestication of peas has occurred for various purposes including dry grain, as a vegetable crop and for forage, resulting in strong selection for specific plant, pod and seed traits (Davies 1976). The most reliable indication for domestication in pea is provided by the surface of the seed coat. Wild peas are characterized by a rough or granular seed surface while the cultivated varieties have smooth seed coat. Subsequently, during centuries of selection and breeding, thousands of pea varieties were developed, many of which are maintained in germplasm collections worldwide.

2.1.2 Studies of pea as genetic model organism

Pea is a self-pollinated, cool-season, annual and diploid crop with 14 chromosomes (2n=14, n=7) and a genome size of ~4300 Mbp (Cool Season Food Legume Crop Database Resources 2018). Pea was used as a model crop for hybridization experiments before Mendel’s genetic discoveries. This might be due to the presence of contrasting observable traits, short life cycle, easy of pollination and seed production from a single cross. The first systematic hybridization of plants between different varieties and species was started in 1694 (Camerer 1694). However, in pea, crossing and the transmission of traits among generations was found by Thomas Andrew Knight (Knight 1799). In his crossing, varieties with grey/pigmented and white/transparent testa produced hybrid seeds with uniformly grey testa. Further, the heterozygous grey seeds produced two types of testa- grey and white but the number of seeds belongs to each colour types was not recorded. In another crossing between deep blue and
yellowish-white cotyledon created yellowish-white F$_1$ and different colours in the segregating generations, but the number of seeds of each colour types were not counted separately either (Goss 1824). Gregor Johann Mendel (1822–1884) studied 22 varieties of pea and recorded seven different traits - flower color (purple or white), flower position (axil or terminal), stem length (long or short), seed shape (round or wrinkled), seed color (yellow or green), pod shape (inflated or constricted), and pod color (yellow or green) in each plant separately in the segregating population (25,174) (Weeden 2016). Finally, he reported data from hybridization experiments on seven traits that differed among the varieties and discovered the laws of inheritance (Mendel 1866), making pea the vehicle for the foundation of modern plant genetics at the beginning of twentieth century. Many plant breeding techniques were then developed on the basis of Mendelian principles of inheritance (Kingsbury 2009). These include pedigree, mass selection, and backcrossing approaches. The understanding of crop improvement based on Mendel’s genetic principles laid a firm foundation to science-based agriculture. Mapping populations, their use in segregation of molecular markers and marker–trait association to map and isolate genes, were developed on the basis of Mendelism.

2.2 Cultivation of pea

2.2.1 Production and utilization

Peas are grown in a wide range of fine and medium textured well-drained soils in temperate to elevated subtropical environments (Redden et al. 2005). It requires moderate temperatures in the range of 12-18 °C and relatively humid climate for optimum growth and development (Khan & Croser 2004). It ranks second in global production after beans among the pulses crop in 2017 (FAOSTAT 2017). In 2017, the world production of green and dry peas were 20.69 and 16.20 million tons, respectively (FAOSTAT 2017). Green peas include immature seeds alone and also immature pods with small immature seeds inside, while dried peas are fully ripened pod on the plant. China is the largest producer of green peas (12.58 Mt) followed by India (5.34 Mt) and USA (0.24 Mt). Major dry
pea producers were Canada (4.62 Mt), Russian Federation (3.28 Mt) and China (1.52 Mt). Dry pea is the third most important pulse crop in Australia after lupin (*Lupinus angustifolius* L.) and chickpea, where in 2017, the pea cultivated area was 0.230 million ha with a total production of 0.441 million tons (FAOSTAT 2017). The majority of Australian peas are exported to Asia and the Middle East. Australia is the 5th largest pea exporter (0.156 Mt) in the world, while Canada stands as the top exporter of dry peas (3.136 Mt) (FAOSTAT 2016).

Peas are grown for two purposes either for green pods as vegetables, which are harvested at their early stage of development or for their grains, which are harvested either as immature or dry mature seeds. Green peas are most often used as vegetables in cooking, while dry peas are mostly used in the feed industry, particularly in the diet of pigs and poultry (Khan & Croser 2004). Whole dry peas are used in confectionery and milled produce are utilized for making soups/dal, flour and canned products (Khan & Croser 2004). Forage peas are also grown as green manure for sustainable agricultural system (Mooleki et al. 2016). Pea seeds are an important dietary component as a good source of protein (21- 33%), starch (37- 49%), soluble fibre (2- 9%), minerals and vitamins (Dahl et al. 2012).

2.2.2 Cropping systems and production constraints

Pea is a major cool-season pulse crop which is grown as a rotational crop for sustainable cropping systems (Nemecek et al. 2008). The rotation of dry pea with non-legume crops increases yield for succeeding crops, as they are capable of fixing nitrogen and adding organic matter (Hu et al. 2017). It is also a break crop for cycles of diseases and pests which affect other crops, reduces incidence of weeds, and increases P, K and S availability, reduces risk of crop failure and improves biodiversity (Stevenson & Kessel 1996). Peas are often intercropped with cereal- barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.) and maize that increases total yields of pea/cereal intercropping compared to growing cereal as monocrop (Carr et al. 1998; Jacobs & Ward 2012; Yang et al. 2018). Pea has been progressively more cultivated to replace fallow in arid and semi-arid regions because it requires less
water to grow compared to cereals (Lenssen et al. 2007; Miller et al. 2015). A green pea crop can replace fallow between rainfed monsoon rice (*Oryza sativa* L.) and irrigated spring rice in the rice based cropping systems of South Asia (Malik et al. 2017), where it is also often grown as relay with monsoonal rice (Ali et al. 2013).

However, like other crops, peas are sensitive to a number of biotic and abiotic stresses. The major abiotic stresses for pea include waterlogging (WL), salinity, drought, heat, cold, frost, boron toxicity and soil pH (Dita et al. 2006). These pose a threat to crop production. Soil WL is a major production constraint that restricts leaf and internode extension, accelerates premature leaf senescence and reduces 6 – 42% seed yield in pea at different stages of growth with 5 days WL (Belford 1980).

### 2.3 Soil Waterlogging (WL)

WL is defined as a condition of the soil in which excess water limits gas diffusion (Setter & Waters 2003). The diffusion of gases is 10,000 times slower in waterlogged soil as compared to air (Jackson 1985) and the flux of O$_2$ into soils is approximately 320,000 times less when the soil pores are filled with water (Armstrong & Drew 2002; Colmer & Flowers 2008). Moreover, rapid consumption of O$_2$ by soil microorganisms reduces the oxygen concentration in the rhizosphere leading to hypoxia/anoxia. As a result of oxygen deficiency in the soil, root respiration is restricted (Jackson & Drew 1984) which leads to a decrease in shoot and root growth and decreased nutrient uptake by plants (Malik et al. 2002; Barret-Lennard 2003).
2.3.1 Waterlogging at germination

Soil WL at germination may occur as result of a combination of factors such as poor drainage, intense rainfall as well as excessive irrigation (Scott et al. 1990). For instance, in irrigated rice-based cropping systems, the top 10-20 cm soil is puddled for rice (Cass et al. 1994). Puddling causes a plough-pan in the upper layer of soil that reduces drainage (Prihar et al. 1985), and lead to WL for succeeding dryland crops in South and South East Asia including Bangladesh (Samad et al. 2001). Importantly, after harvesting of transplanted monsoonal rice (henceforth called T. aman) (late October/November) cultivation of pea as sole crop is delayed (up to three weeks) due to high soil moisture content. This reduces pulse yield and also delays subsequent irrigated boro rice transplanting (February to early March) with knock-on effects on rice yield. Hence, peas are often sown as relay with rice on waterlogged soil that led to WL at germination. By contrast, in dryland agriculture WL mainly occurs in duplex soil types, which are widespread in Australia (Chittleborough 1992), as well as in clay soil (Setter & Waters 2003). Duplex soils comprise coarse sand to clay loam in the A-horizon and light to heavy clay in B-horizon. An impermeable rocks/gravels is present at the boundary between A and B-horizon where rainwater accumulates on compacted clay subsoil (Tennant et al. 1992).

2.3.2 Types of waterlogging

There are two types of WL, transient (i.e. intermittent) and continuous. Both types have significant negative yield impacts over free drained soil, but their effects differ. For instance, the yield reduction was higher in continuous WL compared to intermittent WL in wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and oats (*Avena sativa* L.) (Watson et al. 1976). Similarly, root length and fresh weight were reduced more under continuous WL than intermittent WL in wheat and triticale (*X Triticosecale* Wittmack) (Thompson et al. 1992). However, the effect of transient WL can be more severe than continuous, if it occurs in several cycles (Waldren et al. 1987). Moreover, the effect
depends on the plant’s growth stage. For example, the yield of soybean \[ Glycine \textit{max} \ (\textit{L.}) \textit{Merr} \] was decreased more under continuous WL in early vegetative stage than during reproductive growth (Linkemer et al. 1998).

### 2.3.3 Effects of waterlogging on crop production

WL exerts its adverse effects on plants due to oxygen deficiency and this inhibits root respiration (Jackson & Drew 1984), leading to decreased shoot and root growth and nutrient uptake by plants (Malik et al. 2002). Eventually it causes significant yield losses. Grain legumes are among the more sensitive crop groups to WL and produce lower seed yield under WL than in free-draining situations. For example, under WL at the reproductive stage yield was decreased by 60% in pea and lupin (Pampana et al. 2016), by 35% in chickpea \( (\textit{Cicer} \textit{arietinum} \ (\textit{L.})) \) (Cowie et al. 1996), by 48% in cowpea \( (\textit{Vigna} \textit{unguiculata} \ (\textit{L.})) \) (Minchin et al. 1978), and by 41-58% in soybean (Oosterhuis et al. 1990). Among other crops, significant yield losses (39-44%) have been reported in wheat even with relatively WL-tolerant cultivars (Collaku & Harrison 2002). Two million ha of the wheat-belt in Western Australia are prone to WL that can cause crop losses from 10-15% to more than 50% (Dennis et al. 2000). WL also causes 25-30% yield losses in maize \( (\textit{Zea} \textit{mays} \ (\textit{L.})) \) in South-East Asia (Rathore et al. 1998). In addition, WL triggered ~11% yield loss in cotton \( (\textit{Gossypium} \textit{hirsutum} \ (\textit{L.})) \) that can increase up to 40% under severe waterlogged conditions (Hodgson & Chan 1982).

### 2.4 Crop variation of waterlogging tolerance

#### 2.4.1 Genotypic variation

Tolerance of plants to soil WL varies greatly from species to species (McDonald et al. 2001) and even within species (Sultana et al. 2013). Wetland species are more tolerant to WL due to an effective internal aeration system in the roots (Justin & Armstrong 1987) and strong barriers to radial \( \text{O}_2 \) loss (McDonald et al. 2001). By contrast, dryland species are sensitive to WL with a few days of WL leading to significant yield losses. Among dryland species, the grain legumes are highly sensitive to
WL (Siddique & Sykes 1997; Palta et al. 2010) but significant differences in tolerance have been observed. For example, fababean (*Vicia faba* L.) is more tolerant to WL followed by grasspea (*Lathyrus sativus* L.), narrow-leaf lupin, chickpea, lentil (*Lens culinaris* Medik. subsp. *culinaris*) and pea at the seedling stage (Solaiman et al. 2007; Malik et al. 2015). Among other dryland species, variation in tolerance has also been found among forage legume *Lotus* species (Real et al. 2008); cereal species -where rice is more tolerant to oxygen deficiency than wheat and maize (Mustroph & Albrecht 2003), as well as among Brassica species (Ashraf & Mehmood 1990).

Variation for WL tolerance has also been observed within species i.e. pea, mungbean (*Vigna radiata* L. Wilczek), pigeonpea (*Cajanus cajan* (L.) Millsp.), soybean and forage species *Melilotus siculus*. Beyond legumes, significant variation has also been reported in different cereal species such as rice, wheat and maize, as well as in the model species *Arabidopsis thaliana* (Table 2.1).
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<td>Maize</td>
<td>471</td>
<td>Germination</td>
<td>12, 36, 72, 96 and 120 h</td>
<td>5 cm above soil surface</td>
<td>Zaidi et al. 2012</td>
</tr>
<tr>
<td>Mungbean</td>
<td>530</td>
<td>Seedling</td>
<td>5 d</td>
<td>1.5 cm above soil surface</td>
<td>Islam et al. 2007</td>
</tr>
<tr>
<td>Wheat</td>
<td>5</td>
<td>Seedling</td>
<td>3, 7 and 14 d</td>
<td>1 cm above soil surface</td>
<td>Davis &amp; Hillman 1988</td>
</tr>
<tr>
<td>Melilotus siculus</td>
<td>22</td>
<td>Seedling</td>
<td>24 h</td>
<td>Stagnant</td>
<td>Rogers et al. 2008</td>
</tr>
<tr>
<td>Pea</td>
<td>133</td>
<td>Vegetative</td>
<td>50 d watering</td>
<td>Watered to field capacity</td>
<td>Murfet 1990</td>
</tr>
<tr>
<td>Rice</td>
<td>626</td>
<td>Vegetative</td>
<td>Stagnant flooding from vegetative to maturity</td>
<td>5 to 60 cm above soil surface</td>
<td>Vergara et al. 2014</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>86</td>
<td>Vegetative</td>
<td>1 and 9 h</td>
<td>Plant submergence</td>
<td>Vashisht et al. 2011</td>
</tr>
</tbody>
</table>
2.4.2 Geographical adaptation

Geographical adaptation within a species generally reflects the adaptation of morphological and physiological traits to the environment. It is an indicative of coping ability for genotypes to a specific stress environment. Genotypes with limited acclimatization ability are considered to be at the greatest risk for survival under a stress. However, it has been reported in several crops that WL tolerance is associated with geographical origin. In barley, varieties originating from the middle and lower reaches of the Yangtze River were more WL tolerant than from other areas in China, since barley is grown in winter in rotation with rice on soils very prone to WL (Ma & Gao 1990). Another study also demonstrated that lentil germplasm originating from Bangladesh was more tolerant than that from other regions, because lentil is often sown on waterlogged soil in rice-based cropping systems in Bangladesh (Wiraguna et al. 2017). Other adaptive responses observed in lentil are for boron deficiency (Srivastava et al. 2000), boron toxicity (Yau et al. 2000) and iron deficiency (Erskine et al. 1993). These studies show that environmental stress is a powerful force generating local adaptation via directional selection and rapid evolution (Erskine 1997). The link of such adaptive traits to environment can effectively be used for searching specific adaptive traits from genetic resource collection using focused identification of germplasm strategy (FIGS) approach. FIGS represents a direct and practical approach for identifying specific traits in contrast to core collections.

2.4.3 Variation over growth stages

Variation in WL tolerance has been observed in wheat and barley at different stages of plant growth and development (Setter & Waters 2003). Significant variation has been found at the germination stage, and waterlogged tolerant varieties have been identified in some dryland crop species such as pigeonpea (Sultana et al. 2013), soybean (Hou et al. 1995) and maize (Zaidi et al. 2012). Similarly variation is reported at the developmental stages of seedling, vegetative, flowering and pod growth, but the level of tolerance varies depending on the specific growth stage. For instance, the pre-
flowering stage of growth in pea is more susceptible to WL than any of the vegetative, flowering and pod-filling stages (Cannell & Gales 1979); whereas in mungbean (Ahmed et al. 2002) and chickpea (Cowie et al. 1996) the reproductive stage is the most sensitive growth stage.

2.5 Mechanisms of waterlogging tolerance

2.5.1 Seed reserve metabolism

Seed reserves provide essential energy for germination and coleoptile growth until a seedling becomes photo-autotrophic. In aerobic conditions, seeds germinate when the reserved food such as starch (Galland et al. 2017), protein (Yang et al. 2007; Rosental et al. 2014), and lipid (Sreenivasulu et al. 2008) are mobilized or degraded. However, under WL/anaerobic condition there is variation of germination among genotypes. Generally, carbohydrate reserve seeds, such as rice, wheat, maize, sorghum, and pea are more tolerant to hypoxia during germination than seeds with fatty acid reserve such as lettuce (Lactuca sativa L.), sunflower (Helianthus annuus L.), radish (Raphanus sativus L.), cabbage (Brassica oleracea var. capitata) and soybean (Al-Ani et al. 1985; Raymond et al. 1985). The enzymes amylases in cereal seeds are thought to play a major role in starch breakdown during germination (Murata et al. 1968). For example, amylases in rice seeds are able to break down starch into readily fermentable carbohydrates during germination under hypoxic or even anoxic conditions (Atwell & Greenway 1987). Similarly, Perata et al. (1997) noted that among cereals only the WL tolerant rice seed was able to germinate under anoxia due to combined action of amylolytic enzymes involved in starchy endosperm metabolism. By contrast, dryland crops such as wheat and barley failed to germinate due to non-functioning of amylolytic enzymes during anoxia. Moreover, the variation in the ability to break down starch into usable soluble sugars was also found within rice cultivars. Seeds of rice genotypes tolerant to flooding during germination had higher amylase activity, greater starch depletion, higher soluble sugar concentrations, better shoot and root growth, and higher seedling survival compared to sensitive genotypes during flooding (Ismail et al. 2009). With a
translation experiment, it was found that $\alpha$-amylase increased considerably in a wetland plant Acorus rhizome (*Acorus calamus* L.) to ensure a permanent supply of fermentable sugars for survival and growth, conversely, the impaired translation decreased $\alpha$-amylase in WL intolerant potato (*Solanum tuberosum* L.) tubers during anoxia (Arpagaus & Braendle 2000).

### 2.5.2 Anaerobic metabolism

Decreased oxygen during WL induces a rapid metabolic shift of carbohydrate catabolism, from aerobic to anaerobic pathways to generate adenosine triphosphate (ATP) to maintain plant growth (Banti et al. 2013). However, the production of ATP is 17-fold less in anaerobic pathways than aerobic respiration (Dennis et al. 2000, Fig. 2.1). Nevertheless, the acceleration of glycolysis to enhance ATP synthesis might be one of the most important mechanisms to alleviate the adverse effects of WL. In hypoxic/anoxic tissues, the pyruvate content increases and glycolytic and fermentative enzymes [pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH)] are induced due to the inactivation of oxidative phosphorylation (Borella et al. 2014). The main fermentative pathways active in plants during hypoxia are: alcohol, lactic acid, and alanine (Dennis et al. 2000). Alcohol is the main fermentation product during WL (Fox et al. 1995) and plants, which are more tolerant to flooding, have a more active alcohol fermentation pathway (Kennedy et al. 1992). For example in rice, ADH1 is essential for sugar metabolism via glycolysis to ethanol fermentation in both the endosperm and embryo (Takahashi et al. 2014) as well as the subsequent coleoptile growth during hypoxic conditions (Matsumura et al. 1995; Saika et al. 2006).

During anaerobic metabolism, high concentrations of ethanol, acetaldehyde, and CO$_2$ are produced from seeds and accumulated in the soak water as by-products of ethanol fermentation (Pesis & Timothy 1984). These end-products are toxic and lead to seed death. The amounts of ethanol excreted
during soaking of rice, lettuce (*Lactuca sativa* L.), maize, broadbean and pea seeds were negatively correlated (*r* = -0.98) with subsequent germination (Crawford 1977). This finding agrees with the results of Martin et al. (1988) in maize. Similarly in wheat, seed germination was inhibited by the higher accumulation of ethanol in the sensitive genotypes (Ueno & Takahashi 1997). Another study on 10 wetland plant species indicates that there is a correlation between susceptibility to flooding and ethanol toxicity, suggesting that tolerance to flooding might be related to ethanol tolerance in plants (Maricle et al. 2014).

**Figure 2.1.** Shift in metabolism occurring in plants treated by low oxygen conditions (adapted from Dennis et al. 2000, *Journal of Experimental Botany*, 51:89-97.

### 2.5.3 Molecular mechanisms

Despite knowledge on adaptive metabolic mechanisms, the understanding at the genome level of how plants respond to hypoxia is limited. However, progress has been made in some plants through the discovery of a molecular oxygen sensing mechanism in Arabidopsis (Licausi et al. 2011) and the identification of genes involved in morphological and metabolic adaptations under hypoxic conditions. Hypoxia-induced genes have been identified in various crops at the seedling stage, but
there is no information for the germination stage (Table 2.2). Most of these genes induced during hypoxia at the seedling stage are involved in sugar metabolism, glycolysis and fermentation pathways and ethylene biosynthesis (Sachs et al. 1996). The expression of hypoxia induced genes is controlled predominantly at the transcriptional level as well as during post-transcription (Fennoy & Bailey-Serres 1995). An anaerobic response element (ARE) (Walker et al. 1987) was identified in the promoters of the maize and Arabidopsis alcohol dehydrogenase genes (*ADH1*) and in the promoters of other anaerobically induced genes, indicating that the ARE participates in the control of genes in response to hypoxia stress. Other studies have consistently shown that hypoxia induces the expression of genes such as signal transduction components (Baxter-Burrell et al. 2002) and transcription factors (TFs) (DeVetten & Ferl 1995) which play an important role in waterlogging tolerance. Two TFs, SNORKEL (Hattori et al. 2009) and Submergence-1A (Xu et al. 2006) driven by ethylene have been reported in rice which are involved in internode elongation and metabolic regulation respectively to adapt rice to different types of flooding.
Table 2.2. The role of common differentially expressed genes during hypoxia/anoxia in different plant species

<table>
<thead>
<tr>
<th>Name of species</th>
<th>Hypoxia stage</th>
<th>Role of upregulated genes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td>Coleoptile</td>
<td>Anaerobic carbohydrate metabolism, ethylene response factors and heat shock proteins</td>
<td>Lasanthi-Kudahettige et al. 2007</td>
</tr>
<tr>
<td>Cucumber (Cucumis sativus L.)</td>
<td>Seedling</td>
<td>Carbohydrate mobilization, nitrate assimilation, ethylene hormone production and signaling pathways, transcription factors and cell for adventitious root primordia initiation</td>
<td>Xu et al. 2017</td>
</tr>
<tr>
<td>Maize</td>
<td>Seedling</td>
<td>Glycolysis and ethanolic fermentation, auxin response factor, carbohydrate and energy metabolism</td>
<td>Zhang et al. 2008</td>
</tr>
<tr>
<td>Soybean</td>
<td>Seedling</td>
<td>Alcohol fermentation, ethylene biosynthesis, pathogen defense and cell wall loosening</td>
<td>Komatsu et al. 2009</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>Seedling</td>
<td>Glycolysis and fermentation, ethylene synthesis, calcium signalling, nitrogen utilization, trehalose metabolism and alkaloid synthesis</td>
<td>Liu et al. 2005</td>
</tr>
<tr>
<td>Cotton</td>
<td>Flowering</td>
<td>Anaerobic fermentation, starch and sucrose metabolism, glycolysis and plant hormone signal transduction and ethylene related genes</td>
<td>Zhang et al. 2017</td>
</tr>
</tbody>
</table>
2.5.4 Testa colour/pigmentation

Seed testa pigmentation has frequently been observed to play a major role in tolerance to WL at germination (Table 2.3). For example, pea genotypes with dark green-brown seeds exhibited better WL tolerance than light green or white seeded genotypes; in soybean black seeds exhibited higher WL tolerance than those with a yellow seed coat colour; in wheat, seeds with a red colour showed higher tolerance to flooding compared to white seeds; in rapeseed (Brassica napus L.) flooding tolerance is significantly correlated with seed coat colour and with the melanin pigment content of the testa (Table 2.3). Such differences of tolerance have been associated with phenolic compounds in the testa, as in the rapeseed study dark testa tolerant genotypes had higher levels of phenolic compounds than the sensitive light testa genotypes (Zhang et al. 2008). The high levels of phenolic or tannin compounds in the testa are considered as a barrier to imbibition, since the dark testa genotypes in pea, fababean and Arabidopsis (Arabidopsis thaliana L.) are restricted in imbibition, while the light testa seeds are completely permeable to water and subsequent solute leakage (Kantar et al. 1996; Debeaujon et al. 2000). Therefore, it is likely that testa pigmentation plays a protective role against imbibition damage during WL stress.
Table 2.3. The association between testa colour and waterlogging (WL) tolerance in various crop species subjected to seed submergence with different duration and growth conditions

<table>
<thead>
<tr>
<th>Name of species</th>
<th>No. of tested genotypes</th>
<th>Submergence duration &amp; growth condition</th>
<th>Testa colour</th>
<th>% Mean germination</th>
<th>Range of % germination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td>9</td>
<td>14 d at 8 °C temperature</td>
<td>Brown</td>
<td>58</td>
<td>54 - 62</td>
<td>Uzun &amp; Acikgoz 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>White/green</td>
<td>26</td>
<td>0 - 56</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>730</td>
<td>4 d at 25 °C temperature</td>
<td>Brown/black</td>
<td>67</td>
<td>0 - 100</td>
<td>Hou &amp; Thseng 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yellow/green</td>
<td>10</td>
<td>0 - 100</td>
<td></td>
</tr>
<tr>
<td>Rapeseed</td>
<td>18</td>
<td>24 h at 20 °C temperature</td>
<td>Red/black</td>
<td>93</td>
<td>70 - 100</td>
<td>Zhang et al. 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yellow</td>
<td>64</td>
<td>14 - 100</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>342</td>
<td>8 d at 20 °C temperature</td>
<td>Red</td>
<td>46</td>
<td>0 - 100</td>
<td>Ueno &amp; Takahashi 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>White</td>
<td>23</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
2.5.5 Testa integrity

Testa is the primary defense of seeds against conditions such as mechanical injury and to biotic and abiotic stresses. At germination, the testa protects seeds against leakage of intracellular substances during imbibition (Simon 1974). However, with a short period of seed submergence (i.e. 24 h), rapid imbibition causes electrolyte leakage and the release of seed reserve sugars, amino acids, organic acids and proteins from seeds (Larson 1968; Samad & Pearce 1978; Simon & Wiebe 1975). Leakage was faster with seeds without testa than the seed with intact testa (Larson 1968, pea; Powell & Matthews 1978, pea; Duke & Kakefuda 1981, soybean, navy bean (Phaseolus vulgaris L.), pea, and peanut (Arachis hypogaea L.); Duke et al. 1983, soybean). The greater the leakage, the greater is the damage to seed tissue particularly membranes (Simon 1974; Powell & Matthews 1978; Woodstock & Taylorson 1981). The amount of leakage during imbibition is negatively correlated with seed vigour and field emergence in species such as pea (Larson 1968), soybean (Yaklich 1979) and peanut (Samad & Pearce 1978). Therefore, the leakage might be a symptom for the fundamental dysfunction of testa integrity. Possible mechanisms for dysfunction of the testa integrity are discussed below.

2.5.5.1 Loss of testa integrity by reduced adenosine triphosphate (ATP) production

Plants need un-interrupted access to an energy source for survival in an O₂-deprived environment (Barclay & Crawford 1983). However, in response to ATP deprivation in submergence germination, membrane damage can be induced, which is suggested by the correlation between the leakage of electrolytes from the cells and the release of free fatty acids (FFAs) in anoxic potato tubers (Crawford & Braendle 1996). The role of ATP in maintaining membrane integrity under anoxia has been confirmed by Rawyler et al. (1999) in potato cell culture. In that study potato cells survived temporarily (0–12 hr) under anoxic condition with intact cell membranes by the absence of lipid degradation. But with prolonged anoxia (>12 hr) cell biomass and ATP levels decreased, and the accumulation of free fatty acids increased by the extensive hydrolysis of phospholipids in cell
membrane. This suggests that there is a threshold in fermentation and ATP synthesis below which the integrity of the membranes in anoxic potato cells cannot be preserved (Rawyler et al. 1999). The underlying mechanism was the activation of tuber protein patatin after a threshold time under anoxia which exhibited lipolytic acyl hydrolase (LAH) activity leading to lipid hydrolysis by cleaving both fatty acyl chains from membrane lipids and subsequent membrane damage (Andrews et al. 1988; Rawyler et al. 2002). In contrast, additional studied mechanism in a flood-tolerant rice cultivar revealed that energy consumption was reduced by restricting shoot elongation under submergence (Setter & Laureles 1996). The energy in tolerant plants is preserved during submergence, and upon de-submergence their growth is restarted by using the preserved energy (Singh et al. 2001).

2.5.5.2 Loss of testa integrity by reactive oxygen species (ROS)

ROS are free radicals atoms or groups of atoms having at least one unpaired electron. ROS are highly unstable and tend to pair with other molecules in order to make a stable bond of two electrons (Foyer & Halliwell 1976). Plants constantly produce ROS in chloroplasts, mitochondria, peroxisomes and other sites of the cell during their normal metabolic processes such as photosynthesis and respiration. The generation of ROS is accelerated by various stresses. At the same time, plants have evolved with different anti-oxidants and anti-oxidative enzymes to normalize the ROS reactivity. Imbalance between ROS production and their detoxification by enzymatic and non-enzymatic reactions causes oxidative stress. As a result a higher net ROS catalyzes the degradation of phospholipids in testa membrane and causes ultimate cell death (Halliwell 1991; Barclay & McKersie 1994). This type of cell death is sometimes beneficial to plants, for example, during WL the death of the cell by ROS creates a gaseous space called aerenchyma in the root cortex that helps plants adapt in waterlogged soil (Drew et al. 1981; Jackson & Armstrong 1999; Evans 2003; Haque et al. 2010). Ethylene is mainly involved to induce aerenchyma formation (Jackson & Armstrong 1999; Evans 2003) but ROS are the key factors that transduce signals stimulated by abiotic stresses in plants (Baxter et al. 2014).
For example, in rice, ethylene promotes adventitious root growth, death of epidermal cells in adventitious root primordia, and parenchymal cell death which results in aerenchyma formation. All of these responses are mediated by ROS, therefore, ROS are involved in many adaptive responses of plant in stress environment when the root systems are well developed.

In contrast, at germination when roots and shoots are absent, ROS is lethal to germination during WL stress. The elevated levels of ROS during WL stress (Foyer et al. 1994, Alscher et al. 1997) at germination causes membrane lipid peroxidation and degradation. Lipid peroxidation in the testa membrane leads to membrane damage and subsequent leakage of seed solutes. As a result seeds failed to germinate due to lack of energy necessary for germination in pea (Rahoui et al. 2010).

2.5.6 Role of antioxidant activity for WL tolerance

Plants have evolved different antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidases (APX) and glutathione reductase (GR), enzymes detoxifying lipid peroxidation products (glutathione S-transferases, phospholipid-hydroperoxide-glutathione peroxidase and ascorbate peroxidase), and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds and α-tocopherol) to protect cells from possible ROS damage. The activity of antioxidant enzymes increases in response to different environmental stresses such as WL, salinity, drought, chilling, high light intensities and pathogen infection (Bowler et al.1992; Sairam et al. 2002). Phenolic compounds are the potential source of antioxidants. Several studies showed a positive correlation between phenolic compounds and antioxidants (Jun et al. 2014, Canola seed; Arcan & Yemenicioglu 2009, hazelnuts [Corylus avellana L.], walnuts [Juglans nigra L.] and pistachios [Pistacia vera L.]; Sreeramulu & Raghunath 2011, areca nut [Areca catechu L.], linseed [Linum Usitatissimum L.], mustard [Brassica nigra. L.], safflower [Carthamus tinctorius L.], Milk and Milk products). The external colour of seeds or fruits is a good indicator for phenolic compounds. Seeds with dark testa/pigmentation exhibit higher amounts of phenolic compounds than the seeds.
with a lighter coloured testa (Zhang et al. 2008, rapeseed; Weidner et al. 2018, bean), and dark testa genotypes are tolerant to WL in several studies. Therefore it may be inferred that the antioxidant properties of dark testa seeds are key to WL tolerance by neutralizing ROS activity in cells.

2.6 Genetic basis of waterlogging tolerance

A knowledge of the inheritance of WL tolerance is a pre-requisite to efficiently develop varieties with the desired tolerance. There are, however, only a few reports of the genetic control of WL tolerance in crops including soybean (Hou et al. 1995), wheat (Boru et al. 2001) and rice (Toojinda et al. 2003). The nature of the genetic control varies from species to species and is summarized in Table 2.4. Quantitative trait locus (QTL) mapping is a powerful approach to identify key genomic regions controlling adaptive traits, especially for species where a reference genome is already available. QTLs for tolerance to WL have been reported in soybean (Van Toai et al. 2001), wheat (Yu & Chen 2013), maize (Qiu et al. 2007) and cucumber (Yeboah et al. 2008).
Table 2.4. The inheritance of waterlogging (WL) tolerance in various crop species subjected to WL at different duration and growth stages of crops

<table>
<thead>
<tr>
<th>Name of species</th>
<th>Stage of WL</th>
<th>Submergence /WL duration</th>
<th>Selection criteria for tolerance</th>
<th>Control of WL tolerance</th>
<th>Heritability ($H^2$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td>Vegetative</td>
<td>50 d</td>
<td>Loss of leaflet</td>
<td>Reduced stipule mutant, st</td>
<td>-</td>
<td>Murfet 1990</td>
</tr>
<tr>
<td>Pigeonpea</td>
<td>40 DAS*</td>
<td>8 d</td>
<td>Dead/survival plant</td>
<td>Dominant effect</td>
<td>-</td>
<td>Sarode et al. 2007</td>
</tr>
<tr>
<td>Soybean</td>
<td>Germination</td>
<td>4 d</td>
<td>% Germination</td>
<td>Both additive and dominant effects</td>
<td>47%</td>
<td>Hou et al. 1995</td>
</tr>
<tr>
<td>Wheat</td>
<td>First node</td>
<td>40 d</td>
<td>% Leaf chlorosis</td>
<td>Additive effect</td>
<td>-</td>
<td>Boru et al. 2001</td>
</tr>
<tr>
<td>Maize</td>
<td>Knee-high</td>
<td>7 d</td>
<td>Chlorophyll concentration</td>
<td>Both additive and non-additive effects</td>
<td>-</td>
<td>Zaidi et al. 2010</td>
</tr>
<tr>
<td>Barley</td>
<td>Three leaf</td>
<td>10 d</td>
<td>% Yellow leaves</td>
<td>Additive effect</td>
<td>73%</td>
<td>Zhou et al. 2007</td>
</tr>
<tr>
<td>Rice</td>
<td>Seedling</td>
<td>3 d</td>
<td>Shoot elongation</td>
<td>Additive effect</td>
<td>-</td>
<td>Toojinda et al. 2003</td>
</tr>
<tr>
<td>Cucumber</td>
<td>27 DAS*</td>
<td>7 d</td>
<td>% Wilting plants</td>
<td>Additive effect</td>
<td>74%</td>
<td>Yeboah et al. 2008</td>
</tr>
</tbody>
</table>

* DAS= Days after sowing
2.7 Conclusions

Waterlogging is one of the major constraints to crop production around the world that causes 6-42% yield loses of pea. Improving tolerance is the best way to mitigate WL stress. The genotypic variation of WL tolerance was found in vegetative stage in pea, and different growth stages for other crops. However the literature review indicates a relative lack of information available about WL tolerance at germination. The mechanism of WL tolerance at germination is different than other stages. The morphological mechanism for WL tolerance include testa colour associated with WL tolerance, whereas the adaptive physiological mechanisms include seed reserves metabolism, anaerobic metabolism, induced antioxidant systems and genomic regulation enabling germinating seeds to survive under WL stress. These literature resources will accelerate our understanding of the mechanisms involved in adaptation of the original model species pea used by Gregor Mendel to excess water stress.
2.8 References


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Vergara, G. V., Nugraha, Y., Esguerra, M. Q., Mackill, D. J., & Ismail, A. M. (2014). Variation in tolerance of rice to long-term stagnant flooding that submerges most of the shoot will aid in breeding tolerant cultivars. *AoB Plants, 6*.


Chapter 3

Waterlogging tolerance of pea at germination


This thesis chapter - waterlogging tolerance of pea at germination was designed by M. S. U. Zaman in consultation with W. Erskine, A. I. Malik and P. Kaur. Seed sowing in relay cropping in the field in Bangladesh, soil redox measurement and data recording were handled by M. S. U. Zaman where A. I. Malik was involved in seed sowing. In the glasshouse experiment at the University of Western Australia - soil preparation, seed sowing, soil redox measurement and data recording were conducted by M. S. U. Zaman in consultation with A. I. Malik. The manuscript was written by M. S. U. Zaman and the co-authors W. Erskine, A. I. Malik and P. Kaur were involved in the discussion of results, structure of the manuscript and editorial comments.
Chapter 3

Waterlogging tolerance of pea at germination

3.1 Abstract

Peas (*Pisum sativum* L.) are exposed to waterlogging at germination when grown as relay in rice-based cropping. Ninety one germplasm accessions were evaluated in relay (sown in waterlogged soil), and subsequently 10 diverse genotypes compared under relay and sole cropping (conventional tillage sowing) over two seasons in Bangladesh. Contrasting genotypes, BM-3, NL-2 and Kaspa, were further evaluated in three waterlogging treatments (drained control, 4 and 8 days waterlogging) in the glasshouse. Conspicuous variation in waterlogging tolerance at germination was observed in the field and confirmed under controlled conditions. In relay sowing in 2011, emergence of a few genotypes was affected by waterlogging. In 2012 emergence in relay was severely affected (12 plants m$^{-2}$) compared to sole sowing (37 plants m$^{-2}$). Among genotypes, BM-3 had 6 plants m$^{-2}$ emerge, which all subsequently died, in contrast to NL-2 in which emergence was 13 plants m$^{-2}$ with all plants surviving. In the glasshouse there was 14% emergence in BM-3, 40% in NL-2 and 55% in Kaspa after 8 days of waterlogging. Such marked differences in waterlogging tolerance at germination in the model pea are the first reported and illustrate prospects for selection to improve adaptation to relay sowing in South Asia.
3.2 Introduction

Peas (*Pisum sativum* L.) are adapted worldwide to many soil types, but grow best on fertile, light-textured, well-drained soils (Elzebroek & Wind 2008). In South Asia 1.7 million hectare (FAOSTAT 2013) of pea is grown mainly in rice-based systems either as a sole crop (Awadhwal et al. 2001) following rice or as relay with rice (Gupta & Bhowmick 2005; Ali & Sarker 2013). For sole cropping, cultivation after the harvest of transplanted monsoonal rice (henceforth called T. *aman*) (late October/November) is delayed (up to three weeks) due to high soil moisture content, so sowing as a sole grain legumes is later than as relay (Ali et al. 2005). This reduces pulse yield and also delays subsequent irrigated *boro* rice transplanting (February to early March) with knock-on effects on rice yield. Given the tight cropping window available for crop intensification between rice crops, relay sowing - whereby pea seed is hand-broadcast into the standing T. *aman* rice on wet soil approximately 2 weeks prior to rice harvest - is a timely alternative practice (Ali & Sarker 2013) that does not reduce rice production. During rice cropping a plough-pan is formed from puddling in the upper layer of the soil to create a favourable environment for rice growth and development (Cass et al. 1994). Puddling increases soil bulk density (Prihar et al. 1985) and reduces soil drainage (Scott et al. 1990). As a result, relay sowing of peas with T. *aman* rice are often exposed to soil waterlogging to pea in South Asia including Bangladesh, India, Nepal and Pakistan (Samad et al. 2001). In contrast, cultivation prior to sole crop sowing destroys the plough-pan reducing the risk of waterlogging.

Under waterlogging, plants are exposed to a deficient O$_2$ supply because when soil pores are filled with water the flux of O$_2$ into soils is 320, 000 times less than that of air-filled pores (Armstrong & Drew 2002). In addition to O$_2$ deficiency, harmful levels of ethanol and acetaldehyde are increased from seed as an end products of anaerobic metabolism (Woodstock & Taylorson 1981). As a result, a short period of waterlogging can delay germination and lead to germination failure. For example, genotypes of pea (Crawford 1977), narrow-leaf lupin (*Lupinus angustifolius* L.) (Sarlistyaningsih et
al. 1995) and pigeonpea (*Cajanus cajan* (L.) Millsp.) (Kumetha et al. 2008) failed to germinate following from 3 - 6 days of waterlogging. Germination was also severely affected in soybean (*Glycine max* (L.) Merr.) by 4 days soaking prior to germination (Hou & Thseng 1992). Cereal crops, such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.) and rye (*Secale cereale* L.), also failed to germinate and lost viability in waterlogged soils, resulting in poor crop establishment (Perata et al. 1993, 1997). In addition, waterlogging delayed germination in maize (*Zea mays* L.) (Zaidi et al. 2012) and reduced seedling growth in fababean (*Vicia faba* L.) and peas (Rowland & Gusta 1977). Furthermore, waterlogging at germination reduced coleoptile growth (Biswas & Yamauchi 1997) and root and shoot growth (Ismail et al. 2009) in rice (*Oryza sativa* L.).

Despite the multiple advantages of pulse relay sowing into a standing *T. aman* rice crop, one possible disadvantage is the increased risk of waterlogging damage compared to later-sown sole cropping into tilled land. To illustrate the potential for crop intensification by relaying pulses into double rice cropping, in Bangladesh alone the area of *T. aman – boro* rice is 1.8 million ha (Elahi et al. 1999) and additional major areas exist in adjacent parts of India. To intensify pulses in this region, it is important to exploit the considerable variation in waterlogging tolerance reported both between (McDonald et al. 2001) and within species (Sultana et al. 2013). Species adapted to wetlands are more tolerant to waterlogging (Justin & Armstrong 1987) than dryland species such as legume crops which are highly sensitive to waterlogging. Nevertheless among such species significant differences in tolerance have been observed. For example, pea is more sensitive to waterlogging than lentil (*Lens culinaris* Medik. ssp. *culinaris*), chickpea (*Cicer arietinum* L.), narrow-leafed lupin, grasspea (*Lathyrus sativus* L.) and fababean, respectively (Solaiman et al. 2007). Another experiment also rated pea as more sensitive than lentil and grasspea (Malik et al. 2015). Genetic variation for tolerance to waterlogging at germination has been observed within legume species; for example, lentil (Wiraguna et al. 2017), pigeonpea (Sultana et al. 2013) and soybean (Hou et al. 1995).
Planting waterlogging tolerant varieties may be the most effective means to overcome low germination in waterlogged soil (DeBoer 1970). To our knowledge, however, there is no study on pea of waterlogging tolerance at germination. Therefore, the present study was to identify genetic variation in waterlogging tolerance at germination in the field, confirm this under controlled conditions, and thereby optimized a methodology to assess genotypic variability for the trait.

3.3 Materials and methods

The study had two components: Field experimentation in Bangladesh over three seasons and a glasshouse trial in Australia.

3.3.1 Field experimentation

3.3.1.1 Evaluation of pea germplasm in relay cropping

A total of 91 accessions and breeding lines of pea from the collection at Pulses Research Centre (PRC), Bangladesh Agricultural Research Institute (BARI) and the national gene bank at BARI and the Australian Grains Genebank, Department of Jobs, Precincts and Regions, Victoria were screened under relay sowing into a standing T. aman rice where soil water was visible at the soil surface. Collected seeds were multiplied in 2010 and stored in a cool room at 20 °C at PRC, Ishwardi, Bangladesh. Sowing was performed in November 2011 at PRC, BARI, Ishwardi (24°9′N; 89°4E; 19 m a.s.l.), Bangladesh. The experiment was conducted in a randomized complete block (RCB) design with two replications. Individual plots comprised two rows 1.5 m in length with row-to-row spacing of 35 cm. Seeds were soaked overnight (~12 h) in tap water and then sown by hand into the standing T. aman rice crop on November 3, approximately two weeks prior to rice harvest when the soil was sufficiently wet for germination. Rice was harvested manually on November 17 leaving ~30 cm of stubble, by which time the pea plants had two to three leaves. The land was medium low clay loam soil with pH of 7.5 (1: 5 soil and water suspension) containing 12 g organic matter kg\(^{-1}\) (Walkley &
Black 1934), 17 mg N kg\(^{-1}\), 26 mg P kg\(^{-1}\) and 300 mg K kg\(^{-1}\). Fertilizer was applied as urea, triple superphosphate (TSP) and potassium chloride (muriate of potash [MoP]) at the rate of 20 kg N ha\(^{-1}\), 40 kg P\(_2\)O\(_5\) ha\(^{-1}\) and 20 kg K\(_2\)O ha\(^{-1}\). The TSP and MoP were broadcast over the standing rice plant just before pea seed sowing and urea was top-dressed 20 days after pea sowing. Weeding was done manually 25 days after sowing and as necessary to maintain the plots weed-free. The insecticide Dimethoate (Tafgor 40 EC) was sprayed at 2 ml L\(^{-1}\) of water twice starting from the flowering stage to control aphids. Monthly rainfall and temperature records for the duration of the experiment were collected from the nearest weather station (∼5 km distance).

3.3.1.2 Evaluation of pea cultivar in relay vs. sole cropping

Ten diverse genotypes selected from the germplasm evaluation under relay cropping were compared in field experiments in relay and sole cropping. The genotypes were BARI Motorshuti-1 (BM-1), BARI Motorshuti-3 (BM-3), Natore local-1 (NL-1), Natore local-2 (NL-2), IPSA-3, Jhikorgacha local (JL), Bagha local (BL), BD-4190, BD-4142 and BD-4181. The experiment was conducted in two seasons - 2012-13 and 2013-14 – henceforth called 2012 and 2013 seasons respectively - at the Pulses Research Centre of Bangladesh Agriculture Research Institute (BARI), Ishwardi. The experiments had three sowing treatments for pea into/following T. aman rice - 1. Relay sowing on November 1 – the optimum date for relay cropping with rice; 2. Sole sowing on November 1; and 3. Sole sowing on November 11 after harvesting rice- the optimum date to sow for sole cropping. Treatments 1 and 3 are realistic agronomically as pea is grown either relay with rice or sole after harvesting rice. But Treatment 2 - which necessitated premature harvest/destruction of the rice crop to sow pea as sole - was done to deconstruct the comparison between Treatments 1 and 3 into the individual effects of sowing date and sowing method. All treatments were sown in RCB design with three replications, where the sole sowing treatments were adjacent to each other and the relay sowing was in an adjacent field of T. aman rice.
Treatment 1 was sown in the same field with similar management practices to germplasm evaluation. Thirteen platinum electrodes were inserted into the field to a depth of 100 mm to assay redox potential. Treatments 2 and 3 were in adjacent medium high land sandy loam soil with pH of 7.5. Land preparation was done by tractor and all the fertilizer including urea was applied as a basal dressing during final land cultivation. Soil clods were broken and the plot was levelled by ladder during final land preparation. Seeding was done by hand at a depth of 3 to 5 cm. Immediately after sowing, flood irrigation to field capacity was applied for all the sole cropping treatments in both the seasons. Soil crusting was broken where necessary.

For all treatments, each genotype was sown in 8-row plots 4-m in length with distances row-to-row of 35 cm and plant-to-plant of 7 cm which accommodated 40 seeds m$^{-2}$ of plot. Weeding was done manually 25 days after sowing and as necessary to maintain the plots weed-free. The insecticidal application was as in the germplasm evaluation.

### 3.3.2 Glasshouse experiment

The experiment was conducted with three cultivars only to confirm waterlogging tolerance. Two cultivars, BM-3 and NL-2 selected from field experiment those were markedly contrasting in waterlogging response under relay cropping in Bangladesh and Kaspa - a popular Australian variety was added as a local control. The experiment comprised of three treatments (drained control, 4 and 8 days waterlogging) and the design was factorial with genotypes (3 levels) × waterlogging treatment (3) in a completely randomized design with four replications. The experimental unit was plastic pot - free draining and sealed base. Free draining pots contained gravel at the bottom and 3.5 kg mixed sand and soil collected from Mukinbudin (30°78’ S, 118°31’ E), Western Australia (Kotula et al. 2015) (1: 1) at the top. The soil and sand were dried for 3 days at 65 °C, passed through a 0.5 mm diameter sieve and mixed thoroughly before pot filling. The soil mixture had pH 6.7 and EC 0.46 dS m$^{-1}$ at 1:5 w/v soil/ water. Each free-draining pot (19 cm height × 21 cm diameter) was placed in a sealed base.
pot (24 cm height × 26 cm diameter). A total of ten electrodes were inserted into the soil at a depth of 100 mm for redox measurement. For the waterlogged treatment, DI water was added to sealed base pots to maintain a water table at 10 mm below the soil surface of the free-draining pot. Pots were waterlogged for 4 days prior to sowing to ensure hypoxia at sowing. Water was added to sealed base pots daily as required to maintain the water table. For drained control treatments there was no water in the sealed base pots but the soil moisture in free draining pots were maintained at ~ 80% of field capacity. Seeds were treated with Thiram (Tetramethylthiuram disulfide) at the rate of 3 g kg\(^{-1}\) seeds just before sowing. Twenty seeds of each genotype were sown in a free-draining pot through dibbling to 5 mm soil depth. All pots were covered for 3 days after sowing to ensure darkness for germination. Waterlogged pots were drained as per treatment. Drained control pots were weighed every day and watered to ~ 80% field capacity. Within replicates pots were moved every 5 days to minimize bench locational effects in the glasshouse. The experiment was conducted in the glasshouse at UWA at 25 °C temperature. The trial was terminated 23 days after sowing, when there was no possibility for further emergence.

### 3.3.3 Measurements

In 2011 for germplasm evaluation, data on emergence stand at germination stage (two weeks after sowing) were recorded with 1-9 scoring scale using the pea descriptor (Pavelkova et al. 1986), where 1 = very small, 3 = small, 5 = intermediate, 7 = high and 9 = very high. Time to flowering was recorded when an individual plot reached 50% flowering, while time to maturity was at 80% pod maturity stage. At harvest, plant height, branches per plant, pods per plant, and hundred seed weight were recorded from five randomly selected plants in the guarded center of the plot. Seed yield was measured from the entire plot.

For the experiments of cultivar evaluation in relay vs. sole cropping in 2012 and 2013, the redox potential was measured daily in the relay field in 2012 from sowing for 15 days by using Pt electrodes.
and a silver/silver chloride reference electrodes attached to a millivolt meter. The reading was corrected following the procedure described by Patrick et al. (1996). Plant population at emergence and at harvest were recorded from 2 m² plot area. Root rot disease was measured using 0-5 scoring scale of Davis et al. (1995), where 0: no visible symptoms; 1: a few small discoloured lesions on the entire root system; 2: minor discolouration covering the root system; 3: brown discolouration on entire root system, no symptoms on epicotyl or hypocotyl; 4: brown discolouration on entire root system, shrivelled and brown epicotyl or hypocotyls and 5: plant dead. Percent disease severity was assessed from 2 m² plot area by the following formula, disease severity = Sum of all disease rating/(total no. of rating x maximum disease grade) x 100 (Sharma 2004).

In the glasshouse, the redox potential was measured daily from sowing to the end of the experiment. Seed emergence (%) was recorded by the number of emerged epicotyls expressed as a percentage of total number of seeds sown. Emergence was recorded daily with those seeds with an epicotyl longer than 5 mm considered as emerged.

3.3.4 Data analysis
Data were analyzed by using GenStat 16th edition for Windows statistical software (VSN International, UK). General analyses of variance (ANOVA) were undertaken to determine the effects of the different treatments and least significant differences (l.s.d) at $p > 0.05$ calculated for significant differences between treatments, genotypes and interaction means.
3.4 Results

3.4.1 Field experiment

3.4.1.1 Seasonal weather conditions

The mean seasonal maximum and minimum temperatures were similar in 2011, 2012 and 2013 during the pea growing period from November 1 to February 10 (Fig. 3.1). The highest monthly average temperature during the growing season was 27 °C in 2013 followed by 26 °C in 2012, while the average minimum temperature was almost 14 °C in all the seasons. The average temperature during the germination period (November 1- November 25) was 25 °C in 2011 and 2012 and 24 °C in 2013. Total rainfall during the pea growing season (November – March) was low at 27 mm in 2011 and 69 mm in 2013 compared to 118 mm received in 2012. There was no rainfall during the germination stage (November) in 2011 and 2013, but a total of 87 mm rainfall was recorded during germination in 2012. However, pea growth is primarily dependent on residual moisture from the preceding monsoon rainfall (April to October) – when the T. aman crop is grown and when 1496, 868 and 1089 mm rainfall was received in 2011, 2012 and 2013 respectively (Fig. 3.1). In 2012 in relay sowing, the moisture increased to soil saturation point after rain which started 4 days after sowing and reduced soil redox potential (Fig. S3.1).
Figure 3.1. Monthly rainfall in mm (bars), maximum (●) and minimum (○) temperature (°C) during the experimental period from 2011 to 2014 at PRC Ishwardi, Bangladesh.

3.4.1.2 Germplasm screening

In principal component analysis of 91 germplasm accessions (Table S3.1) the first two principal components (PC) accounted for 60% of the total variation among the genotypes with PC1 accounting for 39% and PC2 contributing 21% of the total variation, respectively. On both these principal axes, a number of characters contributed to the total variation. Mean seed yield was the largest contributor for the variation in PC1 (Table S3.2). The principal-component biplot of the first two components showed a wide range in variation among the 91 germplasm accessions grown under relay cropping in 2011 (Fig. 3.2). Among the biplot axis for each variable, the lines between emergence and seed yield had low angles and a similar direction indicating strong positive correlation. The lines in opposite direction indicate a negative correlation between average seed weight and seed yield. Two groups were established from PCA analysis comprising the top 10% and bottom 10% of accessions based on mean seed yield (Table 3.1). The average seed yield of the 10% best genotypes was 2.81 t ha⁻¹ in contrast to 0.51 t ha⁻¹ in the bottom group. Germplasm adapted to relay sowing (10% best) was significantly taller (19%) and had better stand at emergence, pods plant⁻¹ and seed yield, but had a
smaller seed mass (47%) on average than the ill-adapted germplasm (10% worst). The top and bottom yield groups did not differ significantly in phenology (time to flowering and maturity) and branching (No. branches plant\(^{-1}\)).

**Figure 3.2.** Principal component analysis of 91 pea genotypes. Each closed circle represent accession, line indicates biplot axis for variable. The angles between the biplot axes represent the correlations between the variables. The lines in same directions indicate positive correlation and in opposite direction indicate negative correlation. Ten genotypes - BD-4181, BD-4142, BD-4190, Natore local-1 (NL-1), Natore local-2 (NL-2), Bagha local (BL), Jhikorgacha local (JL), IPSA-3, BARI Motorshuti-1 (BM-1) and BARI Motorshuti-3 (BM-3) were selected to compare in relay vs. sole cropping. Among these, three contrasting genotypes - IPSA-3 sensitive to foot rot, BM-3 sensitive to waterlogging and NL-2 tolerant to waterlogging - are discussed in the text.
Table 3.1. Mean performance of yield and yield contributing traits of ten percent best performing and ten percent worst performing germplasm accessions of pea from among 91 accessions relay sown during 2011 cropping season

<table>
<thead>
<tr>
<th>Selection criteria</th>
<th>Time to flower (days)</th>
<th>Time to maturity (days)</th>
<th>Plant height (cm)</th>
<th>No. branches plant&lt;sup&gt;⁻¹&lt;/sup&gt;</th>
<th>No. pods plant&lt;sup&gt;⁻¹&lt;/sup&gt;</th>
<th>Emergence stand</th>
<th>100 seed weight (g)</th>
<th>Seed yield (t ha&lt;sup&gt;⁻¹&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yield - Top 10%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>55</td>
<td>97</td>
<td>114</td>
<td>1.7</td>
<td>13.9</td>
<td>7.0</td>
<td>10.8</td>
<td>2.81</td>
</tr>
<tr>
<td>Genotype range</td>
<td>51-60</td>
<td>87-112</td>
<td>90-139</td>
<td>1.0-2.8</td>
<td>6.2-23.4</td>
<td>5.0-9.0</td>
<td>5.9-16.5</td>
<td>2.38-2.94</td>
</tr>
<tr>
<td>SE</td>
<td>1.06</td>
<td>2.80</td>
<td>4.78</td>
<td>0.20</td>
<td>1.86</td>
<td>0.33</td>
<td>1.26</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Yield - Bottom 10%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>58</td>
<td>102</td>
<td>96</td>
<td>1.2</td>
<td>7.8</td>
<td>2.0</td>
<td>20.3</td>
<td>0.51</td>
</tr>
<tr>
<td>Genotype range</td>
<td>33-79</td>
<td>75-115</td>
<td>69-128</td>
<td>0.4-3.2</td>
<td>2.4-14.6</td>
<td>1.0-5.0</td>
<td>13.8-25.9</td>
<td>0.0-0.81</td>
</tr>
<tr>
<td>SE</td>
<td>6.02</td>
<td>5.93</td>
<td>6.09</td>
<td>0.27</td>
<td>1.64</td>
<td>0.47</td>
<td>1.36</td>
<td>0.07</td>
</tr>
<tr>
<td>Pr&gt; F</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NS indicates non-significant.
### 3.4.1.3 Relay vs. sole-cropping comparison

Comparing first the agronomically realistic treatments vis. relay sowing on November 1 vs. sole crop sowing on November 11, in 2012 there was a dramatic reduction in plant population at emergence under relay sowing (12 plants \( \text{m}^2 \)) compared to sole crop sowing on November 11 (37 plants \( \text{m}^2 \)) on average (Table 3.2). The plant population of sole crop sowing on November 1 were marginally lower (30 plants \( \text{m}^2 \)) than the later sole cropping showing that the above major reduction in plant stand was primarily a sowing method (relay cropping vs. sole cropping) effect rather than a sowing date effect. These effects were later observed to accentuate as harvest plant population levels with the plant population decreasing to 4.3 plants \( \text{m}^2 \) at harvest from 12 plants \( \text{m}^2 \) at emergence in relay cropping and 25 plants \( \text{m}^2 \) from 30 plants \( \text{m}^2 \) in sole sowing on November 1. In the following season (2013) the plant population levels at emergence and at harvest of the sowing treatments were similar (Table 3.2), showing that relay cropping is much more sensitive to waterlogging than sole cropping.

**Table 3.2.** Plant population at emergence and at harvest under relay sowing (sown on Nov 1) and sole sowing (sown on Nov 1 and Nov 11) during 2012 and 2013 cropping seasons

<table>
<thead>
<tr>
<th>Plant population (plants ( \text{m}^2 ))</th>
<th>Season</th>
<th>Sowing method/time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relay – Nov 1</td>
</tr>
<tr>
<td>At emergence</td>
<td>2012</td>
<td>12 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>34 ± 1.6</td>
</tr>
<tr>
<td>At harvest</td>
<td>2012</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>33 ± 1.6</td>
</tr>
</tbody>
</table>

Values are means ± S. E. of three replicates.
Interactions of genotype, year and sowing method were significant for both emergence and harvest population (Table 3.3). In 2012, both the emergence and harvest population were similar for all treatments for most genotypes with few exceptions, BM-3, NL-2 and IPSA-3, (Fig. 3.3). In relay sowing in 2012, BM-3 had only 6 plants m\(^{-2}\) at emergence which died before harvest. Two other genotypic patterns were noted in 2012: IPSA-3 had 12 plants m\(^{-2}\) at emergence and this was reduced to 6 at harvest; whereas in NL-2 there was almost same number of plants (13 plants m\(^{-2}\)) at emergence as at harvest. In the same season (2012) in sole sowing on November 1, both the emergence and harvest population was similar in BM-3 and NL-2, while there was a significant difference in IPSA-3. Sole cropping on November 11 in 2012 showed similar emergence and harvest populations for all the three genotypes. The following season (2013) there was no difference between emergence and harvest population for any sowing treatment (Fig. 3.3). Variation was also found in regards to disease reaction. The disease severity was mainly observed in relay and sole sowing in November 1, 2012. In relay sowing the highest percent of root rot severity (43%) was found in IPSA-3, while there was no root rot recorded in BM-3 and NL-2 (Fig. 3.4). In sole cropping on November 1, IPSA-3 exhibited the highest percent of severity of root rot followed by NL-2, but there was no root rot in BM-3. The sole sowing in November 11 in 2012 and all the sowing in 2013 were almost free of root rot (Fig. 3.4).

In 2012 there was a strong correlation between plant density and seed yield in relay sowing with poor yields probably attributable to the very low plant population levels (Fig. 3.5). By contrast, in the following season 2013, the correlation between plant density and seed yield in relay sowing was non-significant and plant population in relay sowing was not limiting to yield.
Table 3.3. Significance (Pr> F) and degrees of freedom (DF) of the combined ANOVA for the effects of experimental year (Y), sowing method (SM), genotype (G) and their interactions for plant population (PP) at emergence and at harvest, and root rot in 2012 and 2013 growing seasons

<table>
<thead>
<tr>
<th>Factor</th>
<th>Y</th>
<th>SM</th>
<th>G</th>
<th>Y x SM</th>
<th>Y x G</th>
<th>SM x G</th>
<th>Y x SM x G</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF</td>
<td>1</td>
<td>2</td>
<td>9</td>
<td>2</td>
<td>9</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>PP at emergence</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PP at harvest</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Root rot</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NS indicates non-significant.

Figure 3.3. Plant population of representative pea genotypes IPSA-3, BM-3 and NL-2 at emergence and at harvest in relay sowing on Nov 1 (A) and the sole sowing on Nov 1 (B) and Nov 11 (C) in 2012 and 2013 cropping seasons. Error bars represent the standard error of the mean (n=3). The three genotypes showed contrasting response to soil waterlogging in regards to emergence in relay in 2012. Among them, NL is a popular local cultivar that was tolerant to waterlogging and BM-3 is national short duration variety which was highly sensitive to waterlogging. The variety IPSA-3 was highly sensitive to root rot disease.
Figure 3.4. Percent mean severities of root rot disease for three genotypes with different sowing methods in 2012 (A) and 2013 (B) cropping season. T1, T2 and T3 indicates relay sowing Nov 1, sole sowing Nov 1 and sole sowing Nov 11 respectively. Error bars represent the standard error of the mean (n=3).

Figure 3.5. Relationship between seedling plant density (plant m$^{-2}$) and seed yield (t ha$^{-1}$) in relay sowing in 2012 (A) and 2013 (B) cropping seasons.
3.4.2 Glasshouse experiment

At the start of the experiment, the mean of soil redox potential in drained control pots was 474 ± 23 mV, which increased to 569 ± 8 mV by the end of experiment. By contrast, the redox potential in waterlogged pots was 320 ± 7 mV throughout the waterlogged period; and after draining pots the mean redox potential increased to 560 ± 11 mV by Day 23.

An analysis of variance indicated that the interaction between waterlogging (WL) duration and genotype (G) was significant (P<0.01) for emergence, though the contribution of WL duration was higher than genotype. Genotype contributed more than the interaction effects for percent emergence (Table 3.4).

Table 3.4. Variance components, degrees of freedom (DF), coefficient of variance (CV), sum of squares (SS) and residual, for emergence at different duration of WL stress at germination for pea genotype (G)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Emergence (%)</th>
<th>Total SS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>2</td>
<td>1323**</td>
<td>8.1</td>
</tr>
<tr>
<td>WL</td>
<td>2</td>
<td>13266**</td>
<td>81.6</td>
</tr>
<tr>
<td>G x WL</td>
<td>4</td>
<td>400**</td>
<td>4.9</td>
</tr>
<tr>
<td>Residual</td>
<td>24</td>
<td>72</td>
<td>5.3</td>
</tr>
<tr>
<td>CV (%)</td>
<td></td>
<td>3.6</td>
<td></td>
</tr>
</tbody>
</table>

** Indicates significance at P<0.01.

Emergence was reduced significantly by the waterlogging treatment (Fig. 3.6). All three genotypes showed close to 100% emergence when grown in drained soil. Four days of waterlogging reduced emergence to 75% for BM-3, to 89% for NL-2 and to 94% for Kaspa at the end of experimental period. With the increase of waterlogging duration to 8 days, emergence percentage was further reduced for BM-3 to 14%, for NL-2 to 40% and for Kaspa to 55%. Percent cumulative emergence increased consistently for all three genotypes with 4 days waterlogging (Fig. 3.6). However genotypic response was different with the prolongation of waterlogging duration to 8 days. In BM-3, maximum
cumulative emergence (14%) was found during the waterlogging period and there was no subsequent emergence during the recovery period. NL-2 showed most emergence (30%) during waterlogging and only 10% in recovery period. In Kaspa, by contrast, only 15% emergence was recorded during waterlogging while most emergence (40%) was seen in the recovery period (Fig. 3.6). Genotypic variability for waterlogging tolerance was greatest with the treatment of 8 days of waterlogging.

Figure 3.6. Percent mean cumulative emergence of BM-3 (A), NL-2 (B) and Kaspa (C) in drained control (○), 4 days (●) and 8 days (■) waterlogging. Arrows indicates start of draining. Emergence started from 3 days after sowing and was completed by 23 days after sowing.
3.5 Discussion

Conspicuous genetic variation in waterlogging tolerance at germination in the model plant pea was observed for the first time in both the field and controlled conditions. Through diverse germplasm screening as relay cropping in 2011, a few genotypes including BM-3 had very low plant population at emergence that all died by harvest. Clearly plants encountered stress at germination - most probably due to waterlogging as a result of the high monsoon rainfall (1496 mm, almost 2-fold than other season) received in rice in that year as well as slow drainage of the puddled rice soil. In the following year (2012), relay cropping was clarified as considerably more sensitive to waterlogging as sole cropping as evidenced by the comparison of T1 vs. T2 in 2012 and by reduced soil redox potential from heavy rainfall. In 2013, there was no difference in plant population between relay and sole sowing. This indicates that the population variation in relay in 2012 was caused by waterlogging from the intense rain. In this case soil structure in relay was the key driver for waterlogging because of the formation of a plough-pan on the upper layer of soil from puddling of the rice crop (Cass et al. 1994). This compact structure of soil reduces drainage and causes waterlogging from rain at the time of sowing of the dryland crop pea (Samad et al. 2001). By contrast in traditional sole cropping the plough-pan is broken by soil cultivation that increases soil drainage and reduces the risk of waterlogging damage. Additionally, plant density was strongly correlated with seed yield in relay 2012. In the following season 2013, the correlation was weak in relay sowing indicating that plant population in relay cropping was no longer a limiting factor to yield. This means that the poor yield in 2012 was due to poor population in the relay crop caused by soil waterlogging. This emphasizes the need for WL tolerance in relay to reduce the risk of waterlogging.

Obvious variation in waterlogging tolerance was observed among genotypes (i.e. BM-3 vs. NL-2) due to soil waterlogging. Further, genotype IPSA-3 was found specifically susceptible to root rot which is a serious disease of pea throughout the world (Kraft et al. 1998). The disease is aggravated by WL due to breakdown of host resistance mechanisms during hypoxia (Moslemi et al. 2018). It
affects plant stand and causes considerable crop losses in India (Sen & Mazumdar 1974). By contrast, there was no difference in plant population at emergence and at harvest in genotype NL-2 indicating its superior tolerance to waterlogging. Genotypic variability in waterlogging tolerance at germination has also been found in such crops as rice (Ismail et al. 2009), maize (Zaidi et al. 2012), lentil (Wiraguna et al. 2017), pigeonpea (*Cajanus cajan* (L.) Millsp.) (Sultana et al. 2013) and soybean (Hou et al. 1995).

Germination ability in waterlogged condition is considered a component of waterlogging tolerance (Sayama et al. 2009). Seeds with a prolonged soaking (i.e. waterlogging) time had reduced germination percentage in grasspea (Sharma & Pandey 2001), maize and fababean (Crawford 1977). However, the ability to tolerate prolonged waterlogging stress varies, for example, greatest variation was observed for maize following 4 days of waterlogging in a pot soil in open field experiment (Zaidi et al. 2012), for lentil it was 6 days in a glasshouse trial (Wiraguna et al. 2017), and for pigeonpea 8 days in a field experiment (Sultana et al. 2013). In the current glasshouse experiment the emergence rate in sensitive genotypes started deterioration within 4 days of waterlogging, while after 8 days there was no emergence. The greatest differentiation between tolerant and sensitive genotypes was observed following 8 days of waterlogging. Extension of the hypoxia period can lead to germination failure and seed death (Crawford 1977). Both in field and glasshouse the extended period of waterlogging reduced germinability but to a much lower extent in the tolerant genotypes. Probably, difference in enzymatic action and seed reserve (Carbohydrate) metabolism in hypoxia is the key factor determining the germination of the studied genotypes. Perata et al. (1997) noted that among cereals only the tolerant rice seed was able to germinate under anoxia due to combined action of amylolytic enzymes involved in starchy endosperm metabolism. By contrast dryland crops like wheat and barley failed to germinate due to absence or non-functioning of amylolytic enzymes. Alternatively, rapid absorption of excessive water by seeds resulting in leakage of seed solutes reduced seed germination (Powell & Matthews 1978). In the glasshouse experiment, waterlogging (8
days) caused seed deterioration and subsequent germination failure in BM-3 in which the leakage of seed solutes (visual observation) was seen after 3 days of waterlogging.

Pea was domesticated in a rainfed Mediterranean ecosystem (Zohary & Hopf 1973). Despite the striking contrast between the environment of origin with that presented by relay-sowing into moist soils with rice, the presence of genotypic variation in pea for waterlogging tolerance at germination raises the prospect of selection for this trait. Climate change predictions for South Asia suggest alterations in the intensity of rainfall events, an increase in inter-annual precipitation variability (Sivakumar et al. 2010) and delayed monsoon rains (Li et al. 2017) which could significantly change crop productivity. We anticipate that selection for waterlogging tolerance at an early stage of crop growth could significantly improve adaptation to relay sowing and stabilize crop yield as part of a strategy to enhance productivity in South Asian countries. Additionally, the contrasting genotypes identified in this research might help to identify gene(s) responsible for waterlogging tolerance as well as the mechanisms involved to facilitate future breeding programs. The tolerant cultivar NL-2 and Kaspa can also be used as a tolerant varieties in waterlogging prone areas to reduce crop damage and for relay sowing.

In conclusion, relay cropping of pea is more prone to soil waterlogging at germination than traditional sole cropping. Waterlogging reduced plant population and subsequent productivity of dryland peas. However, genotypic variation in response to waterlogging has been identified in the field and also under controlled condition. The selection of tolerant genotypes at germination can improve adaptation to relay sowing in South Asia.

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3.6 References


### 3.7 Supporting information

Additional supporting information may be found online in the supporting information section at the end of the article- https://doi.org/10.1111/jac.12230
Chapter 4

Changes in gene expression during germination reveal pea genotypes with either 'quiescence' or 'escape' mechanisms of waterlogging tolerance

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This thesis chapter- Changes in gene expression during germination reveal pea genotypes with either “quiescence” or “escape” mechanisms of waterlogging tolerance was designed by M. S. U. Zaman in consultation with A. I. Malik, W. Erskine and P. Kaur. For the phenotyping experiment, seed sowing soil redox measurement and data recording were conducted by M. S. U. Zaman; and for the transcriptomic experiment, seed sowing and sample collection were conducted by M. S. U. Zaman under the supervision of P. Kaur. Kaur contributed during RNA extractions and library preparation. Data from the phenotypic experiment were analysed by M. S. U. Zaman. From the transcriptomic data, the primary analysis i.e. quality control of sequencing reads, alignment and differential expression was conducted by P. Kaur; and the downstream analysis - GO term enrichment analysis and pathway analysis were accomplished by M. S. U. Zaman. The manuscript was written by M. S. U. Zaman and the co-authors W. Erskine, A. I. Malik and P. Kaur were involved in the discussion of results, structure of the manuscript and editorial comments.
Chapter 4

Changes in gene expression during germination reveal pea genotypes with either 'quiescence' or 'escape' mechanisms of waterlogging tolerance

4.1 Abstract

Waterlogging causes germination failure in pea (*Pisum sativum* L.). Three genotypes (BM-3, NL-2 and Kaspa) contrasting in ability to germinate in waterlogged soil were exposed to different durations of waterlogging. Whole genome RNAseq was employed to capture differentially expressing genes. The ability to germinate in waterlogged soil was associated with testa colour and testa membrane integrity as confirmed by electrical conductivity measurements. Genotypes Kaspa and NL-2 displayed different mechanisms of tolerance. In Kaspa, an energy conserving strategy was indicated by a strong upregulation of tyrosine protein kinsase and down regulation of LOX5, a fat metabolism gene. In contrast, a faster energy utilisation strategy was suggested in NL-2 by the marked upregulation of a subtilase family protein and PNC2, a fat metabolising gene. Waterlogging susceptibility in germinating seeds of genotype BM-3 was linked to upregulation of a kunitz-type trypsin/protease inhibitor that blocks protein metabolism and may lead to excessive lipid metabolism and the membrane leakage associated with waterlogging damage. Pathway analyses based on gene ontologies showed seed storage protein metabolism as upregulated in tolerant genotypes and downregulated in the sensitive genotype. Understanding the tolerance mechanism provides a platform to breed for adaptation to waterlogging stress at germination in pea.
4.2 Introduction

Soil waterlogging is a common abiotic stress that impacts on crop production (Jackson & Colmer 2005). Pulse crops are generally susceptible to waterlogging which may occur at different stages of growth and development. In pea (*Pisum sativum* L.), the severity of the effect of waterlogging depends on the growth stage of the plant (Cannell et al. 1979; Belford et al. 1980). Other pulse crops such as chickpea (*Cicer arietinum* L.) (Cowie et al. 1996; Palta et al. 2010), mungbean (*Vigna radiate* L.) (Islam et al. 2008) and fababean (*Vicia faba* L.) (Munir et al. 2018) have also demonstrated diverse responses to waterlogging at different stages of development. Waterlogging is a common problem at the germination stage as pea is often sown as relay with monsoonal rice in Bangladesh (Ali & Sarker 2013), India and Nepal. In relay cropping, pea seeds are hand-broadcasted on waterlogged soil into the standing rice 2-3 weeks prior to rice harvest. This causes a reduction of gas exchange between seeds and the aerial environment, particularly oxygen that diffuses very slowly in water, ~10,000 times slower than in air (Armstrong 1979). In addition to O$_2$ deficiency, harmful levels of ethanol and acetaldehyde are accumulated in the seed as end-products of anaerobic metabolism (Woodstock & Taylorson 1981). As a result, a short period of waterlogging can delay germination and/or cause germination failure (Crawford 1977) and lead to reduced pea plant population and ultimately yield (Zaman et al. 2018).

The mechanism of waterlogging tolerance at germination is equivocal when root and photosynthetic systems are absent and seed storage provides the energy necessary for germination. Several reports have shown that the testa (seed coat) colour and testa integrity (i.e. by stopping leakage of seed) are important characteristics in imbibition damage. In soybean (*Glycine max* (L.) Merill), varieties with a black testa germinated well, whereas yellow seeds failed to germinate after 8 d of soaking (Hou & Thseng 1991). Likewise in wheat (*Triticum aestivum* L.), red coloured testa exhibited higher tolerance to waterlogging than white (Ueno & Takahashi 1997); however, a few red testa genotypes were
sensitive and a few white seeded were tolerant. In rapeseed (Brassica napus L.), red or black-seeded genotypes - which had higher testa melanin content - were tolerant, showed slower water uptake and lower leakage compared to waterlogging sensitive yellow-seeded rapeseed accessions (Zhang et al. 2008). The leakage of seeds by rapid absorption of excessive water leads to reduced seed germination in peas (Powell & Matthews 1978). The germination percentage in soybean was also negatively correlated with the amount of sugar exudate from seeds (Zheng & Watabe 2000). Another response to low oxygen was found in the metabolic level where the amount of ethanol excreted from seeds during soaking significantly correlated with the subsequent germination of rice (Oryza sativa L.), lettuce (Lactuca sativa L.), maize (Zea mays L.), fababean and pea seeds (Crawford 1977). Proteomic analysis showed that enzymatic activity was responsible for germination in tolerant rice and the absence of an amylolytic enzyme for carbohydrate metabolism led to germination failure in the dryland crops - wheat and barley (Hordeum vulgare L.) (Perata et al. 1997).

To-date studies on the molecular mechanisms for waterlogging tolerance are limited, but progress has been made in various crops through the discovery of a molecular oxygen sensing mechanism (Licausi et al. 2011) in Arabidopsis (Arabidopsis thaliana L.) and the identification of genes involved in morphological and metabolic adaptations under hypoxic conditions. Hypoxia-induced genes in the seedling stage have been identified in Arabidopsis (Liu et al. 2005), maize (Zou et al. 2010), rice (Lasanthi-Kudahettige et al. 2007), soybean (Komatsu et al. 2009) and other species (Qi et al. 2012). Most of these genes are involved in sugar metabolism, glycolysis and fermentation pathways (Sachs et al. 1996). Subsequent studies have identified genes involved as signal transduction components (Baxter-Burrell et al. 2002) and transcription factors (TFs) (Vetten & Ferl 1995) as playing an important role in waterlogging tolerance. Two TFs, SNORKEL (Hattori et al. 2009) and Submergence-1A (Xu et al. 2006) driven by ethylene have been reported in rice which are involved in internode elongation and metabolic regulation respectively to adapt rice to different types of
flooding. In short, the waterlogging tolerance of germinating seeds is presumably related to several mechanisms that remain unexplained.

We used pea as a model for waterlogging tolerance at germination, as phenotypic contrasts and comprehensive genomic information (Franssen et al. 2011; Kaur et al. 2012; Chen et al. 2013; Duarte et al. 2014; Kerr et al. 2017; Zhernakov et al. 2017) are available. The objective of this study was to understand how the stress causes significant changes in gene expression in tolerant versus sensitive genotypes using high-throughput RNA-sequencing to explore the mechanisms that play a significant role in waterlogging tolerance at germination.

4.3 Materials and methods

The study had two components: Phenotyping and transcriptomics.

4.3.1 Phenotyping

4.3.1.1 Study the germination in waterlogged soil

Three genotypes of pea BARI Motorshuti-3 (BM-3), Natore local-2 (NL-2) and Kaspa with contrasting testa colours were used for this experiment with BM-3 as greenish white, NL-2 as yellow and Kaspa - reddish brown (Wilson 1942). BM-3 is sensitive to waterlogging, NL-2 is moderately tolerant while Kaspa is tolerant during germination (Zaman et al. 2018). The experiment comprised three treatments (drained control, 4 and 8 d of waterlogging) and the design was factorial with genotypes (3 levels) × waterlogging treatment (3) in a completely randomized design with four replications. The experimental unit was plastic pot - free draining with a sealed base. Free draining pots contained gravel at the bottom and 3.5 kg mixed sand and soil collected from Mukinbudin (30°78’ S, 118°31’ E), Western Australia (Kotula et al. 2015) (1: 1) at the top. The soil and sand were dried for 3 d at 65 °C, passed through a 0.5 mm diameter sieve and mixed thoroughly before pot filling. 
The soil mixture had pH 6.7 and EC 0.46 dS m\(^{-1}\) at 1:5 w/v soil/water. Each free-draining pot (19 cm height × 21 cm diameter) was placed in a sealed base pot (24 cm height × 26 cm diameter). A total of ten electrodes were inserted into the soil at a depth of 100 mm for redox measurement. The redox potential was measured daily from sowing to the end of the trial. For the waterlogged treatment, DI water was added to sealed base pots to maintain a water table at 10 mm below the soil surface of the free-draining pot. Pots were waterlogged for 4 d prior to sowing to ensure hypoxia at sowing. Water was added to sealed base pots daily as required to maintain the water table. For drained control treatments there was no water in the sealed base pots, but the soil moisture in free draining pots was maintained at ~80% of field capacity. Seeds were treated with Thiram (Tetramethylthiuram disulfide) at the rate of 3 g kg\(^{-1}\) seeds before sowing to eliminate fungal infection. Twenty seeds of each genotype were sown in a free-draining pot through dibbling to 5 mm soil depth. All pots were covered for 3 d after sowing to ensure darkness for germination. Waterlogged pots were drained as per treatment. Within replicates pots were moved every 5 d to minimize the effects of varying conditions in the glasshouse in different positions. Seed germination was recorded daily both during waterlogging and in the recovery period and expressed as a percentage of the total number of seeds sown. Seeds with an epicotyl longer than 5 mm were considered as germinated (i.e. emerged). The experiment was conducted in the glasshouse at UWA at 25 °C temperature, and was terminated 23 d after sowing, when there was no sign of further emergence. Data were analysed using GenStat 16th edition for Windows statistical software (VSN International, UK).

4.3.1.2 Determination of electrical conductivity of seed leakage

We confirmed visual observation of seed leakage in a separate experiment. The experiment comprised five treatments (12, 24, 48, 96 and 192 h waterlogging) with three replications in a completely randomized design. Seeds were sterilized with 1% commercial bleach (available active ingredients 4% m/v) for 1 minute, then washed 3-5 times with deionized water. Twenty seeds of each genotype
(Kaspa, NL-2 and BM-3) were submerged in a glass bottle containing 75 ml of 0.5 mM CaSO$_4$ solution and incubated in a germination cabinet at 25 °C temperature. Electrical conductivity of the same solution was measured at each time point of seed submergence using an AQUA- PH v1.0 conductivity meter (TPS, Brisbane Australia).

4.3.2 Transcriptomics

4.3.2.1 Experimental Design

This experiment comprised two factors in a completely randomized factorial design with two replicates as follows: 1. Waterlogging factor with two levels (waterlogged and drained control) and 2. Genotypes (3 as above) with four time points (12, 24, 48 and 96 h waterlogging). The initial time point 12 h was considered based on farmer’s practice where they soak seeds for 10-12 h before sowing as relay with rice, followed by 24 h when pea seeds with excess water start to exhibit germination failure (Matthews & Whitbread 1968). The last treatment 96 h was as in the phenotyping experiment for comparison. The genotypes, experimental unit and sowing methods were as in the phenotyping experiment. A total of 48 samples were collected for RNA extraction. Each sample was collected by forceps from the soil and rinsed rapidly with Milli Q water. Seeds of the controls were also harvested at the corresponding time point. All samples were collected into 2 ml tubes and immediately placed in liquid nitrogen and stored at −80 °C prior to RNA extraction.

4.3.2.2 RNA extraction and library preparation

Total RNA from all the tissue samples was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) following manufacturer instructions. A slight modification of the procedure was undertaken in order to extract RNA from mature seeds, which involved the addition of polyvinylpyrrolidone (PVP-40) (Sigma-Aldrich) to 450 μl of lysis solution containing 10 μl ml$^{-1}$ β-mercaptoethanol (Sigma-Aldrich) to a final proportion of 2% (w/v) with an off-column DNAase
treatment. Aliquots of purified RNA were stored at −80 °C. The concentration of RNA was confirmed using a Qubit fluorometer with the Qubit RNA assay kit (Life Technologies, Carlsbad USA). The integrity of total RNA was determined by electrophoretic separation on 1.2% (w/v) denaturing agarose gels. Sequencing libraries were constructed using 500 ng of total RNA with a TruSeq® Stranded Total RNA Sample Prep Kit with Ribo-Zero (Illumina Inc., San Diego, USA) following the manufacturer’s instructions. The amplified libraries were pooled in equimolar amounts and quality was assessed with Agilent high-sensitivity DNA chips (Agilent Technologies, Santa Clara, USA). All reads were 100 bp pair-end sequenced using the HiSeq 2000 platform (Illumina Inc., San Diego, USA). The sequencing data has been made available (under embargoed) at https://zenodo.org, doi: 10.5281/zenodo.1209073.

4.3.2.3 Differential gene expression analysis

Illumina reads were quality filtered using in-house scripts (part of the open-source toolkit Friedrich, https://bitbucket.org/jtnystrom/friedrich). Filtered reads were then mapped to the published pea reference transcriptome (Sudheesh et al. 2015) using Tophat (v2.1.0) with default parameters and passing the reference annotation with the -G option. Paired and single-end reads were mapped separately and then outputs merged into a single BAM file for each sample, as advised on the Tophat website. Read counts for each gene were called using HTSeq (v0.6.0). The matrix of read counts for each gene (rows) and each sample (columns) was analysed in R (v3.1.1) using edgeR (v3.8.6). A generalised linear model (GLM) was fitted to the data with time as the sole variable; common, trended and tagwise dispersions were estimated following the procedure detailed in the edgeR manual. Differentially expressed genes (DEGs) with false discovery rate (FDR) less than 5% were output for each time point.
4.3.2.4 Functional annotation and classification of the DEGs

The DEGs were aligned to several databases using blastp v 2.2.31+ (Altschul et al. 1990) (minimum e-value 1e⁻¹⁰). The databases used were Swiss-Prot and TrEMBL downloaded on 15 October 2016 (Boeckmann et al. 2003) and all DEGs downloaded from Phytozome v11 on 20 October 2016 (Goodstein et al. 2012). For each DEGs, the hit with the highest score and lowest e-value was chosen as annotation. GO-terms were assigned to proteins by manually transferring the GO-terms for Swiss-Prot IDs using the UniProt-GOA database downloaded on 10 November 2016 (Huntley et al. 2014). KEGG K numbers were assigned to all predicted proteins using BLASTKOALA (taxonomy group: Plants, KEGG GENES database: family_eukaroytes) (Kanehisa et al. 2016). Top forty-three DEGs (logFC ≥4) were verified with another pea RNA-Seq gene atlas (Alves-Carvalho et al. 2015). Few non-matched genes were searched in Phyre2 web portal (Kelley et al. 2015) to find out predicted protein structure.

4.3.2.5 Functional characterisation and GO enrichment analyses

DEGs were grouped into the three main GO categories: biological processes, molecular function and cellular components. GO enrichment analysis further characterised the DEGs, and enriched functional groups were identified with agriGO, an integrated web-based GO analysis toolkit (Du et al. 2010). REVIGO web server (Supek et al. 2011) was used using the default settings in the singular enrichment analysis tool and a reference background of Arabidopsis gene model (TAIR9) and the results were visualized graphically. REVIGO analyses were conducted to further reduce the number of enriched GO terms by semantic similarity, with an allowed similarity of 0.7 (medium) and using the SimRel semantic similarity measure.
4.4 Results

4.4.1 Testa colour and physiological process for waterlogging tolerance

In this study we used genotypes differing in testa colour and four soil waterlogging treatments. At the start of the experiment, the mean of soil redox potential in drained control pots was $474 \pm 23$ mV, which increased to $569 \pm 8$ mV by the end of experiment. By contrast, the initial redox in waterlogged pots was $320 \pm 7$ mV which was almost similar during the waterlogging period, and it increased to $560 \pm 11$ mV after draining by 23 d. An analysis of variance indicated that the interaction between waterlogging (WL) duration and genotype (G) was significant ($P<0.01$) for germination (Table S4.1) and testa colour was associated with waterlogging tolerance (Fig. 4.1-A & B). There was close to 100% germination in all genotypes when grown in the drained control soil. Germination was reduced by waterlogging in all genotypes; however, the reduction in germination was greater in greenish white testa seeds (BM-3) than in seeds with yellow (NL-2) and reddish brown (Kaspa) testa colour (Fig. 4.1B). After 4 d of waterlogging, germination was reduced to 94% in reddish brown testa genotype - Kaspa, to 89% in yellow testa NL-2 and to 75% for greenish white testa, sensitive BM-3 by the end of experimental period. In this 4 d-waterlogging treatment most of the germination occurred during the period of recovery. With an increase in waterlogging duration to 8 d, germination percentage was reduced to 55% in Kaspa, 40% in NL-2 and only 14% in sensitive BM-3. In Kaspa, only 15% germination was recorded during the period of waterlogging and most germination (40%) was observed during the recovery period (after draining). In contrast, for BM-3 all germination (14%) was during the waterlogging period with no subsequent emergence during recovery (Fig. 4.1B). At recovery following 8 d of waterlogging, whitish seed solute from seed leakage was observed on the seed surface of the sensitive genotype BM-3 (Fig. 4.1A). Seed solute was not visible on the testa of the tolerant NL-2 and Kaspa genotypes.
The electrical conductivity (EC) from seed leakage was significantly higher in the sensitive genotype BM-3 than in tolerant NL-2 and Kaspa across all time points (12, 24, 48, 96 and 192 h waterlogging) (Fig. 4.1C). After 12 h waterlogging, the waterlogging sensitive genotype BM-3 already had the highest EC (282 μS cm\(^{-1}\) g\(^{-1}\) seed) compared with tolerant NL-2 (98 μS cm\(^{-1}\) g\(^{-1}\) seed) and Kaspa (65 μS cm\(^{-1}\) g\(^{-1}\) seed). After 192 h of waterlogging, the EC of sensitive BM-3 had increased to 1711 compared to 539 and 179 μS cm\(^{-1}\) g\(^{-1}\) seed in tolerant NL-2 and Kaspa respectively. Between tolerant genotypes Kaspa and NL-2, the EC was only significantly higher in NL-2 at the longest duration of waterlogging stress (192 h).
**Figure 4.1.** Seed morphology, physiological process and transcriptional changes in response to waterlogging (WL) stress in pea. (A) Different testa colours and testa membrane integrity after 8 d waterlogging. No visible seed solutes on seed surface from tolerant yellow testa NL-2 and reddish brown Kaspa, but visible seed solutes indicated by yellow arrow was found on waterlogging sensitive greenish white testa genotype BM-3. (B) Percent mean total germination of BM-3, NL-2 and Kaspa in drained control, 4 and 8 d waterlogging. Emergence started from 3 d after sowing and was completed by 23 d after sowing. Germination was recorded for each seeds daily in both waterlogged and recovery period. Error bars represent the standard error of the mean (n=4). (C) Electrical conductivity of seed leakage at different time points (12, 24, 48, 96 and 192 h) of waterlogging stress. Seeds of each genotypes (Kaspa, NL-2 and BM-3) were sterilized with 1% commercial bleach, washed with deionized water then submersed in a glass bottle containing 75 ml of 0.5 mM CaSO4 solution and incubated in a germination cabinet at 25 °C temperature. EC of the same solution was measured after each time point. Error bars represent the standard error of the mean (n=3). (D) Represents number of genes differentially expressed (logFC>2 and FDR < 0.05) both up (bars above x-axis) and down (bars below x-axis) regulated across the time points (12, 24, 48 and 96 h) in different genotypes. Blue arrow indicates the transcriptomic treatments which are the representation of 4 d WL phenotypic experiment.

### 4.4.2 Comparative analysis of DEGs between waterlogging tolerant and sensitive genotypes

To explore the molecular mechanisms for waterlogging tolerance, RNA sequencing was conducted on seed samples collected from waterlogged soil. Waterlogging induced differentially expressed genes (DEG) with a log2 fold-change (logFC) of ≥2 were studied for each genotype across the time
points (12, 24, 48 and 96 h). In tolerant genotypes (Kaspa and NL-2) the highest numbers of genes (11,923 and 11,719 respectively) showing upregulation were observed at longer waterlogging durations (96 h) (Fig. 4.1D). In contrast, in the sensitive BM-3 the highest number of genes (14,579) showing upregulated expressions were after the shortest period of waterlogging stress (12 h) and this decreased to 9,343 at 96 h waterlogging duration (Fig. 4.1D). The highest number of downregulated genes was found at 96 h in sensitive BM-3 (23,222) and in tolerant NL-2 (25,012), while the maximum number (25,171) were at 48 h in Kaspa.

For functional predictions, DEGs for each genotype were analysed to identify genes whose differential regulation was common among the time points with logFC ≥2 and FDR < 0.05 to understand the mechanisms in the tolerant and susceptible genotypes (Fig. 4.2). In tolerant Kaspa and NL-2 approximately equal number of genes; 248 and 241, respectively were consistently differentially expressed across all time points (Fig. 4.2 and Tables S4.2 & S4.3). In contrast, in the sensitive genotype BM-3 a higher number of genes (1,421) were expressed across all time points (Fig. 4.2 and Table S4.4).

To understand the mechanisms of tolerance we analysed the most significant DEGs (logFC ≥4 and FDR < 0.05) across all time points for both tolerant and sensitive genotypes (Table S4.5). Among the genes with upregulated expression in Kaspa the highly expressed DEGs the tyrosine protein kinase family protein whose expression (logFC > 7.99) increased gradually with the waterlogging duration, and the other outstanding DEGs; hypoxia-responsive family protein, wound-responsive family protein, structural protein, leucine-rich repeat transmembrane protein and Serine/threonine/tyrosine-protein kinase showed greatest expression of upregulation at 48 and 96 h (Table S4.5).
In NL-2 among highly expressed DEGs, PNC2 (peroxisomal adenine nucleotide carrier 2) showed greatest upregulated expression (logFC > 10.72) based on the difference of logFC between 12 h and 96 h waterlogging stress. The other most upregulated DEGs in NL-2 whose expression showed the greatest upregulation at 48 and 96 h, were EDA35 (embryo sac development arrest 35), amino acid dehydrogenase-like N-terminal domain, subtilase family protein, LNS2 and ethylene-responsive family protein. The two other most upregulated genes were uncharacterized (Table S4.5). Regarding downregulation in tolerant genotypes, highly differentially expressed genes increased down regulation with waterlogging duration: For example, LOX5 (Linoleate 9S-lipoxygenase 5) (logFC > -12.72), phospholipase D delta (logFC > -10.20) and pfkb-type carbohydrate kinase protein family (logFC > -5.63) in Kaspa, and emsy n terminus (ent) domain-containing protein (logFC > -9.5) and tesmin TSO1-like CXC domain-containing protein (logFC > -6.23) in NL-2 (Table S4.5).

In the sensitive BM-3 the most highly differentially expressed upregulated functional genes were kunitz type trypsin and protease inhibitor (logFC > 11.36) and protein phosphatase (logFC > 11.09) whose expression gradually increased with waterlogging duration (Table S4.5). Turning to the important downregulated genes, the expression of copper amine oxidase, UDP-glucoronosyludpglucosyl transferase family protein and carboxyl esterase lipase were gradually increased their downregulation with the length of the waterlogging treatment (Table S4.5).
Figure 4.2. Venn diagram showing the numbers of common and specific differentially expressed genes (log (fold change) (logFC) > 2; false discovery rate (FDR) < 0.05) at 12, 24, 48 and 96 h waterlogging treatment in pot soil with three different pea genotypes. Kaspa and NL-2 are waterlogging tolerant while BM-3 is sensitive.

4.4.3 GO term enrichment analysis

4.4.3.1 GO enrichment analysis from consistently DEGs

GO enrichment analysis was performed for DEGs with known function and consistent expression (logFC ≥ 2 and FDR < 0.05) across the time points (12, 24, 48 and 96 h) (Fig. 4.2). Functionally unknown DEGs were annotated as NA that may allow for the identification of novel genes in the response to waterlogging stress. A total of 248 and 241 genes from tolerant Kaspa and NL-2 were analysed, respectively. Upregulated genes were enriched with 160 and 43 GO terms in biological processes category for Kaspa (Table S4.6) and NL-2 (Table S4.7) respectively, while downregulated genes of both Kaspa (Table S4.8) and NL-2 (Table S4.9) contributed 170 enriched GO terms. In sensitive BM-3, 161 GO terms came from the enrichment of upregulated genes (Table S4.10), while downregulated genes gave 167 GO terms (Table S4.11) through enrichment.
The enriched GO terms with up and down regulated genes were categorized with representative terms by Revigo TreeMap (Supek et al. 2011) for both tolerant Kaspa (Tables S4.12 & S4.13) and NL-2 (Tables S4.14 & S4.15) as well as sensitive BM-3 (Tables S4.16 & S4.17) and plotted in Fig. 4.3. In tolerant Kaspa, the largest groups of upregulated representative GO terms were response to endogenous stimulus comprising 23 enriched GO terms, regulation of biological quality (19), system development (17), cofactor metabolism regulation (17), secondary metabolism (15) and polysaccharide localization (12) (Fig. 4.3A). In contrast, downregulated clusters of the tolerant Kaspa were polysaccharide metabolism (25), response to chemical (24), anatomical structure development (22), cellular ketone metabolism (16), establishment of localization in cell (12) and primary metabolism (11) (Fig. 4.3A). In the other tolerant genotype NL-2, the most upregulated representative terms were cellular metabolism (8), response to carbohydrate (8), developmental growth (4), monocarboxylic acid metabolism (3) and regulation of multicellular organismal process (3), while the most downregulated terms were regulation of biological quality (35), response to other organism (28), anatomical structure development (24), lipid metabolism (18), cellular localization (11), polysaccharide metabolism (10), cellular catabolism (5) and primary metabolism (4) (Fig. 4.3B). Three downregulated terms polysaccharide metabolism, anatomical structure development and cellular ketone/lipid metabolism were common to both tolerant genotypes.

In the sensitive genotype BM-3, key upregulated GO terms were for cellular ketone metabolism (31 enriched GO terms), response to stress (29), metal ion transport (12), regulation of biological quality (12), cellular modified amino acid metabolism (11) and multicellular organismal development (10) (Fig. 4.3C). In contrast, regarding downregulation important clusters were cellular ketone metabolism (28 GO terms), response to chemical (23), anatomical structure development (18), regulation of biological quality (12), cellular protein modification process (12) and cation transport (11) in the sensitive genotype BM-3 (Fig. 4.3C).
Figure 4.3. GO enrichment analysis for biological processes in waterlogging tolerant Kaspa (A) and NL-2 (B) as well as waterlogging sensitive BM-3 (C). The enrichment was conducted using consistently expressed genes from both tolerant and sensitive genotypes across time points (12, 24, 48 and 96 h) with logFC > 2 and FDR < 0.05. The enriched GO terms from both up and down regulated genes were further categorized into 39, 41 and 49 representative GO terms in Kaspa, NL-2 and BM-3 respectively by Revigo TreeMap. Up and Down refers to up and down regulated enriched GO terms.
The enriched GO terms from consistently expressed DEGs for upregulated tolerant pathways and downregulated sensitive pathways are presented in Fig. 4.4 for both biological processes and molecular function. Two highly significant biological process terms - protein metabolism (GO: 0019538, P=4.01e-10) and phosphorus metabolism (GO: 0006793, P=3.50e-10) - were upregulated in tolerant pathways (Fig. 4.4A & 4.4C) and downregulated in sensitive pathways (Fig. 4.4E). Importantly seed germination (GO: 0006807, P=3.47e-10) is also downregulated in sensitive pathways. In the molecular function category, oxidoreductase activity, transferase activity and binding were upregulated in the tolerant genotypes pathways (Fig. 4.4B & 4.4D), while importantly in sensitive genotype pathways the same terms were highly enriched in downregulation (Fig. 4.4F). Comparing the tolerant genotypes, significantly enriched GO terms protein metabolism, primary metabolism and metabolic process were common in both tolerant genotypes, but the terms regulation of metabolism and regulation of primary metabolism were only observed in Kaspa (Fig. 4.4A). Additionally, phosphorus metabolism and phosphate-containing compound metabolism were mostly enriched in Kaspa, whereas in NL-2 (Fig. 4.4C) cellular macromolecule metabolism and nitrogen compound metabolism were mostly enriched.
Figure 4.4. Pathway analysis for consistently differentially expressed genes across the time points (12, 24, 48 and 96 h) with logFC > 2 and FDR < 0.05. (A) Biological process and (B) molecular function from upregulated genes in tolerant Kaspa; (C) Biological process and (D) molecular function from upregulated genes in tolerant NL-2 and (E) biological process and (F) molecular function from down regulated genes in sensitive BM-3. Bubble colour indicates the p-value (legend in the lower right-hand corner), and bubbles that are close together represent similar processes. The smaller the bubble and the darker red the colour, the higher the enrichment level of the term. Arrows indicate up and down regulated pathways.
4.4.3.2 GO enrichment analysis of DEGs at individual time points

GO enrichment analysis in relation to the biological processes category was also performed separately for DEGs with known function expression of logFC ≥ 2 and FDR < 0.05 at individual time points for both tolerant and sensitive genotypes. In the comparison between genotypes, the enriched GO terms were categorized with representative terms by Revigo TreeMap. In tolerant Kaspa (Table S4.18-S4.25), it was found that the representative GO term cellular ketone metabolism was expressed in both up and downregulation at the shortest duration (12 and 24 h) of waterlogging stress (Fig. 4.5A). With the increase in stress duration of 48 and 96 h, new processes of purine nucleotide biosynthesis and purine nucleotide metabolism were represented with the highest number of GO terms were seen in downregulation, while the GO term macromolecule catabolism relating to DEGs with upregulation were mostly expressed at 48 and 96 h (Fig. 4.5A). In the other tolerant cultivar NL-2 (Table S4.26-S4.33), most representative GO terms associated to DEGs with upregulation were cellular amine metabolism at 12 and 24 h; cellular ketone metabolism at 24 and 48 h and macromolecule catabolism at 96 h (Fig. 4.5B). However, GO terms relating to DEGs with downregulation in NL-2 were homeostatic process, water-soluble vitamin metabolism, macromolecule metabolism and vitamin biosynthesis at 12, 24, 48 and 96 h respectively (Fig. 4.5B). By contrast in the sensitive genotype BM-3 (Table S4.34-S4.41), upregulation of DEGs corresponding to representative GO term cellular lipid/ketone metabolism was observed across all time points; with lower number of terms at 12 h which gradually increased at 24 and 48 h (Fig. 4.5C). Another upregulated GO term cellular macromolecule catabolism was found the most expressive at 96 h. On the other side of downregulated DEGs, the GO term protein phosphorylation; the most important mechanisms regulating metabolism was observed across 24, 48 and 96 h time points with the highest number of GO terms at 48 h (Fig. 4.5C).
Figure 4.5. Time point GO enrichment analysis for biological processes in the waterlogging tolerant Kaspa (A) and NL-2 (B) as well as the waterlogging sensitive BM-3 (C). The enriched GO terms at individual time point from both up and down regulated genes were further categorized into representative GO terms through Revigo TreeMap. Up and Down refers to up and down regulated enriched GO terms.

4.5 Discussion

This study was conducted to elucidate the survival of the model pea crop under waterlogging stress at germination. To explore the underlying mechanism, differentially expressed genes identified at germination under waterlogging stress have been linked with morphological traits; testa color and testa membrane integrity/seed leakage associated with waterlogging tolerance.

4.5.1 Association of testa colour and testa membrane integrity with waterlogging tolerance

We found coloured testa genotypes (reddish brown and yellow testa; Kaspa and NL-2 respectively) had greater germination percentage following exposure to both 4 and 8 d of waterlogging than
sensitive BM-3 with its greenish white testa. Previous studies have shown that red coloured genotypes of wheat (Ueno & Takahashi 1997) and rapeseed (Zhang et al. 2008) and brown and black coloured genotypes of soybean (Hou & Thseng 1991) are tolerant to waterlogging compared to less pigmented seeds of respective crops. This difference in tolerance is presumably due to the presence of higher levels of phenolic compounds in reddish seeds (and tolerant genotypes) compare to the sensitive yellow seeded lines (Zhang et al. 2008). In the present study, reddish brown testa Kaspa was tolerant as in other crops but the yellow testa NL-2 also showed tolerance to waterlogging in contrast to rapeseed and soybean, suggesting an additional factor might also be involved in waterlogging tolerance. The visual observation of solute leakage showed that there was no apparent leakage of seed solutes in tolerant genotypes reddish brown Kaspa and yellow NL-2 both at 4 and 8 d after waterlogging (Fig 4.1A). In contrast, the sensitive genotype (BM-3) was able to tolerate 4 d of waterlogging without apparent solute leakage, but after 8 d of waterlogging there was clear whitish seed solutes on the seed surface, indicative of cell membrane damage and subsequent seed death (Fig 4.1A). These observations of solute leakage were confirmed by EC measurements which showed sensitive BM-3 had three-fold higher EC than both tolerant NL-2 and Kaspa, since solute leakage of pea seeds is associated with electrical conductivity (Rahoui et al. 2010). Clearly testa colour and testa membrane integrity; and its corollary solute leakage play a major role in tolerance to waterlogging at germination in pea.

4.5.2 Gene activation linked with testa colour and testa membrane integrity under waterlogging stress

Transcriptomic profiling showed that the most significant DEGs are involved in seed reserve materials - protein, fat and carbohydrate metabolism, suggesting that seed reserve metabolism plays a major role in cell membrane integrity. The main seed reserve carbohydrate whose metabolism is often restricted under anoxia in dryland crops like wheat and maize; due to the inactivation of
amylolytic enzymes (Perata et al. 1997). During the restriction of carbohydrate metabolism, protein and fat metabolism appears to provide energy for germination during abiotic stress like soil waterlogging. Several studies (Crawford & Braendle 1996; Rawyler et al. 1999) have showed that fat metabolism is strongly correlated with membrane leakage as it is the major component of phospholipid bilayer in cell membrane.

In the present study the two tolerant genotypes used contrasting metabolic strategies to survive under waterlogging stress. In rice at seedling two tolerance strategies - escape and quiescence - have been identified with submergence (Sone et al. 2012). A few rice varieties exhibit limited or no stem elongation during submergence and show tolerance to flash flooding. Tolerant Kaspa showed higher percent of germination (73%) during recovery phase compared to during waterlogging (27%). This suggests that the energy in Kaspa seeds was preserved during waterlogging. After draining at recovery, germination was restarted by using the conserved energy similar to quiescence strategy in rice. The tolerant rice cultivars were not responsive to the ethylene promoted shoot elongation during submergence causing quiescence in growth (Jackson et al. 1987). Most differentially expressed genes in Kaspa were involved in metabolic regulation during waterlogging. The gene for tyrosine protein kinase was upregulated which is known to be induced by abiotic stresses such as cold and salt treatments in peanut (Arachis hypogaea) (Rudrabhatla & Rajasekharan 2002), and the overexpression of this gene in Arabidopsis increases tolerance to salt stress (Bing et al. 2013). The underlying function of this gene is to phosphorylate Ser, Thr, and Tyr residues on target proteins, the most important biochemical mechanism to regulate enzyme activities and many other cellular processes (Hunter 1987). In peanut, this gene regulates lipid metabolism during seed germination through phosphorylation of oleosin protein which was shown to involve both biosynthesis and catabolism of lipids (Parthibane et al. 2012). Other upregulated genes in Kaspa; hypoxia-responsive family protein is considered to regulate transcription during waterlogging stress and the leucine-rich repeat receptor-
like kinase (LRR-RLKs) play a fundamental role in sensing external signals and regulating gene expression responses at the cellular level (Lease et al. 1998). LRR-RLKs are mainly induced by abiotic stresses (Chae et al. 2009). The responses of this gene increased the salinity and abscisic acid tolerance in the germination and early root growth as well as to oxidative stress in Arabidopsis (Wang et al. 2017). In *Medicago truncatula* this gene was found to regulate the adaptation of roots to salt stress (De Lorenzo et al. 2009). Moreover, highly downregulated genes, such as LOX5 (Lipoxygenase), alpha beta fold family protein and phospholipase D delta are involved in the hydrolysis of glycerol-phospholipids that are related to diverse catalytic activities of lipid (Bannenberg et al. 2009; Vijayakumar & Rajasekharan 2016; Li et al. 2008 respectively). Another gene for pfkb-type carbohydrate kinase family protein was down regulated and is involved in the growth and development of Arabidopsis (Gilkerson et al. 2012). The GO enrichment from consistently expressed DEGs also showed metabolic regulation where cellular ketone and polysaccharide metabolism were mostly downregulated, similar to most downregulated genes. Individual time point GO enrichment also revealed cellular ketone metabolism downregulated with short waterlogging stress (12 and 24 h) however, with the increase in stress to 48 and 96 h, another term corresponds to purine nucleotide metabolism known to provide energy at seed germination in legumes (Ashihara 1983) was highly downregulated. Furthermore, two most enriched GO terms in tolerant pathways- regulation of metabolism and regulation of primary metabolism showed upregulation. These clearly indicate energy preservation in tolerant Kaspa through metabolic regulation during waterlogging period. Additionally, the phenolic compound in this reddish brown Kaspa is considered to provide defensive support to the seeds during waterlogging. The most regulated gene for wound responsive family protein was upregulated during waterlogging in accordance with wound responsive genes being induced from diverse environmental stresses (Zhou & Thornburg 1999) including genes encoding for phenylalanine ammonia lyase, chalcone synthase and chalcone isomerase. The function of these genes is to provide the cell with lignin and phenolic
precursors to the wounded surface and provide defence to plant. This is consistent with most expressed upregulated GO terms secondary metabolism and polysaccharide localization. Secondary metabolism represented steroid biosynthetic process (GO: 0006694), phenylpropanoid metabolic process (GO: 0009698) and pigment biosynthetic process (GO: 0046148) while polysaccharide localization depicted for polysaccharide localization (GO: 0033037), callose localization (GO: 0052545) and defence response by callose deposition (GO: 0052542). These imply that metabolic regulation and wound sealing by phenolic compounds provided rigid membrane integrity in tolerant Kaspa.

In the other tolerant genotype NL-2, 75% germination was found under waterlogging and 25% during the recovery phase out of total germination which suggests that this genotype used considerable energy during waterlogging to escape the stress. This is similar to the escape strategy of rice cultivars whereby they elongate their leaves and stem to get oxygen at the water surface under submergence. This shoot elongation requires large amounts of energy to escape the waterlogging stress. DEGs showed that two most upregulated genes are involved in catabolism, one for Subtilase-type proteinase considered to be involved in protein catabolism, the most important processes associated with leaf senescence through nutrient mobilization, especially nitrogen (Martinez et al. 2015). Another upregulated gene PNC2 (Peroxisomal adenine nucleotide carrier 2) was highly expressed which is known to have a key role to catabolize seed reserved triacylglycerols into sucrose during postgerminative growth of seedlings in soybean and Arabidopsis by supplying ATP to peroxisomes (Arai et al. 2008; Graham 2008; Linka et al. 2008). This implies that during waterlogging in NL-2 energy supplies come from both protein and fat metabolism leading to high percent of germination under waterlogging. GO enrichment from consistently expressed genes showed that representative GO term cellular metabolism of protein metabolic process (GO: 0019538), primary metabolic process (GO: 0044238) and cellular macromolecule metabolic process (GO: 0044260) was upregulated which
is consistent with the function of the most upregulated gene Subtilase-type proteinase, but the GO term relating to lipid metabolism was downregulated which was just opposite from most upregulated gene PNC2. This could be explained by the time point GO enrichment analysis where lipid/cellular ketone metabolism was mostly downregulated with only a short duration (12 h) of waterlogging stress. However, ketone metabolism was mostly upregulated at 24 and 48 h, which is consistent with most upregulated gene PNC2. Cellular amine metabolism was also mostly upregulated at 12 and 24 h probably to provide nitrogen to plants for growth. Furthermore, the tolerant pathways showed that the most top enriched GO terms were relating to metabolism including protein and nitrogen compound metabolism. All of these evidence suggest extensive energy utilization through metabolism for rapid germination during waterlogging.

Turning to the sensitive genotype BM-3, only 14% germination was found after 8 d of waterlogging and there was no further germination during the subsequent recovery phase. A highly differentially expressed downregulated gene in this genotype was copper amine oxidase, which is involved in cell wall maturation and lignification during development as well as in wound-healing and cell wall reinforcement during stress (Cona et al. 2006). Another gene UDP-glucosyltransferase (UGT) family protein represented five isoforms were also mostly downregulated (Table S4.5). This gene is involved in the biosynthesis of plant natural products, such as flavonoids, phenylpropanoids, terpenoids and steroids, and the regulation of plant hormones (Liu et al. 2015). The downregulation of UGT might have negative effect on seed germination in sensitive BM-3 as its down expression contributed to delay in seed germination in Arabidopsis by increasing abscisic acid level (Liu et al. 2015). Moreover, the top upregulated genes such as Kunitz type trypsin inhibitor contained in legume seeds function as a protease inhibitor resulting in a complex resistance to proteolysis and protein metabolism (Meester et al. 1998). In the shortage of protein metabolism under waterlogging stress the sensitive genotype may be employing the alternate fat metabolism which can be supported by GO enrichment analysis.
The enriched GO for protein metabolism (GO: 0019538), cellular macromolecule metabolic process (GO: 0044260) and protein autophosphorylation (GO: 0046777) under highly expressed representative GO term cellular protein modification process were downregulated whereas the highly expressed ketone/fat metabolism was upregulated. Time-point analysis also showed upregulation of fat metabolism for all the time points with most upregulation at 24 and 48 h waterlogging stress. These indicate that the sensitive genotype largely depends on lipid metabolism for energy during waterlogging. With prolonged waterlogging stress the excessive lipid metabolism induced membrane damage, which is suggested by the correlation between the leakage of electrolytes from the cells and the release of free fatty acids in anoxic potato (*Solanum tuberosum* L.) tubers (Crawford & Braendle 1996). Rawyler et al. (1999) also revealed that potato cells survive temporarily (0-12 h) under anoxic condition with intact cell membrane by the absence of lipid degradation. But, following 12 h of anoxia cell biomass and ATP levels decreased and the accumulation of free fatty acids was increased by the hydrolysis of phospholipids in cell membrane. This is suggestive that excessive lipid metabolism; in contrast to the tolerant genotypes might be responsible for cell membrane damage and solute leakage in sensitive BM-3 under waterlogging stress.

In summary, our results suggest two contrasting tolerance mechanisms in Mendel’s model plant pea under waterlogging at germination. One of them is a quiescence strategy whereby the seed reserved energy is preserved through metabolic regulation in reddish brown testa Kaspa during waterlogging. Another tolerance mechanism with NL-2 is an escape strategy, which involves rapid germination utilizing energy from both protein and lipid metabolism during waterlogging. In contrast, in the sensitive genotype BM-3 inhibition of protein metabolism led to excessive lipid metabolism for its energy requirement under waterlogging leading to membrane leakage and subsequent seed damage confirmed by EC measurements. Such mechanisms and associated genes identified with this study provide a platform to breed pea for adaptation to waterlogging stress at germination.
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4.6 References


### 4.7 Supporting information

Additional supporting information may be found online in the supporting information section at the end of the article- https://doi.org/10.1111/pce.13338
Chapter 5

Waterlogging tolerance at germination in field pea: Variability, genetic control and indirect selection


This thesis chapter- waterlogging tolerance at germination in field pea: Variability, genetic control and indirect selection was designed by M. S. U. Zaman in consultation with A. I. Malik, P. Kaur and W. Erskine. For preparing plant materials, a bi-parental crossing in response to WL tolerance was conducted by M. S. U. Zaman while F. M. Ribalta was involved for advancing F₂ – F₆ generation to develop RIL population. A diversity panel of pea germplasm was collected from the Australian Grains Genebank, Department of Jobs, Precincts and Regions, Victoria by the help of W. Erskine. Seed sowing, soil redox measurement and data recording for all the experiments were handled by M. S. U. Zaman. The data were analyzed by M. S. U. Zaman under the supervision of W. Erskine. The manuscript was written by M. S. U. Zaman and the co-authors W. Erskine, A. I. Malik and P. Kaur were involved in the discussion of results, structure of the manuscript and editorial comments.
Chapter 5

Waterlogging tolerance at germination in field pea: Variability, genetic control and indirect selection

5.1 Abstract

In the Eastern Gangetic Plain of South Asia field pea (*Pisum sativum* L.) is often grown as a relay crop where soil waterlogging (WL) causes germination failure. To assess if selection for WL tolerance is feasible, we studied the response to WL stress at germination stage in a recombinant inbred line (RIL) population from a bi-parental cross between WL-contrasting parents and in a diversity panel to identify extreme phenotypes, understand the genetics of WL tolerance and find traits for possible use in indirect selection. The RIL population and the diversity panel were screened to test the ability of germination under both waterlogged and drained soils. A total of fifty, most waterlogging tolerant and sensitive genotypes from both the RIL population and the diversity panel were further evaluated to assay testa integrity/leakage in CaSO$_4$ solution. Morphological characterisation of both populations was undertaken. A wide range of variation in the ability to germination in waterlogged soil was observed in the RIL population (6 - 93%) and the diversity panel (5 - 100%) with a high broad-sense heritability ($H^2 > 85\%$). The variation was continuously distributed indicating polygenic control. Most genotypes with a dark coloured testa (90%) were WL tolerant, whereas those with a light coloured testa were all WL sensitive in both the RIL population and diversity panel. Testa integrity, measured by electrical conductivity (EC) of the leakage solute, was strongly associated with WL tolerance in the RIL population ($r_G = -1.00$) and the diversity panel ($r_G = -0.90$). Therefore, testa integrity can be effectively used in indirect selection for waterlogging tolerance. Response to selection for WL tolerance at germination is confidently predicted enabling the adaptation of the ancient model pea to extreme precipitation events at germination.
5.2 Introduction

Peas (*Pisum sativum* L.) are an important pulse crop, ranks second in global production after beans among the pulse crops (FAOSTAT 2017). Pea seeds are rich in protein, slowly digestible starch, soluble sugars, fiber, minerals and vitamins (Dahl et al. 2012). It has an economic and agronomic importance in cropping systems (Yang et al. 2018). The crop is also an important component of agroecological cropping systems in diverse regions of the world. In South Asia, there is a history of relay-sowing of pea into standing rice on waterlogged soil (Ali & Sarker 2013). Waterlogging (WL) can cause germination failure (Crawford 1977) and lead to reduced plant population in pea (Zaman et al. 2018).

Global climate change causes waterlogging events to be more frequent, severe, and unpredictable (Intergovernmental Panel on Climate Change [IPCC] 2014). Climate change predictions for South Asia suggest alterations in the intensity of rainfall events, an increase in inter-annual precipitation variability (Sivakumar & Stefanski 2010) and delayed monsoon rains (Li et al. 2017). This constitutes a major threat to regional crop production. Pea is very prone to WL, even more than other grain legumes (Solaiman et al. 2007; Pampana et al. 2016). In recent years, unseasonal rain during sowing exposed the pea crop to waterlogging stress (Zaman et al. 2018). Therefore, it is crucial to develop stress-resistant peas and to improve agricultural practices to cope with WL stress.

Developing pea genotypes tolerant to WL might be an effective strategy to mitigate WL stress. Variation in WL tolerance at germination among three pea genotypes was demonstrated by Zaman et al. (2018) indicative of valuable diversity within the species. WL tolerance at germination has also been identified in lentil (*Lens culinaris* Medik. ssp. *culinaris*) (Wiraguna et al. 2017), pigeonpea (*Cajanus cajan* (L.) Millsp. (Sultana et al. 2013), soybean (*Glycine max* (L.) Merr.) (Hou & Thseng 1991), wheat (*Triticum aestivum* L.) (Ueno & Takahashi 1997), maize (*Zea mays* L.) (Zaidi et al. 2012) and barley (*Hordeum vulgare* L.) (Takeda & Fukuyama 1986). However, the long history of
focused breeding on high yield and food quality has led to a loss of genetic diversity and stress resistance. Therefore, breeders have to undertake more efficient methods of selection and take advantage of the large genetic diversity present in pea genepool. Recently, Simple Sequence Repeat marker panels have been developed that could be useful for identifying markers linked to WL tolerance and marker-assisted selection (Burstin et al. 2015), but no markers linked to WL tolerance have been identified yet. The value of morpho-physiological traits as indirect selection criteria for WL tolerance is also worthy of evaluation. Several traits are associated with WL tolerance at germination. Small seeds in soybean showed a higher germination rate than large seeds during seed submergence (Sayama et al. 2009). Testa (seed coat) colour is sometimes associated with WL tolerance (Hou & Thseng 1991; Ueno & Takahashi 1997; Zhang et al. 2008). Several studies on the role of the testa in preventing cellular damage during imbibition showed that seeds with cracked testa and seeds without testa had rapid imbibition and higher solute leakage than those with intact testa and no cracks (Larson 1968, pea; Powell & Matthews 1978, pea; Duke & Kakefuda 1981, soybean, navy bean (*Phaseolus vulgaris L.*), pea, and peanut (*Arachis hypogaea L.*); Duke et al. 1983, soybean). Furthermore, a short period (i.e. 24 h) of seed submergence showed rapid imbibition leading to solute leakage, and was associated with low seedling vigour (Perry & Harrison 1970, pea; Yaklich et al. 1979, soybean; and Kantar et al. 1996, fababean). Testa integrity appears to be a key trait for WL tolerance at germination.

Here, to assess if selection for L tolerance is feasible in peas, we studied the response to WL stress at germination stage in a recombinant inbred line (RIL) population from a bi-parental cross between WL-contrasting parents and a diversity panel to: (i) identify extreme phenotypes for WL tolerance, (ii) understand the genetic basis of WL tolerance and (iii) find traits for possible use in indirect selection for WL tolerance.
5.3 Materials and methods

5.3.1 Plant materials

A recombinant inbred line (RIL) population and a diversity panel of pea germplasm were used in this study.

The RIL population (108 lines) was from a bi-parental cross between waterlogging (WL) tolerant genotype Kaspa and sensitive BM-3 (Zaman et al. 2018). Hybridization was done at the University of Western Australia (UWA) in 2015. The F₁ generation was allowed to self-pollinate and 250 F₂ seeds were produced in the glasshouse at an average temperature of 25 °C in 2016. Generation advancement from F₂ to F⁶ was undertaken by a rapid generation system using single seed descent from May 2016 to June 2017. In this system, flower induction was accelerated by growing plants under far red enriched LED light (AP67 spectrum) from B series Valoya lights (Helsinki, Finland) (Croser et al. 2016). The temperature was at 24/20 °C with a 20 h photoperiod. Seeds were sown in plastic pots (19-cm height × 21-cm diameter) filled with steam pasteurised potting mix (UWA Plant Bio Mix – Richgro Garden Products Australia Pty Ltd). Potting mix was of composted pine bark, coco peat and brown river sand in a 5:2:3 ratio. Plants were watered daily and fertilised weekly with a water soluble N-P-K fertiliser (19-8.3-15.8) with micronutrients (Poly-feed, Greenhouse Grade, Haifa Chemicals Ltd.) at a rate of 0.3 g pot⁻¹. Immature seed harvesting, in vitro germination and transplantation were conducted as described in Ribalta et al. (2017).

The diversity panel of 110 genotypes comprised five Australian varieties and germplasm accessions from the Australian Grains Genebank, Department of Jobs, Precincts and Regions, Victoria. The panel included the WL contrasting genotypes - Kaspa and BM-3. The germplasm represents global pea diversity and originates from the geographic regions of South Asia (21 genotypes), former USSR (18), Northern Europe (18), Mediterranean (17), North America (12), Australia (9), South America (8) and Africa (7).
5.3.2 Methods

Three types of experiments and within each a RIL population and Diversity panel trial were conducted. The same source of seeds were used for all the experiments.

5.3.2.1 Experiment 1: Studies on waterlogging tolerance

RIL population

The experiment to assay WL tolerance was conducted in a glasshouse of the Plant Growth Facility at UWA in July 2017 as in Zaman et al. (2018) using the 108 RIL population and the parents - tolerant Kaspa and sensitive BM-3. The experimental design was split-plot with three replicate blocks. Main plots were WL treatments (2 levels: drained control and 8 d WL) while the genotypes (108 RILs and two parents) were in sub-plots. The experimental unit was plastic pot—free draining with a sealed base. Free-draining pots contained gravel at the bottom and 3.5 kg sand and soil mixed (1:1) (pH 6.7 and electrical conductivity (EC) 0.46 dS m$^{-1}$ at 1:5 w/v soil/water) at the top. Soil was collected from Mukinbudin (30°78′ S, 118°31′ E), Western Australia (Kotula et al. 2015). Each free-draining pot (19-cm height × 21-cm diameter) was placed in a sealed base pot (24-cm height × 26-cm diameter). Platinum (Pt) electrodes were inserted in the substrate at a depth of 100 mm in 10 pots for redox measurement (Patrick et al. 1996). For the waterlogged treatment, DI water was added to sealed pots so that free-draining pots could be waterlogged from the bottom to maintain a water table at 10 mm below the soil surface. Pots were waterlogged for 4 days prior to sowing to ensure hypoxia at sowing. Water was added to sealed base pots daily as required to maintain the water table. For drained control treatments, there was no water in the sealed base pots, but the soil moisture in free-draining pots was maintained at ~ 80% of field capacity. Seeds were treated with Thiram (Tetramethylthiuram disulphide) at the rate of 3 g/kg seeds just before sowing. Twenty seeds of each genotype were sown in a free-draining pot by dibbling at 5 mm soil depth. The seed rate for WL screening followed the WL-screening protocol of Zaman et al. (2018). All pots were covered for 3 d after sowing to ensure
darkness for germination. Waterlogged pots were drained after 8 d of WL treatment. Drained control pots were weighed every day and watered to ~ 80% field capacity. Within replicates, pots were moved every 5 d to minimize the effects of varying conditions in the glasshouse. The experiment was conducted at 25 °C temperature and was terminated 23 d after sowing, when there was no sign of further emergence.

**Diversity panel**

The experiment to assay WL tolerance was conducted on 110 genotypes of the diversity panel including the WL controls – WL tolerant Kaspa and WL sensitive BM-3 at germination under similar growth conditions and management practices in June 2016 as described above for the RIL population. The experimental design was split-plot in three replicate blocks with WL treatments (as above) as main plots and genotypes as sub-plots.

Seed emergence was recorded daily during WL and during the recovery period (draining of pots after 8 days WL); and expressed as a percentage of the total number of seeds sown. The emergence was assessed till 23 day, the end day of experiment. Seeds with an epicotyl longer than 5 mm were considered as germinated (i.e. emerged). The redox potential of the soil was recorded daily from 10 pots with a Pt electrode and silver/ silver chloride reference electrode attached to a millivolt-meter following the procedure described by Patrick et al. (1996).

**5.3.2.2 Experiment 2: Agro-morphological traits and WL tolerance**

**RIL population**

The RIL population (108 lines + 2 parents) was screened for agro-morphological traits in the UWA glasshouse from August to December 2017 in a randomized complete block design with two replications in non-stressed condition. The experimental unit was plastic pot (diameter 260 mm and height 230 mm). Each pot was filled with gravel at the bottom with 4.0 kg of potting mix (composition described above) on top. Five seeds of each genotype were sown in each pot. After 3 weeks, plants
were thinned to 2 plants per pot. Four weeks after sowing, a water soluble N-P-K fertiliser (19-8.3-15.8) with micronutrients (Poly-feed, Greenhouse Grade, Haifa Chemicals Ltd.) at a rate of 0.3 g per pot were applied and this concentration was doubled after 6 weeks. The fertiliser was applied weekly till the end of grain filling. Insecticide Spinetoram (DOW Agrosciences Australia Limited) was applied as required to control fungal gnats (Orfelia and Bradysia sp.). Pots were watered to ensure the plants had access to adequate moisture. Watering was stopped to individual pots when pod colour turned to light yellow. The average temperature of the glasshouse was 22 °C from August to December 2017.

**Diversity panel**

The diversity panel (110 genotypes including 2 controls - WL tolerant Kaspa and WL sensitive BM-3) was screened for agro-morphological traits in the UWA glasshouse in a randomized complete block design with two replications. Seed sowing and other management practices were the same as for the RIL population above. The experiment was conducted in the UWA glasshouse with an average temperature of 23 °C from September to December 2016.

Stem base width and plant height of five plants were measured three weeks after sowing using digital Vernier caliper (Kincrome, Australia) and 30-cm plastic scale (Promotion products, Australia), respectively. Flower colour and leaf axil pigmentation were noted at flowering using UPOV pea descriptors (UPOV 2009) were used with 1-3 (1= white, 2= pink and 3= purple) and 1-2 (1= absent and 2= present as single ring) scoring scales, respectively. Time to 50% flowering (d) was recorded for individual plants. Testa colour and seed weight were recorded after drying for three months at room temperature following harvest. Testa colour was scored with a 1-9 (1= light yellow, 2= yellow pink, 3= waxy, 4= yellow-green, 5= grey-green, 6= dark green, 7= light brown, 8= brown and 9= black) scoring scale (Pavelkova et al. 1986). The colour of flower, leaf axil and testa were observed and confirmed by horticultural colour chart (Wilson 1942).
5.3.2.3 Experiment 3: Testa leakage and WL tolerance

RIL population
To assay for testa integrity/leakage under waterlogging conditions 50 genotypes with contrasting responses (i.e. 25 tolerant and 25 sensitive RIL lines) to WL treatment were selected from the 108 RIL population. The testa of tolerant parent (Kaspa) was dark in colour, whereas the sensitive parent BM-3 had a light coloured testa. The 50 genotypes were subjected to a submergence treatment with eight replications in a completely randomized design. An individual seed representing a replicate of each genotype was submerged in a 50 ml centrifuge tube (SARSTEDT, Germany) containing 40 ml of 0.5 mM CaSO$_4$ solution and incubated in a germination cabinet at 25 °C temperature with 12:12 light-dark cycle for 6 d.

Diversity panel
A total of 50 genotypes with contrasting responses (i.e. 25 tolerant and 25 sensitive) to WL were selected from the 110-genotype diversity panel. The tolerant 25 genotypes comprised 20 dark and 5 light coloured testa, whereas sensitive 25 genotypes comprised of 23 light and 2 dark testa. Experimental design and growth conditions were similar to that of the RIL population.

Electrical conductivity (EC) of submergence solution was measured after 6 d of treatment with an AQUA- PH v1.0 conductivity meter (TPS, Brisbane Australia). Seeds were germinated in CaSO$_4$ solution so the germination was counted at the end of the experiment on day 6. Seeds with a radicles longer than 3 mm were considered as germinated. Germination was reported in percent based on the number of seeds germinated from 8 seeds of each genotypes.
5.3.3 Statistical analysis and response to selection

Data were analysed using GenStat 16th edition for Windows statistical software (VSN International, UK). Analyses of variance (ANOVA) were undertaken to determine the effects of the different treatments, and least significant differences (l.s.d) at $p > 0.05$ calculated for significant differences between treatments, genotypes and interaction means. A one-way ANOVA was also conducted by region of origin. Spearman’s rank correlation coefficient was calculated by STAR statistical software, version 2.0.1 2014 (Biometrics and Breeding Informatics, PBGB Division, International Rice Research Institute, Los Baños, Philippines). Chi-square tests for goodness-of-fit was conducted to measure the inheritance of testa colour.

Response to selection: The broad-sense heritability was estimated by: $H^2 = \left(\sigma^2_g\right) / \left(\sigma^2_g + \sigma^2_e\right)$ where $\sigma^2_g$ and $\sigma^2_e$ are the estimated genotypic and error variances, respectively (Nyquist 1991). The estimated genotypic and error variances were calculated as: $\sigma^2_g = (MS_g - MS_e)/r$ while $\sigma^2_e = MS_e/r$, where MS$_g$ is mean square of the population, MS$_e$ is the residual error and $r$ the number of replicates.

Genetic correlations between traits were computed as: $r_{G12} = r_{P12} / \sqrt{H^2_1 \times H^2_2}$ (Cooper et al. 1996) where $r_{G12}$, $r_{P12}$, $H^2_1$ and $H^2_2$ are the genotypic correlation between traits 1 and 2, phenotypic correlation between the same pair of traits, and heritability of traits 1 and 2, respectively.

The efficiency of indirect selection was estimated as (Cooper et al. 1996; Kumar et al. 2008):

$$\frac{CR_G}{DR_G} = r_G \sqrt{H^2_G / H^2_g}$$

where $CR_G$ is the correlated response to indirect selection for germination based on secondary traits, $DR_G$ indicates direct response to selection for germination, $r_G$ is the genotypic correlation, $H^2_G$ and $H^2_g$ represents heritability for the secondary trait and germination respectively under waterlogged stress.
5.4 Results

5.4.1 Redox measurements

In drained control soil the redox potential in the RIL population was 585 ± 5 mV throughout the experimental period. By contrast, the redox potential in waterlogged pots was 318 ± 6 mV throughout the WL period and this increased on draining the pots to 565 ± 13 by 23 d. In the experiment with the diversity panel the redox potential in drained and waterlogged pots followed the same trend.

5.4.2 Variation of waterlogging tolerance

In the RIL population, all the genotypes including parents showed close to 100% germination in drained soil. However, in waterlogged soil the RIL parents showed contrasting responses in germination (measured as emergence) - tolerant Kaspa 73% and sensitive BM-3 20% (LSD$_{P=0.05}$ of 22). The population of 108 RIL lines exhibited segregation from 6 to 93% germination (Fig. 5.1a, b). The mean germination of the RIL population was 41%, mid-way between the parents. Significant transgressive segregation was not recorded in either direction. A high broad-sense heritability of H$^2 = 89\%$ was found for germination under waterlogged conditions for this RIL population.

In the experiment with the diversity panel, in drained soil all the genotypes (including controls) showed close to 100% germination. However, in waterlogged soil a wide range in germination was observed from 5 to 100% exhibiting a continuous distribution (Fig. 5.1c, d). The mean for germination in the diversity panel was 48%, mid-way between tolerant control Kaspa (68%) and sensitive control BM-3 (22%) (LSD$_{P=0.05} = 25$). Five genotypes significantly ($P < 0.05$) exceeded the tolerant parent Kaspa in germination under waterlogging, but no genotype was significantly less tolerant than the sensitive BM-3 control. In the diversity panel the broad-sense heritability for germination in waterlogged soil was high at H$^2 = 87\%$. 

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Figure 5.1. Variation of germination/WL tolerance on 8 d waterlogged soil in RIL population (a, b) and diversity panel (c, d). Emergence started from 3 d after sowing and was completed by 23 d. Germination was recorded daily for each seed in both the waterlogged and recovery periods. Seeds with an epicotyl longer than 5 mm were considered as germinated. The l.s.d. is at $P = 0.05$ and $n = 324$ and 330 for RIL population and diversity panel respectively.

In the diversity panel, a one-way ANOVA by region of origin showed that geographical distribution accounted for significant ($P < 0.001$) variation in WL tolerance at germination (Fig. 5.2). Genotypes from Africa (i.e. Ethiopia in the current study) showed highest germination (80%) on average when exposed to soil waterlogging. The poorest performance under waterlogged conditions was from genotypes from the former USSR.
Figure 5.2. Association of percent germination/WL tolerance with geographic region of origin after 8 d of soil WL. Box plot represents mean germination (mid-point of box plot), standard error (box plot length), together with minimum and maximum values (whisker bars). Multiple comparison was carried out by Fisher’s Protected LSD \((P = 0.05)\) based on one-way ANOVA \((P < 0.001)\) by geographic region of origin. Means followed by different letters are significantly different at \(P = 0.05\). The number in brackets denotes the number of genotypes in a region.

5.4.3 Morphological traits and waterlogging tolerance

Correlation coefficients showed pair-wise associations between WL tolerance and morphological traits as well as among the morphological traits (Table 5.1). In the RIL population the strongest positive correlations with WL tolerance were found for the three traits - flower colour \((r= 0.62)\), leaf axil pigmentation \((r= 0.66)\) and testa colour \((r= 0.59)\) (Table 5.1a). Furthermore, a detailed analysis of testa colour showed two distinct parental groups: dark like WL tolerant Kaspa (Fig. 5.3a) and light testa like WL sensitive BM-3 (Fig. 5.3b). Overall 52 of the 108 genotypes had dark coloured and the rest 56 was light-coloured testa that clearly segregated in 1: 1 ratio \((\chi^2= 0.15, P < 0.001)\), indicating single gene controlling the trait (Fig. 5.3c). The average germination of dark testa RIL genotypes was 58%, which was significantly \((P < 0.001)\) higher than the mean for genotypes with light coloured
testa (26%). The range of percent germination was from 8 - 92% for dark and 8 - 65% for light testa genotypes.

In the diversity panel correlations with WL tolerance were similar to the RIL population with \( r = 0.57 \) for flower colour, leaf axil pigment \( (r = 0.51) \) and testa colour \( (r = 0.51) \) again showing strong positive correlations (Table 5.1b). The trait stem base width exhibited a weaker correlation with WL tolerance. Analysis of testa colour showed that 34 out of 110 genotypes had dark coloured testa and the rest 76 had light-coloured testa (Fig. 5.3d). The mean germination of dark testa coloured genotypes was 71%, which was significantly \( (P < 0.001) \) higher than the mean for genotypes with light coloured testa (37%). However, the range of percent germination was similar for both dark and light testa genotypes.
Table 5.1. Spearman’s rank phenotypic correlation coefficients (r) between morphological traits for a) RIL population (n= 108) and b) diversity panel (n= 110 genotypes)

(a) RIL population

<table>
<thead>
<tr>
<th>Traits</th>
<th>Waterlogged germination</th>
<th>Flower colour (FC)</th>
<th>Leaf axil pigment (LAP)</th>
<th>Testa colour (TC)</th>
<th>Seed weight (SW)</th>
<th>Stem base width (SBW)</th>
<th>Plant height (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td><strong>0.62</strong>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LAP</td>
<td><strong>0.66</strong>*</td>
<td><strong>0.95</strong>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TC</td>
<td><strong>0.59</strong>*</td>
<td><strong>0.86</strong>*</td>
<td><strong>0.88</strong>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SW</td>
<td>0.10</td>
<td>0.13</td>
<td>0.13</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SBW</td>
<td>-0.00</td>
<td>-0.06</td>
<td>-0.06</td>
<td>-0.04</td>
<td>0.18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PH</td>
<td>-0.01</td>
<td>-0.04</td>
<td>-0.04</td>
<td>0.00</td>
<td><strong>0.36</strong>*</td>
<td>0.18</td>
<td>-</td>
</tr>
<tr>
<td>TF</td>
<td>-0.04</td>
<td>-0.06</td>
<td>-0.06</td>
<td>-0.01</td>
<td>0.18</td>
<td>0.02</td>
<td>0.10</td>
</tr>
</tbody>
</table>

(b) Diversity panel

<table>
<thead>
<tr>
<th>Traits</th>
<th>Waterlogged germination</th>
<th>Flower colour (FC)</th>
<th>Leaf axil pigment (LAP)</th>
<th>Testa colour (TC)</th>
<th>Seed weight (SW)</th>
<th>Stem base width (SBW)</th>
<th>Plant height (PH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td><strong>0.57</strong>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LAP</td>
<td><strong>0.51</strong>*</td>
<td><strong>0.86</strong>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TC</td>
<td><strong>0.51</strong>*</td>
<td><strong>0.76</strong>*</td>
<td><strong>0.73</strong>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SW</td>
<td>-0.18</td>
<td>-0.36***</td>
<td>-0.16</td>
<td>-0.29**</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SBW</td>
<td><strong>-0.25</strong></td>
<td><strong>-0.43</strong>*</td>
<td><strong>-0.27</strong></td>
<td><strong>-0.29</strong></td>
<td><strong>0.40</strong>*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PH</td>
<td>0.17</td>
<td>0.23*</td>
<td>0.21*</td>
<td>-0.05</td>
<td>0.00</td>
<td><strong>-0.29</strong></td>
<td>-</td>
</tr>
<tr>
<td>TF</td>
<td>0.02</td>
<td>0.12</td>
<td>0.12</td>
<td>-0.04</td>
<td>0.12</td>
<td>-0.08</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Notes: TF, time to flower. Significant correlations are shown in bold: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
5.4.4 Solute leakage/EC and WL tolerance

In a sub-group of the RIL population comprising contrasting tolerant and sensitive genotypes (i.e. 25 tolerant and 25 sensitive) selected from waterlogging tolerance experiment (described section 5.4.2), germination and electrical conductivity (EC) of 0.5 mM CaSO₄ solution following 6 d of submergence were measured and were found strongly correlated (r= -0.94) (Fig. 5.4a). In this association there was a clear boundary of EC value of 200 μS cm⁻¹ g⁻¹ seed between tolerant and sensitive groups. All the genotypes in the tolerant group had a dark testa with a low EC (61- 161 μS cm⁻¹ g⁻¹ seed). However, all the WL sensitive samples, composing 22 light and 3 dark testa genotypes had higher EC (220- 498 μS cm⁻¹ g⁻¹ seed). Visual observation showed that genotypes in the WL tolerant group had intact testa and low EC (Fig. 5.4b). Conversely, in the WL sensitive group many of the genotypes showed dissolved testa and higher EC (Fig. 5.4c).
Similarly, in a sub-group of the diversity panel (25 tolerant and 25 sensitive genotypes), germination and electrical conductivity (EC) of 0.5 mM CaSO$_4$ solution following 6 d of submergence were measured and were found strongly correlated ($r= -0.89$) (Fig. 5.4d). This association again had a clear boundary of EC value of 200 μS cm$^{-1}$ g$^{-1}$ seed separating WL tolerant and sensitive genotypes. In the WL tolerant group (i.e. 20 dark and 5 light testa), all the dark testa genotypes had low EC (25- 172 μS cm$^{-1}$ g$^{-1}$ seed) but the 5 light testa genotypes showed higher EC (222- 374 μS cm$^{-1}$ g$^{-1}$ seed) as sensitive group. In contrast, all the genotypes in the WL sensitive group (i.e. 23 light and 2 dark testa) had higher EC (240- 588 μS cm$^{-1}$ g$^{-1}$ seed). The genotypes in the tolerant group again had visually intact testa and low EC (Fig. 5.4e), in contrast, many of the genotypes in the sensitive group exhibited dissolved testa and high EC values (Fig. 5.4f).
Figure 5.4. Germination versus EC of 0.5 mM CaSO₄ solution following 6 d of seed submergence. In RIL population- (A) association between EC and germination, (B) intact testa with germination in tolerant genotypes and (C) dissolved testa without germination in sensitive genotypes. Similarly in diversity panel- (D) association between EC and germination, (E) intact testa with germination in tolerant genotypes and (F) dissolved testa without germination in sensitive genotypes. Germination was counted by observing the radical longer than 3 mm in the respective solution. The values on the centrifuge tube refer to EC values (μS cm⁻¹ g⁻¹ seed) measured at 6 d seed submergence. Brown and green circles in (A) and (D) refer to tolerant and sensitive genotypes respectively.

5.4.5 Direct and indirect response to selection for waterlogging tolerance

Direct and indirect responses to selection for WL tolerance were estimated from germination on waterlogged soil, as there was a strong concurrence between the germination in waterlogged soil and germination of seed submerged in CaSO₄ solution in both RIL population (r= 0.95) and diversity panel (r= 0.95) (Fig. S5.1). The direct response to selection for WL tolerance was based on germination values, while the indirect responses to selection for WL tolerance were based on four secondary traits (EC, testa colour, flower colour and axil pigment). All the secondary traits exhibited even higher heritability (H²= 0.95 to 1.00) values than that of germination % (H²= 0.89_RIL; 0.87_diversity)
when grown on waterlogged soil (Table 5.2). Among the four secondary traits, EC had the highest genetic correlation with germination in RIL population ($r_G = -1.00$) and diversity panel ($r_G = -0.90$). Comparing the efficiency of indirect selection for germination under waterlogged conditions, among the four secondary traits EC had the highest efficiency for selection in both RIL population ($CR_G/DR_G = -1.10$) and diversity panel ($CR_G/DR_G = -0.98$) (Table 5.2).

**Table 5.2.** Heritability ($H^2$), genetic correlation ($r_G$) of secondary traits with germination on waterlogged soil and the efficiency of indirect selection for germination ($CR_G/DR_G$) were estimated in the RIL population and the diversity panel. Germination was on the basis of data from waterlogged soil.

<table>
<thead>
<tr>
<th>Traits</th>
<th>RIL population</th>
<th>Diversity panel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$H^2$ $r_G$ CR$_G$/DR$_G$</td>
<td>$H^2$ $r_G$ CR$_G$/DR$_G$</td>
</tr>
<tr>
<td>Germination</td>
<td>0.89 -1.00 -1.10</td>
<td>0.87 -0.90 -0.98</td>
</tr>
<tr>
<td>EC</td>
<td>0.95 0.63 0.67</td>
<td>0.98 0.55 0.59</td>
</tr>
<tr>
<td>Testa colour</td>
<td>1.00 0.67 1.00</td>
<td>1.00 0.61 0.66</td>
</tr>
<tr>
<td>Flower colour</td>
<td>0.99 0.72 1.00</td>
<td>1.00 0.55 0.59</td>
</tr>
<tr>
<td>Axil pigment</td>
<td>1.00 0.70 0.75</td>
<td>1.00 0.55 0.59</td>
</tr>
</tbody>
</table>

CR$_G$ is the correlated response to indirect selection for germination based on secondary traits and DR$_G$ is the direct response to selection for germination.

### 5.5 Discussion

Waterlogging is a major constraint to crop production globally. Genetic variation is prerequisite for any crop to mitigate WL stress, which is predicted to be more frequent and extreme with climate change in temperate-tropical cropping regions (Lobell et al. 2008). In pea variation for WL tolerance at germination has been reported for only three cultivars (Zaman et al. 2018). From these three, the present study identified the extended variation of germination/WL tolerance (5-100%) first to a RIL population from a bi-parental cross and then to a broad germplasm diversity panel. During WL, due to shortage of oxygen (Armstrong & Drew 2002), ATP formation is inhibited (Jackson & Drew 1984) and the oxidation-reduction state between cell membranes becomes unbalanced and membrane permeability is increased. This leads to increased solute leakage (Hsu et al. 2000) (i.e. increased EC.
in current experiments) and decreased germination in our study. Thus, testa integrity is an indirect evaluation of seed vigour. Furthermore, high broad-sense heritability estimates for WL tolerance at germination were found in both the RIL population \( (H^2=0.89) \) and the diversity panel \( (H^2=0.87) \) indicating that most of the variation observed is genetic (Visscher et al. 2008). The frequency distribution of RIL lines for germination under WL showed a continuous variation indicating polygenic control for the trait. This was reinforced by the continuous distribution for WL germination expressed in the diversity panel.

Environmental stress is a powerful force to generate local adaptation through strong directional selection and rapid evolution (Erskine 1997; Hoffmann & Parsons 1997). We found that the germplasm most tolerant to WL was from Africa (i.e. Ethiopia), where peas are generally sown at the start of the rains (mid-June to July) at elevations from 1800 to 3000 m a.s.l. (Telaye 1979; Tsidu 2012). The prevailing temperature at germination in Ethiopia is warmer than at the pea’s domestication region in the Near East where germination occurs during cool wet winter conditions. With the rains in Ethiopia being more intense than those in a Mediterranean winter, the tolerance to WL of Ethiopian genotypes is probably due to their adaptation to excess soil moisture during germination. Similar adaptive potential has been identified in lentil genotypes from Bangladesh, where the crop is often sown onto waterlogged soil in the rice-based cropping system (Malik et al. 2016; Wiraguna et al. 2017). However, such directional selection causes genetic bottleneck in plant breeding, thus we linked WL tolerance to some phenotypic traits.

Testa colour is associated with WL tolerance, for example, dark (red/black/brown) testa genotypes in wheat (Ueno & Takahashi 1997), rapeseed (Zhang et al. 2008) and soybean (Hou & Thseng 1991) are tolerant to WL compared to light (white/yellow) testa genotypes. The difference in tolerance between dark and light testa genotypes is probably due to the levels of phenolic compounds in the testa, as in the rapeseed study dark testa genotypes had higher levels of phenolic compounds than the
sensitive light testa genotypes (Zhang et al. 2008). Higher levels of phenolic or tannin compounds in the testa are considered as a barrier to imbibition, since the dark testa genotypes of pea, fababeans (*Vicia faba* L.) and Arabidopsis (*Arabidopsis thaliana* L.) are restricted in imbibition, whereas light testa is completely permeable to water and subsequent solute leakage (Marbach & Mayer 1974; Kantar et al. 1996; Debeaujon et al. 2000). The present study showed that dark testa genotypes both in RIL population and diversity panel had high percent of germination with lower solute leakage; in contrast, light testa genotypes had low percent of germination and higher solute leakage. Furthermore, genes of wound responsive family protein are highly upregulated in dark testa genotypes in pea during WL stress, which are involved in providing the cell with lignin and phenolic precursors to the wounded surface and provide defense to plants (Zaman et al. 2019). Therefore, it is likely that testa pigmentation plays a protective role against imbibition damage during WL stress. Additionally, in the current study, among RIL population, lines with a dark testa - similar to WL tolerant parent Kaspa - all had pigmented leaf axils and purple/pink flowers, while the light testa lines - similar to sensitive parent BM-3 - had green un-pigmented leaf axils and white flowers. Similarly in the diversity panel, dark testa genotypes predominantly had pigmented leaf axils and purple/pink flowers, whereas the light testa genotypes had non-pigmented axils and white flowers. The exceptions were a few (6%) genotypes with dark testa and pigmented leaf axils but white flowers, indicating that the effects are not pleiotropic. Such exceptions suggest that the loci for the three traits (flower colour, leaf axil pigmentation and testa colour) are linked, and thus any of the traits could be a potential marker/indicator to identify WL tolerance. This finding is consistent with genes for testa and flower colour which are located in the linkage group II reported by Reid and Ross (2011). Similarly, Statham et al. (1972) found that flower colour in *Pisum* is controlled by six major genes, where *A* gene is necessary for general flavonoid production in the plant, and for anthocyanin production in the flowers, axils and pods. However, Mendel observed that colored seed coats were always associated with colored (purple) flowers, and these colored varieties possessed pigmentation in the leaf axils. By
contrast, a colourless testa was always associated with white flowers and the absence of pigmentation in the leaf axils, indicating that these were pleiotropic effects of a single gene. Flower colour and leaf axil pigment are reported for the first time to be associated with WL tolerance in the model crop pea in our study.

Testa integrity is a pre-requisite for germination under waterlogged stress. In the present study, testa integrity measured as EC in submerged solution showed a very strong correlation with germination for both RIL population (r= -0.94) and diversity panel (r= -0.89), indicating that testa integrity might be an effective trait for WL tolerance selection. Visual observation in the RIL population and diversity panel showed that all the genotypes in the tolerant group had intact testa with low EC; in contrast, around 90% genotypes in the sensitive group had dissolved testa with higher EC. This is consistent with sudangrass (*Sorghum sudanense* Stapf) where testa integrity is associated with germination (Hsu et al. 2000). During WL the integrity of testa is lost due to the lipid peroxidation in the testa membrane (Crawford & Braendle 1996) by the two possible pathways- enzymatic and non-enzymatic. In the enzymatic pathway, due to decreased ATP formation during WL stress, different lipid metabolic enzymes such as lipase and lipoxygenase are induced and cause membrane damage (Rawyler et al. 1999) which is supported by the highly upregulated lipid metabolic genes in the sensitive genotype in pea during WL (Zaman et al. 2019). In the non-enzymatic pathway, excessive amount of reactive oxygen species (ROS) are accumulated during WL stress that reacts with lipids in the cell membranes cause oxidative damage and eventually cell death in the testa membrane. As a result of membrane damage in the testa, the electrolytes - in particular potassium ion (K+), along with seeds solutes including sugars and amino acids - are released from seeds (De Vos 1993). Thus, we can use the amount of electrolyte leaked from the seeds as a proxy for the extent of testa leakage and tolerance under waterlogging stress. However, tolerant genotypes control the lipid peroxidation/testa integrity by neutralizing ROS activity in cells by producing increased antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase during waterlogging.
(Kumutha et. al 2009). The concentrations of antioxidants are positively correlated with phenolic compounds (Canola seed, Jun et al. 2014; hazelnuts (*Corylus avellana* L.), walnuts (*Juglans nigra* L.) and pistachios (*Pistacia vera* L.), Arcan & Yemenicioglu 2009); and seeds with dark testa/pigmentation exhibit higher levels of phenolic compounds, lower leakage and higher WL tolerance than the seeds with a lighter coloured testa (Zhang et al. 2008, rapeseed). In the present study 90% dark testa genotypes showed tolerant with intact testa whereas all the light testa genotypes were sensitive with dissolved testa. Therefore, it may be inferred that the antioxidant properties of dark testa seeds are key to testa integrity/WL tolerance in pea.

Peas are a major pulse crop globally, but are more sensitive to WL than other pulses (Solaiman et al. 2007). Predictions of global warming and climate variability in South Asia suggest a change in the inter-annual precipitation pattern - alterations in the intensity of rainfall events, (Sivakumar & Stefanski 2010) and delayed monsoon rains (Li et al. 2017), which could significantly change crop productivity - particularly in pea which is highly sensitive to WL and sown as a relay crop in waterlogged soil. However, as the present study has illustrated the extent of variation in WL tolerance at germination in pea, its polygenic control and the possibilities for indirect selection for WL tolerance, the prospect is raised of adapting the pea, which originates from a rainfed Mediterranean environment, into stable production under relay-sowing in moist soils with rice. We anticipate that selection for WL tolerance at an early stage of crop growth could significantly improve the reliability of relay sowing and help climate-proof production as part of a strategy to enhance productivity in South Asia.

**Acknowledgements**

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5.6 References


### 5.7 Supporting information

Additional supporting information may be found online in the supporting information section at the end of the article- doi: 10.3389/fpls.2019.00953
Chapter 6

General Discussion

Peas (*Pisum sativum* L.) are exposed to waterlogging when grown as relay with monsoonal rice in the rice based cropping system in South Asia particularly in Bangladesh (Ali & Sarker 2013), India and Nepal. Climate change predictions for South Asia indicate alterations to the temporal rainfall events, an increase in inter-annual precipitation variability (Sivakumar et al. 2010) and delayed monsoon rains (Li et al. 2017), which could significantly trigger the possibility of WL in peas sown as relay in damp soil at the end of monsoonal rains. Moreover, extreme rainfall events are expected to increase due to changes in extreme weather and climate events. It is therefore essential to develop tolerant pea cultivars in order to adapt pea to this waterlogged stress environment. However, a long history of domestication and crop improvement has led to a decrease in the overall genetic diversity present within the genepool (Kassa et al. 2012), which also includes traditional farmer varieties and wild related species (Vincent et al. 2013). Reintroducing wild ancestors might be the potential source of tolerance to waterlogging. Different wild soybean accessions showed markedly greater waterlogging tolerance than cultivated cultivar *Glycine max* (Valliyodan et al. 2016). A wide variation in WL tolerance exists in several crops such as lentil (Wiraguna et al. 2017), pigeonpea (Sultana et al. 2013), soybean (Hou & Thseng 1991), maize (Zaidi et al. 2012) and barley (Takeda & Fukuyama 1986). But at the start of this project, there were no information available about the variation of WL tolerance at germination in the model crop pea. The present research has contributed knowledge on (i) the genotypic variation of WL tolerance at germination, (ii) the underlying molecular mechanisms of WL tolerance at germination, (iii) traits associated with WL tolerance at germination and (iv) the genetic basis of WL tolerance at germination in pea. This Chapter has discussed the key traits that had significant contribution to tolerance during WL.
Testa colour is associated with WL tolerance. Dark testa genotypes in several crops showed tolerance to WL while light testa genotypes are WL sensitive, with a few exceptions in both dark and light testa genotypes (Hou & Thseng 1991, soybean; Ueno & Takahashi 1997, wheat; Zhang et al. 2008; rapeseed). The experiments described in Chapters 3 demonstrate higher germination for dark testa genotype Kaspa than the light testa genotype BM-3. However, in pea the other light testa genotype NL-2 showed tolerance to WL with higher percent of germination than sensitive BM-3. Further, in a wider range of germplasm with the association between testa colour and WL tolerance in Chapter 5, the dark testa genotypes were tolerant and the light testa genotypes were sensitive in a RIL population and the diversity panel, however, again a few exceptions were found in both dark and light testa genotypes after 8 d of WL with the lower water level maintained (5 mm below the seed surface). In both RIL population and diversity panel, dark testa genotypes predominantly had pigmented leaf axils and purple/pink flowers, whereas the light testa genotypes had non-pigmented axil and white flower. The genes of flower colour, testa colour and axil pigment are linked and closely located on the same chromosome (Mendel 1866; Reid & Ross 2011). However, there was exception with 6% genotypes in both RIL population and diversity panel which showed dark testa and pigmented leaf axil but white flowers, indicating that the effects are not pleiotropic and more than one genes are associated with pigmentation (Statham et al. 1972). This result clearly suggest that much of variation in WL tolerance associated with testa pigmentation but not all variation, there are other additional factors are involved.

Testa integrity play a major role in preventing cellular damage and solute leakage during imbibition (Larson 1968, pea; Powell & Matthews 1978, pea; Duke & Kakefuda 1981, soybean, navy bean, pea, and peanut; Duke et al. 1983, soybean). However, the role of testa integrity/solute leakage has not been extensively studied in WL tolerance at germination. The experiments described in Chapter 3 showed no visual solute leakage in the most tolerant dark testa genotype Kaspa, however, whitish seed solutes was observed on the surface of the seeds in light testa sensitive BM-3 after 8 d WL. In
Chapter 4 with differential gene expression study, similarly the most tolerant genotype Kaspa had no solute leakage during WL but the sensitive BM-3 again had visual solutes leakage on seed surface. The solute leakage was then confirmed by EC measurement, which was 3-fold higher in sensitive BM-3 than tolerant Kaspa (Chapter 4). The role of testa integrity for WL tolerance was further tested in a RIL population and a wide diversity panel. There was a very strong correlation between EC and WL tolerance. The tolerant genotypes showed visually intact testa and low EC. In contrast, most of the sensitive genotypes showed visually dissolved testa and higher EC. Clearly testa integrity is prerequisite for germination under WL stress which can be used as indirect selection for WL tolerance at germination. The selection of WL tolerance at this early growth of pea could significantly accelerate the breeding of pea for WL tolerance to improve the potential adaptation in relay sowing in waterlogged soil as part of the strategy to increase the reliability and productivity of pea in South Asia. The tolerant genotypes identified from the diversity panel can also be utilized in the cropping system of Australia and other countries where peas are exposed to WL at germination.

In conclusion, the results of the present study indicate that:

- WL causes germination failure in pea when grown on waterlogged soil as relay crop in rice based cropping. However, the genotypic variation of WL tolerance was found at germination in pea (Chapter 3).
- The molecular mechanism of WL tolerance has also been identified by differentially expressed genes where testa colour and testa integrity contributed the key role for WL tolerance (Chapter 4).
- Finally, the experiments with a RIL population and the diversity panel in Chapter 5 showed that only testa integrity or both testa integrity and testa colour/flower colour/axils pigment could potentially be used as indirect selection for WL tolerance at germination in pea.
The findings of the present research looking ahead the following studies:

- Exploration of more sources of genetic variation need to continue to combat more frequent and extreme WL stress under climate change.
- Dark testa genotypes are tolerant with some exceptions being sensitive, further research is required to know the specific phenolic compounds that particularly contributing to tolerance during WL.
- We found lipid metabolism the key mechanism for membrane leakage and subsequent WL damage by phenotyping and differential gene expression, so further research is required in the metabolic level on how much seed stored lipid, carbohydrate and protein are being metabolised during WL stress.
- Further investigation is required for the genes responsible for lipid metabolism and subsequent WL damage.
- The pathways and genes associated with tolerance warrant further validation and are promising potential candidates to develop markers for breeding.
- The RIL population and the diversity panel from this study can be used to map the genomic regions of tolerance, and genetic association between different traits linked to tolerance.
6.4 References


# Appendix

**Table S1.** Accession number, accession name, geographical origin and source of 110 genotypes from diversity panel

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Notes: ETH, Ethiopia; AUS, Australia; USSR, former Soviet Union; S., South; N., North