Seed Priming of Italian Ryegrass (*Lolium multiflorum* L.) with Exogenous Amino Acids: Enhanced Tolerance to Salinity Stress During Germination and Early Seedling Growth

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List of abbreviations, symbols and nomenclature

AAP	Amino acids priming
ABA	Abscisic acid
APX	Ascorbate peroxidase
CAR	Carotenoids
CAT	Catalase
CH	Casein hydrolysate
Chl	Chlorophyll
DHAR	Dehydroascorbate reductase
GA	Gibberellic acid
GB	Glycine betaine
GP	Germination percentage (%)
GR	Glutathione reductase
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
HP	Hydro-priming
IAA	Indole-3-acetic acid
L-met	L-methionine
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase
NO_3^-	Nitrate
NP	Non-priming
NH_4^+	Ammonium
$^1O_2$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical
POD	Peroxidase
P5C	Δ'-pyrroline-5-caboxylate
P5CS	Δ'-pyrroline-5-carboxylate synthetase
P5CR	Δ'-pyrroline-5-carboxylate reductase
ROS	Reactive oxygen species
SA	Salicylic acid
SOD	Superoxide dismutase

Seed priming of Italian Ryegrass (*Lolium multiflorum* L.) with exogenous amino acids: Enhanced tolerance to salinity stress during germination and early seedling growth

Abstract

Climate changes such as erratic rainfall patterns and unpredictable temperatures have brought about environmental stresses, leading to reduction in crop, horticulture, and forage productivities and natural resources in agriculture worldwide. In addition, legacies of anthropognic activities such as contamination and salinisation cause abiotic stress that consequently induces decrease in ariguclaural production. Among the different environmental stresses, salt stress has been an important factor affecting plant growth, which constrains water availability in the soil. As a consequence, high saline soil causes poor seed germination and early seedling growth as well as damages plant cells by inducing generation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2).

It could be expected that New Zealand pasture lands for dairy farming are steadily salinised due to increase in drought duration and excessive irrigation management. This would greatly influence yields of the dairy products and the New Zealand economy. Therefore, in order to maintain sustainable agricultural production, it is necessary to study the resistance of pastoral species to salt tolerance. Italian ryegrass (*Lolium multiflorum*) as a biennial species has been considered to be moderately salt stress resistant and useful for dairy pasture required by cows in New Zealand.

When considering plant response to salinity, seed priming has been used for improving germination performance, uniformity, and stress tolerance. Understanding cellular mechanisms of seed priming would advance seed germination under saline soil. Various materials are used for seed priming, but there is little information relating to seed priming with amino acids under salt stress. Plants utilise synthesis of amino acids for growth and development as well as defence under environmental stress conditions.

Hence, the aims of the present PhD research were to investigate the effect of priming *L. multiflorum* seeds with twenty L-amino acids applied singly as well as casein hydrolysate mainly as a mixture of amino acids on germination and seedling growth under salt stress. When the seedlings were exposed to salt stress, those developed from seeds primed with casein hydrolysate exhibited higher germination percentage, activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), proline level, and biomass than those from control (non-seed priming) and hydro-priming. The hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents were markedly lower in the roots of the seedlings from seeds primed with casein hydrolysate when the seedlings were grown under salt stress compared to those from the control and hydro-priming. Histochemical staining with diaminobenzidine (DAB) and nitro-blue tetrazolium (NBT) revealed less staining in the roots of the seedlings from seeds primed with the casein hydrolysate than control, hydro-priming, and L- and D-methionine priming when the seedlings were grown under salt stress. The beneficial effects of seed priming with casein hydrolysate shown in the laboratory experiments were validated in pot experiments when the plants from the different seed priming treatments were grown in soil added with NaCl solutions under glasshouse conditions. Moreover, in additional glasshouse experiments, there was

evidence that seed priming with casein hydrolysate was also beneficial for the plants exposed to another stressor, namely water stress.

Therefore, the beneficial effect of casein hydrolysate priming could potentially enhance functions of physiological and biochemical response under soil salinity. It is essential that priming of *L. multiflorum* seeds with casein hydrolysate might be potentially considered as an effective priming method leading to improved plant performance under stress conditions.

Keywords: Amino acids, Casein hydrolysate, Climate change, *Lolium multiflorum*, Seed priming, Salt-tolerance

Chapter 1. General introduction and literature review

1.1. General introduction

Since the industrial revolution in 18th century, indiscriminate natural development and population increase have caused environmental changes around the world. In particular, increasing greenhouse gas emission such as carbon dioxide (CO_2) and methane (CH_4) from anthropogenic activities leads to global warming and climate change in a wide range of regions.

In agriculture, global warming and climate change are important variables in securing crop productivity. An increase in local climatic extremes due to changes in rainfall pattern and irradiance increases environmental stress to plants, consequently degrading productivity in agricultural soils. Salinisation is one of the most major environmental stresses to plants linked to climate change (i.e. low precipitation) and intensive irrigation. Salinisation has caused concerns mainly in arid and/or semi-arid regions (Rozema and Flowers, 2008). About 1% of New Zealand terrestrial area is classified as having semi-arid soils found mostly in Canterbury and Otago (Hewitt, 2013). Primary causes of salinisation are due to the weathering process of rocks which release soluble salts such as sodium (Na^+) and calcium (Ca^{2+}) (Hewitt 2013). Also, accumulation of the salts occurs on surface soil by irrigation; salts in subsoil are dissolved by the irrigated water, and then distributed upward back to surface soil (Hewitt, 2013).

In New Zealand as a dairy industry country, pasture productivity including that of Italian ryegrass (*L. multiflorum*) is crucial for the national economy. However, it is expected that recently increasing drought season and salinisation areas have adversely

impacted on production of biomass and seeds of this species. Salt stress appears to be a major constraint to the productivity of crops as it could cause biochemical changes including production of excess reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). In addition, increased soil salinity causes osmotic stress, limiting water use efficiency in plant rhizosphere. It is possible that plant tolerance to salt stress can be generated with antioxidant defence reactions during plant growth.

Seed germination and seedling establishment that are important stages for crop production are most vulnerable under saline conditions. For improving rapid seed germination and seedling establishment upon salt stress, seed priming techniques have been developed. Following priming, seeds can become more tolerant to salt stress as well as exhibit improved germination uniformity and seedling development (Ibrahim, 2016).

Amino acids as the primary products of nitrogen metabolism are induced during abiotic stress. Therefore, there are significantly increased building blocks of protein biosynthesis in plant cells under abiotic stress. Among the 20 common protein L-AAs, a few selected amino acids have been used in experiments for seed priming with the view of improving salt stress tolerance of the seedlings raised from the seeds following seed priming. For example, the effect of cysteine priming of wheat (*Triticum aestivum* L.) seeds was investigated in a previous study (Nasibi et al., 2016). In addition, seeds of mung bean (*Vigna radiata* L.) primed with β-amino butyric acid (BABA) as a non-protein amino acid showed increased salt-tolerance (Jisha and Puthur, 2016). These studies showed that there were higher antioxidant enzyme activities such as ascorbate peroxidase (APX), CAT, SOD, or POD and a decrease of ROS in the seedlings raised from the seeds primed with the respective amino acids.

In general, amino acids are widely involved in cellular reactions that regulate a number of physiological responses such as plant growth and development, intracellular pH control, the metabolic energy demand or redox power in addition to resistance to abiotic stress (Hildebrandt et al., 2015). Hence, the aim of this research was to investigate the effect of priming *L. multiflorum* seeds with 20 L-AAs singly and casein hydrolysate (CH) containing several L-AAs in relation to germination and seedling growth under salinity stress. CH has been used in plant tissue culture medium but not as a seed priming agent before. Besides, it is of interest to better understand how seed priming using L-AA and CH could lead to salt stress tolerance mechanism.

1.2. Objectives

Italian ryegrass has been chosen in this study as this species is economically important pasture production in New Zealand. Increased salinity as well as drought stress due to climate change and/or excessive irrigation could pose significant threats to Italian ryegrass biomass production and seed production. In this proposed research, the main aim was to evaluate the following hypotheses:

(1) Priming of Italian ryegrass seeds with specific L-AAs could induce salt stress tolerance mechanism during germination and early seedling growth.

(2) Italian ryegrass primed with L-AAs would also outperform unprimed seeds or those primed with water only as far as germination rate and seedling vigour under drought stress are concerned. It was hypothesised that the underlying physiological mechanisms of plant tolerance to both salt and drought stress were expected to be highly similar (Tavili et al., 2011). For example, both salt and drought stress might impose water uptake limitation and induce oxidative stress (Jisha et al., 2013) during early seedling

growth (crop stand establishment). Priming seeds with specific L-AA may alleviate osmotic and oxidative stress during early seedling growth.

In this research, Italian ryegrass seeds were primed with each of the 20 common L-AAs, CH as a mixture of AAs, and D-methionine to determine if any AAs would be useful as a suitable priming agent for protecting Italian ryegrass seedlings under high salt stress. The possible involvement of AAs priming-induced antioxidative defence mechanisms for alleviation of NaCl stress on seed germination and early seedling growth was also be investigated.

More on the organisation and objectives of the PhD research project

Objective 1. Identification of influence of priming Italian ryegrass (*Lolium multiflorum* L.) on seedling growth and salt tolerance **[Chapter 3]**

Seed priming techniques could enhance plant growth and tolerance to various forms of environmental stress including salinity. Also, exogenous application of amino acids during plant growth could play a beneficial role in improvement of metabolic and antioxidant systems in plants. This experimental chapter aimed to investigate the effectiveness of priming *L. multiflorum* seeds with each of the 20 L-amino acids and casein hydrolysate in germination rate and radicle establishment of *L. multiflorum* under salt stress. All the experiments were carried out in Petri dishes under laboratory conditions.

Objective 2. Investigation of effects of seed priming with L-methionine and casein hydrolysate on abiotic stress tolerance mechanism in *L. multiflorum* seedlings under salt stress **[Chapter 4]**

Based on the results in Chapter 3, casein hydrolysate and L-methionine were selected as effective regents for seed priming. In addition to germination and radicle length, seed priming with casein hydrolysate and L-methionine could lead to activating activities of antioxidant enzymes (SOD, CAT, and POD) and reducing ROS accumulation in the seedling roots. The objective of this experimental chapter was to investigate effects of priming *L. multiflorum* seeds with casein hydrolysate and L-methionine on alleviating salinity-induced oxidative stress in the seedling roots in the different seed priming treatments. All the experiments were carried out in Petri dishes under laboratory conditions. Also, to see difference between the effects of L-and D-amino acids, D-methionine was included part of this investigation.

Objective 3. Investigation of effects of seed priming with casein hydrolysate and L-methionine on abiotic stress tolerance mechanism in *L. multiflorum* seedlings in salt-affected soil **[Chapter 5]**

Results of the previous microcosm study in Chapter 4 showed that seeds of *L. multiflorum* primed with casein hydrolysate and L-methionine exhibited significant enhancement of germination capacity, radicle elongation, and tolerance mechanism to salt stress during four days after germination. This chapter study aimed to confirm the efficiency of seed priming with casein hydrolysate and L-methionine in physiology and productivity of the Italian ryegrass grown in salt-affected soil through pot experiment in greenhouse.

Objective 4. Investigation of effects of seed priming with casein hydrolysate on biomass production of *L. multiflorum* seedlings under drought stress **[Chapter 6]**

Drought causes inhibition of plant water uptake, which reduces crop growth and yields. Indeed, plant stress in saline soil can be increased by drought due to high temperature and water evaporation. The objective of this chapter was to reveal a potential benefit of casein hydrolysate used in seed priming on biomass production of *L. multiflorum* in response to drought stress via a greenhouse experiment.

1.3. Literature review

1.3.1. Salinity stress

Urbanisation and industrialisation have resulted in increase of global population in the past two centuries. The global increase in human population requires more food production (Yeo, 1999). However, global warming, rainfall pattern change, and pollution, which have continuously featured in the 21st century, are causing difficulties in agriculture. Elevated atmospheric CO_2 concentration due to de-forestation and anthropogenic fossil fuel combustion is attributed to an increasing global average temperature and shifts in rainfall pattern. Such environmental changes have significantly influenced sustainable agriculture (Ahuja et al., 2010). Thus, crop plants must overcome abiotic stresses relative to the climate change such as drought and salinity (Ahuja et al., 2010).

Occurrence of salinisation in soils related to climate change has brought about reduction in crop yield (Kang and Banga, 2013; Munns and Gilliham, 2015), indicating that cropping would be impossible in agricultural land if climate change was to continue unchecked. Indeed, secondary salinisation resulted from anthropogenic activities has immensely increased degree of salinity. Salt stress is predicted to worsen plant life cycle, which leads to breakdown in metabolic homeostasis during all major processes of plant

growth such as germination, seedling establishment, and reproduction and plant metabolism such as photosynthesis, repiration, nutrient assimiliation, and hormonal balance (Parihar et al., 2015). In particular, seed germination and seedling establishment are considerably sensitive to salt stress, leading to reduction in crop production (Ibrahim, 2016). Reduced food production due to salinity causes farmer's income and agricultural economy to deteriorate. The global loss in crop production due to salinity is annually more than US 12 billion dollars, which is gradually increasing (Bose et al., 2014). Thus, growing salt-tolerant crops or grasses at the early growth stages is necessary for increasing crop yields and enlarging effectiveness land usage in saline regions. In addition, some strategy of increasing salt-tolerant crops is needed for agricultural and pasture production in marginal land with high salinity.

1.3.2. Origins of increased soil salinity

Salt stress has been the most widespread in arid or semi-arid regions as well as coastal wetland and inland. It is estimated currently that about 1 billion ha of more than 100 countries and about 23% of cultivated land around the world are salt-affected (FAO and ITPS, 2015; Zhang et al., 2016). Increased soil salinity in agricultural land generally may result from two different original sources (Munns and Tester, 2008; Parihar et al., 2015). Firstly, soil salinity may result from natural processes including accumulation of salts via weathering parent materials or deposing oceanic salt carried by wind and rainfall for a long time. These processes release various types of soluble salts, which are mainly NaCl and Na_2SO_4 in saline soil (Parihar et al., 2015). Also, significant amount of sodium (Na^+), calcium (Ca^{2+}), potassium (K^+), and magnesium (Mg^{2+}) as chlorides, sulfates, or bicarbonates are distributed in some saline soils (Masters et al.,

2007). Secondly, salinisation is caused by anthropogenic activities such as irrigation, land-clearance, or disposal of wastewater effluents (Herbert et al., 2015). Of the 230 million ha which is the global irrigated land, 45 million ha (about 20%) is affected by salinity (Parihar et al., 2015). Although only 15% of the global cultivated lands is irrigated, about one third of the world's food is produced from the irrigated land (Parihar et al., 2015). Additionally, elevated evapotranspiration due to climate change (i.e. drought) lowers water potential in soil and accelerates salinisation, inhibiting crop growth especially in dry regions (Kang and Banga, 2013).

1.3.3. Drought stress

Drought is one of the major constraints restricting agricultural production around the world. It is expected that frequency and severity of drought will steadily increase over time as consequences of decreasing local precipitation and increasing evaporation by global warming (Sheffield et al., 2012). Additionally, unpredictable global climate change, increase of anthropogenic activity, and water pollution have gradually exacerbated shortage of water resource, which would subsequently lead to drought stress in the environment (Fang and Xiong, 2015; Trenberth et al., 2014). Indeed, more than 80% of the freshwater used by humans is used as agricultural water (Morison et al., 2008). Thus, water scarcity due to such environmental changes adversely affects food security worldwide. Water scarcity has been deleterious to plant growth, development, and reproduction, which leads to devastating crop yields and food security (Fang and Xiong, 2015; Farooq et al., 2009). In addition, since drought causes soil erosion, non-sustainable cultivated land (i.e. desertification) and ecological damage, it contributes to enormous economic losses in agriculture (Fang and Xiong, 2015).

Depending on the extent of drought stress, below average rainfall is more aggravated to survival of plant species. Indeed, plant growth stages including germination, vegetative, flowering, and seed filling are susceptible for drought stress (Farooq et al., 2009). Thus, many plant species cannot cope well with severe water deficiency and drought in soil.

In general, drought stress hampers water uptake from the root system and negatively influences transpiration stream leading to stomatal closure in leaves (Anjum et al., 2011). Response of plants to drought stress has been studied for more than two decades at different levels including morphological, structural, physiological, biochemical and molecular traits in plants (Fang and Xiong, 2015). Plants exposed to drought stress differently have various adaptative responses depending on plant species and developmental stages, which regulate physio-biochemical mechanism including osmolyte and antioxidant defence mechanism (Anjum et al., 2011). For example, plants under drought stress have tolerance by generating compatible solutes such as accumulation of proline, soluble sugars, glycine betaine, ions of calcium and potassium in plant cells and by detoxifying ROS for maintaining their cell turgor pressure (Farooq et al., 2009). Additionally, a large number of antioxidant enzymes including SOD, APX, CAT, and glutathione reductase (GR) are often observed in drought-stressed plants (Fang and Xiong, 2015). With these stress signal mechanisms, effects of drought stress may be counteracted resulting in drought tolerance.

1.3.4. The effect of salinity stress during seed germination and early seedling growth

Salt stress adversely affects seed germination and seedling growth, involving osmotic and oxidative stress and ion toxicity such as Na^+ and Cl^-, and nutrient imbalance (Jahromi et al., 2008; Parihar et al., 2015). Salt stress causes a low osmotic potential and therefore a decrease in water uptake by seeds and seedlings (Farooq et al., 2015; Parihar et al., 2015). Consequently, this is accompanied by accumulation of excessive salts and then the plants are withered. In particular, influence of the toxicity of Na^+ and Cl^- ions results in loss of seed viability as well as destruction of enzymes and macromolecular structures, damage of organelles, and plasma membranes within seeds and seedlings (Farooq et al., 2015; Jahromi et al., 2008). As a result, salt stress negatively affects germination, growth rate, respiration, photosynthesis, protein synthesis and lipid metabolism (Farooq et al., 2015; Jahromi et al., 2008).

Seed germination and seedling emergence are susceptible to salinity (Ashraf and Foolad, 2005). In regions of hot and dry climate, there is a significantly higher evaporation for water loss from soil surface. This deleterious phenomenon stimulates salinisation around the rhizosphere. Seeds are generally sown within 10 cm deep from the soil surface, in which a lot of salts would be accumulated relative to the lower layer of the soil (Ibrahim, 2016). In saline soil, seeds may show non-uniform germination rate and seedling growth deterioration. For instance, canola (*Brassica napus* L.), okra (*Abelmoschus esculentus* L.), maize (*Zea mays* L.), radish (*Raphanus sativus* L.), cabbage (*Brassica oleraces capitata* L.), mustard (*Brassica juncea* L.), water spinach (*Impomoea aquatic* L.), celery (*Apium graveolens* L.), fennel (*Foeniculum vulgare* L.), parsley (*Petroselinum crispum* L.), cowpea (*Vigna unguiculata* L.) and milk thistle (*Silybum marianum* L.) showed low germination rates under salinity (Bybordi, 2010;

Dkhil et al., 2014; Khodarahmpour et al., 2012; Sarker et al., 2014; Soliman and El-Shaieny, 2014; Thiam et al., 2013; Zavariyan et al., 2015). Since seed germination as well as seedling establishment are important in crop production, it is crucial to have techniques for improving tolerance of crop species during germination and seedling establishment in salt-affected agricultural land.

1.3.5. Reactive oxygen species (ROS) production in response to salinity

Reactive oxygen species are formed in aerobic metabolism occurring in the chloroplasts, mitochondria, peroxisomes by conversion of molecular oxygen (O_2) (Das and Roychoudhury, 2014). About 1-2% of O_2 consumption is contributed toward production of ROS in plant tissues (Gill and Tuteja, 2010). ROS comprise superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) (Das and Roychoudhury, 2014; Gill and Tuteja, 2010). $O_2^{\bullet-}$ is the first ROS produced by O_2 reduction in photosystem I (PS I) by the Mehler reaction in thylakoid membrane and the $O_2^{\bullet-}$ is transformed to more reactive and toxic $OH^{\bullet}$ and $^1O_2$, which lead to lipid peroxidation (Bose et al., 2014; Das and Roychoudhury, 2014). $^1O_2$ produced during photosynthesis severely damages to PS I and II, as well as the whole photosynthesis-associated organ (Gill and Tuteja, 2010).

Salt and drought stresses induce stomatal closure and low intercellular CO_2 level in the chloroplasts, which contribute to production of the $^1O_2$ (Gill and Tuteja, 2010). H_2O_2 is generated from univalent reduction and protonation of $O_2^{\bullet-}$ mostly through superoxide dismutase (SOD) catalysis (Das and Rochoudhury, 2014; Gill and Tuteja, 2010). H_2O_2 at a low concentration in plant cells plays a beneficial role in regulating senescence, photorespiration, photosynthesis, stomatal movement, cell cycle, and growth and development. H_2O_2, is known to inhibit a variety of enzymes activities such

as Calvin cycle enzymes, Cu/Zn SOD, and Fe-SOD. It could also oxidise residues of cysteine (-SH) and methionine (-SCH_3), and cause plant cell death at high concentrations (Das and Rocyoudhury, 2014). $OH^{\bullet}$ is the most reactive and active toxic ROS, which is produced from H_2O_2 and $O_2^{\bullet-}$ by a Fenton reaction including Fe^{2+} and Fe^{3+} (Das and Roychoudhury, 2014; Pospíšil, 2016). Hydroxyl radicals cause damage in all cellular components such as DNA, proteins, lipids, and membranes and at high concentration could lead to cell death due to the absence of an effective enzymatic system for scavenging the radicle (Das and Rochoudhury; 2014; Gill and Tuteja, 2010).

ROS are constantly being produced and scavenged by a variety of antioxidant defence mechanisms at a basal level in plant cells during growth upon favorable growth conditions. Also, ROS can act as signaling molecules mediating tolerance to salt stress as well as developmental processes such as root hair growth (Zhang et al., 2016). However, it can be elevated to deleterious levels under severe environmental stresses including salinity that cause protein degradation, DNA damage, lipid peroxidation and cellular damage (Gill and Tuteja, 2010). The elevated ROS induced by salt stress could inhibit redox metabolism, particularly disrupting balance between ROS generation and ROS scavenging in organelles and cellular compartments (Cunha et al., 2016; Gill and Tuteja, 2010). Therefore, a drastic rise of ROS could inhibit radicle emergence during germination and seedling root growth under salinity stress (Manai et al., 2014). For example, under salinity stress (150 mM NaCl), the amount of ROS was considerably increased in the root of *Arabidopsis thaliana* (Boursiac et al., 2008). Roots of *Panicum miliacium* and *P. sumatrense* exhibited an increase in malondialdehyde (MDA) content under severe salinity (200 mM NaCl) (Bhaskaran and Panneerselvam, 2013). Broccoli (*Brassica oleracea* L.) roots had higher levels of H_2O_2 and MDA under 80 mM NaCl stress (Hernandez et al., 2010).

However, opposite to these results, there are several exceptional examples of decreasing ROS such as $O_2^{\bullet-}$ and H_2O_2 contents in the roots with increasing NaCl concentrations (Table 1.1). The antioxidant enzyme activities such as APX, SOD, CAT, or POD, etc. in these studies might be sufficient to detoxify salt stress-triggered toxic ROS for maintaining redox homeostasis under salinity stress.

Table 1.1. Examples of decreasing reactive oxygen species ($O_2^{\bullet-}$ or H_2O_2) contents in plant roots response to salt stress.

Plant species	NaCl concentration	References
Tomato (*Lycopersicon pennellii* L.)	100 mM	Mittova et al. (2004)
Tomato (*Solanum lycopersicon* L.)	120 mM	Manai et al. (2014)
Barley (*Hordeum vulgare* L.)	200 mM	Kim et al. (2005)
Rice (*Oryza sativa* L.)	7 and 14 dS m^{-1}	Mishra et al. (2013)
	150 mM	Cunha et al. (2016)

1.3.6. Antioxidant enzymes

Plant species have antioxidant defence mechanism within plant tissue against salinity stress condition (Gill and Tuteja, 2010). For regulating oxidative stress triggered under salt stress, plant species have several antioxidant enzymes including SOD (EC. 1.15.1.1), CAT (EC. 1.11.1.6), POD (EC. 1.11.1.7), APX (EC. 1.1.11.1), monodehydroascorbate reductase (MDHAR (EC. 1.6.5.4)), dehydroascorbate reductase (DHAR (EC. 1.8.5.1)), and GR (EC. 1.6.4.2) which detoxify ROS (Das and Roychoudhury, 2014). These enzymes are localised in the different subcellular compartments including chloroplasts, mitochondria, and peroxisomes.

1.3.6.1. Superoxide dismutase (EC. 1.15.1.1)

Superoxide dismutase (SOD) enzyme converts $O_2^{\bullet-}$ into H_2O_2 and O_2. Upregulation of SOD plays a role in controlling/regulating (is responsible for) plant survival for reducing oxidative stress produced by abiotic stress environment (Das and Roychoudhury, 2014). SOD occurs as different forms of isoenzymes. The isozyme structure is based on the metal ion associated with the enzyme protein (Bose et al., 2014). Fe/SOD is localised mainly in the chloroplasts. Mn/SOD is localised in mitochondria and peroxisomes, while Cu/Zn SOD is localised in the cytosol and chloroplasts. SOD enzyme activity acts as 'the first defence line' to alleviate a wide range of oxidative stresses (Alscher et al, 2002; Bose et al., 2014). For example, elevated SOD enzyme activity under salinity stress was shown in seedlings of canola (*Brassica napus*), chickpea (*Cicer arietinum*), foxtail millet (*Setaria italica*), and maize (*Zea mays*) (Ashraf and Ali, 2008; Eyidogan and Öz, 2007; Kukreja et al., 2005; Sreenivasulu et al., 2000; Tuna et al., 2008). Yang et al. (2008) found that Dragon spruce (*Picea asperata*) seedlings had an increase in SOD enzyme activity under combined effect of drought and high light stress. Tomato

(*Lycopersicon esculentum*) leaves under heat stress at (45˚C) had a higher activity of Fe/SOD (Camejo et al., 2007).

1.3.6.2. Catalase (EC. 1.11.1.6)

Catalase (CAT) is responsible for the conversion of H_2O_2 into O_2 and H_2O mainly in peroxisomes (Bose et al., 2014), as by-products due to photorespiration, fatty acid β-oxidation, purine catabolism, and oxidative stress (Das and Rocyoudhury, 2014). However, CAT is also found in cytosol, chloroplasts, and mitochondria. CAT has an indispensable capacity to detoxify ROS caused by abiotic stress. It can convert about 6 million molecules of H_2O_2 into O_2 and H_2O per minute (Gill and Tuteja, 2010). There are various experimental studies that showed enhanced CAT enzyme activity in plant tissue under environmental stresses. For example, seedlings of canola (*B. napus*), chickpea (*C. arietinum*), and sea plantain (*Plantago maritima*) showed a higher CAT enzyme activity under salinity stress (Ashraf and Ali 2008; Eyidogan and Öz, 2007; Kukreja et al., 2005; Sekmen et al., 2007). Dragon spruce (*P. asperata*) had an increase in CAT enzyme activity under a combined effect of drought and light stress (Yang et al., 2008). Under cadmium stress, additionally, increased CAT activity in rice (*Oryza sativa*) and mustard (*B. juncea*) was reported by Hsu and Kao (2004) and Mobin and Khan (2007).

1.3.6.3. Peroxidase (EC. 1.11.1.7)

Peroxidase (POD) is a family of isoenzymes consisting of 40-50 kDa monomers, which is capable of scavenging excess H_2O_2 production during normal and abiotic stress conditions in the apoplastic space of plants (Bose et al., 2014; Das and Rochoudhury, 2014). POD enzyme uses aromatic compounds such as guaiacol and pyragallol as electron donors (Das and

Roychoudhury, 2014). This enzyme decomposes indol-3-acetic acid (IAA) and removes H_2O_2, which plays an important role in catalysing lignin biosynthesis and scavenging oxidative stress under unfavorable/stressed conditions (Das and Rochoudhury, 2014; Gill and Tuteja, 2010). For example, enhanced POD enzyme activity in response to salt condition was found in plants of canola (*B. napus*), chickpea (*C. arietinum*), and maize (*Z. mays*) (Ashraf and Ali, 2008; Kukreja et al., 2005; Tuna et al., 2008). Hsu and Kao (2004) noted that rice (*O. sativa*) had an increase in POD activity under Cd-stressed condition.

1.3.6.4. Ascorbate peroxidase (EC. 1.1.11.1)

Ascorbate peroxidase (APX) is a vital component of the ascorbate-glutathione (ASC-GSH) cycle, which scavenges H_2O_2 mainly in the cytosol and chloroplast by converting to H_2O and dehydroascorbate (DHA) using ascorbic acid (AA) (Das and Roychoudhury, 2014). A family of APX comprises of different isoforms: thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), chloroplast stromal soluble form (sAPX), and cytosolic form (cAPX) (Gill and Tuteja, 2010). APX has a greater capacity for scavenging H_2O_2 caused by abiotic stress since it has a better affinity for H_2O_2 compared to CAT and POD enzymes (Das and Roychoudhury, 2014; Gill and Tuteja, 2010). Increased activity of APX activity was found in salt-stressed plants including tomato (*L. pennelli*), sea plantain (*P. maritima*), and foxtail millet (*S. itallica*) (Mittova et al., 2004; Sekmen et al., 2007; Sreenivasulu et al., 2000).

1.3.6.5. Monodehydroascorbate reductase (EC. 1.6.5.4)

Monodehydroascobate reductase (MDHAR) plays a role in regenerating AA from the short lived monodehydro ascorbate (MDHA), which uses NADPH as a reduction agent (Das and Roychoudhury, 2014). Like APX scavenging H_2O_2, this enzyme is localised in the

peroxidsomes and mitochondria (Gill and Tuteja, 2010). There are several isozymes of MDHAR that are found in the chloroplast, cytosol, glyoxysomes in addition to the peroxidsomes and mitochondria (Das and Roychoudhury, 2014).

1.3.6.6. Dehydroascorbate reductase (EC. 1.8.5.1)

When MDHAR is unsuccessful to reduce MDHA to AA, DHA is produced (Miller et al., 2010). However, dehydroascorbate reductase (DHAR) can act as reducing DHA to AA, using reduced glutathione (GSH) as an electron donor (Miller et al., 2010). This enzyme is found in all plant organs such as seeds, roots, and either green and etiolated shoots (Das and Roychoudhury, 2014). DHAR plays a role in maintaining redox homeostasis of the plant cells by regulating the AA pool size within the cytosol and apoplast (Chen and Gallie, 2006; Das and Roychoudhury, 2014). Therefore, over-expression of DHAR would enhance plant tolerance to a variety of abiotic stress (Gill and Tuteja, 2010).

1.3.6.7. Glutathione reductase (EC. 1.6.4.2)

Glutathione reductase (GR) is a flavoprotein oxidoreductase which is responsible for reducing oxidised glutathione (GSSG) to GSH using NADPH as a reductant (Das and Roychoudhury, 2014). This enzyme is localised in chloroplasts, and a small amount is also found in cytosol, and mitochondria (Gill and Tureja, 2010). GR is one of important enzymes involved in ASC-GSH cycle that sustains a ratio of higher cellular GSH/GSSG by catalysing the formation of disulfide bond (S_2^{2-}) in glutathione disulfide (Das and Roychoudhury, 2014). By maintaining the reduced status of GSH, GR plays a role in regulating antioxidant mechanism to ROS caused by abiotic stresses (Gill and Tuteja, 2010). It is known that GSH, as a non-enzymatic antioxidant, has a function to determine plant tolerance under various abiotic stresses by

scavenging ROS (Das and Roychoudhury, 2014). Eyidogan and Öz (2007) and Kukreja et al. (2005) reported that seedlings of *C. arietinum* showed an increase in GR enzyme activity in response to salt stress.

1.3.7. Non-enzymatic antioxidants

1.3.7.1. Proline

Proline has a protective function in plants against a variety of environmental stresses such as drought, salinity, heavy metals, and extreme temperatures, etc. (Verbruggen and Hermans, 2008). It is considered as a non-enzymatic antioxidant to scavenge $OH^{\bullet}$ and $^1O_2$ caused by abiotic stress. Also, proline plays beneficial roles as a compatible osmolyte, a protein stabiliser, a redox homeostasis, a metal chelator, and a sub-cellular structure stabiliser under unfavorable conditions (Ashraf and Foolad, 2007; Gill and Tuteja, 2010; Iqbal et al., 2014). Under salt or drought stresses, proline is generally accumulated in cytosol, in which it mediates the cytoplasmic osmotic adjustment (Ashraf and Foolad, 2007).

Proline synthesis in plants occurrs mainly via the reductive pathway of glutamate pathway (Fig. 1.1) (Iqbal et al., 2014; Szabados and Savouré, 2010). Firstly, glutamate is reduced to glutamate-semialdehyde (GSA) by pyrroline-5-carboxylate synthetase (P5CS) enzyme and spontaneously reduced to pyrrolidine-5-carboylate (P5C). Then, the P5C is converted to proline by pyrroline-5-carboxylate reductase (P5CR) enzyme. Increase of proline biosynthesis contributes to maintaining a low ratio of NADPH: $NADP^+$, which results in reduction of $^1O_2$ generation from PSI degrading photoinhibition, and stabilising redox homeostasis (Bose et al., 2014; Szabados and Savouré, 2010).

Elevated proline content in plant tissues under salinity and drought stresses has been reported by previous studies: shoots of *Agrostis stolonifera* (Ahmad et al., 1981); roots and leaves of durum wheat (*Triticum durum* Desf.) (Bouthour et al., 2015); roots of alfalfa (*Medicago sativa* L.) (Fougère et al., 1991); embryogenic callus of lemon (*Citrus limon* L.) (Piqueras et al., 1996); roots and leaves of mustard (*B. juncea* L.) (Rais et al., 2013); root apical meristem of maize (*Z. mays*) (Ober and Sharp, 1994; Voetberg and Sharp, 1991).

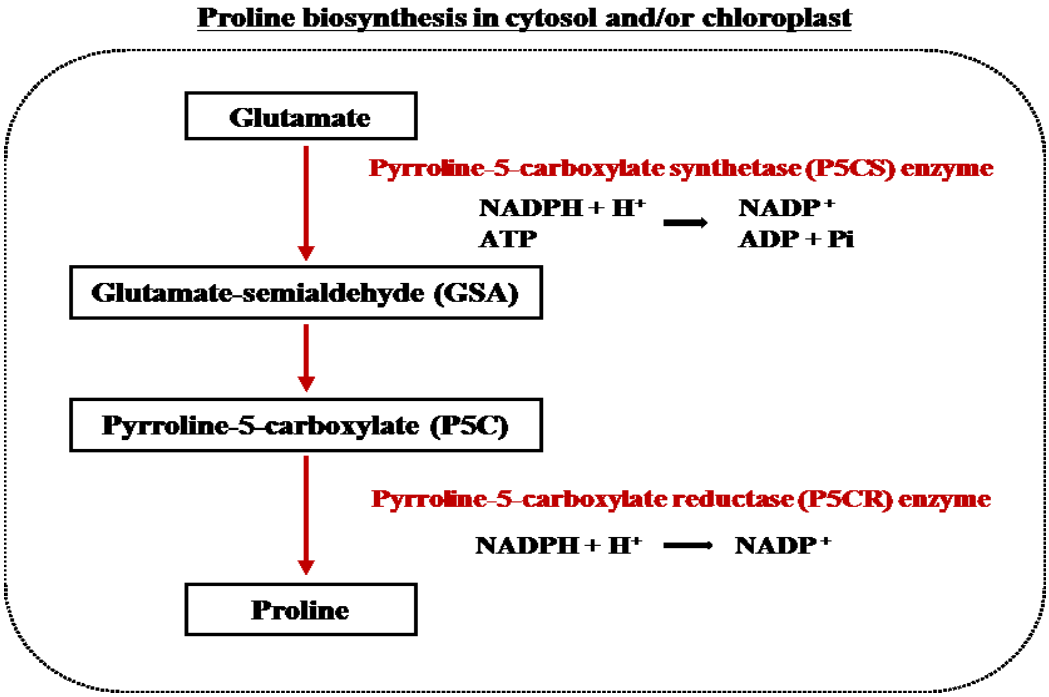


Fig. 1.1. Proline biosynthesis pathway in the cytosol and/or chloroplast (modified from Szabados and Savouré, 2010).

1.3.7.2. Carotenoids

Carotenoids (CAR) are involved in a group of antennae molecules, which have a function to absorb light from the solar emission spectral region (450-570 nm) and then translocate the light energy to chlorophyll molecule (Das and Roychoudhury, 2014). CAR also have an antioxidant function to protect the photosynthetic apparatus from photo-oxidative stress, by deactivating

triplet chlorophyll (^{3}Chl) and $^1O_2$ naturally produced in the photosynthetic systems and dissipating the excess excitation energy through the xanthophyll cycle (Das and Roychoudhury, 2014; Gill and Tuteja, 2010; Jahns and Holzwarth, 2012; Telfer, 2014) (Fig. 1.2). Additionally, they contribute to stabilising structures of light harvesting complex proteins and thylakoid membrane (Gill and Tuteja, 2010). However, when plants are exposed to severe abiotic stress, homeostasis is disrupted, including interrupting the photosynthetic process. Subsequently, oxidative damage occurrs in plant membranes and proteins. For example, a significant reduction in CAR content in perennial ryegrass (*Lolium perenne* L.) was found under the treatment of heavy metals such as Pb and Cd (Bai et al., 2015; Wang et al., 2013b). Under salt stress, seedlings of tomato (*L. esculentum* Mill.) and wheat (*T. aestivum* L.) had a considerable decrease in CAR content (Doganlar et al., 2010; Qiu et al., 2014). However, enhanced salt tolerance in the wheat seedlings following exogenous treatment of jasmonic acid had increases in antioxidant enzymes and CAR content (Qiu et al., 2014). It may indicate that a high content of CAR played an antioxidant role by detoxifying $^1O_2$ and lipid peroxidation products caused by the oxidative stress (Bose et al., 2014; Gill and Tuteja, 2010).

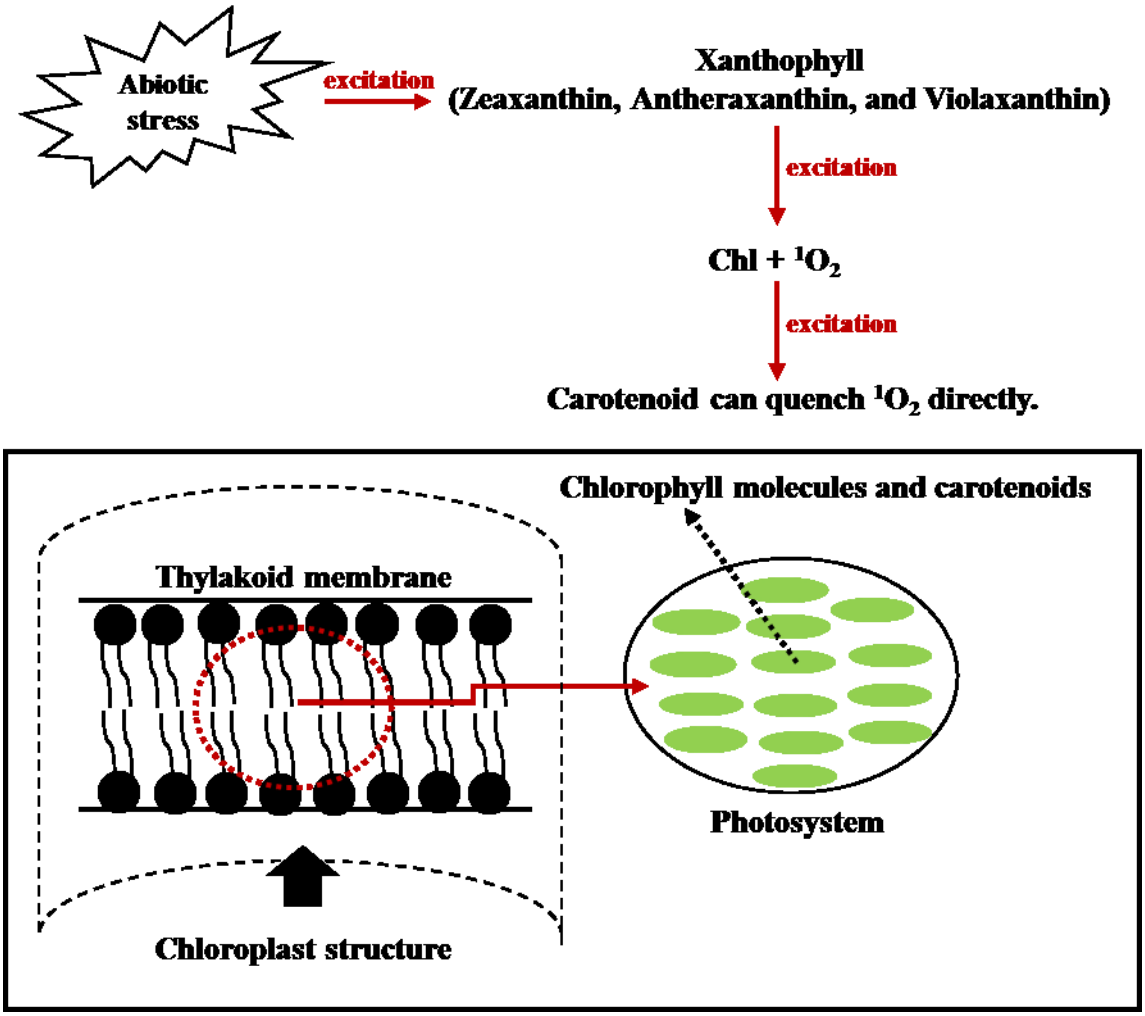


Fig. 1.2. Response of chlorophylls and carotenoids in the chloroplast under abiotic stress.

1.3.7.3. Reduced glutathione (GSH)

Glutathione as a tripeptide form (γ-glutamyl-cysteinyl-glycine) is one of essential metabolites in plants and is found in all the cell compartments such as cytosol, endoplasmic recticulum, vacuole, mitochondria, chloroplasts, peroxisomes, and apoplast as reduced form (GSH) (Ashraf, 2009; Gill and Tuteja, 2010). GSH has functions not only to scavenge ROS (i.e. $^1O_2$, H_2O_2, $OH^{\bullet}$, and $O_2^{\bullet -}$), but also to regulate plant growth and development including differentiation, death, and senescence of cell, enzyme regulation, and pathogen resistance (Das and Roychoudhury, 2014; Gill and Tuteja, 2010). Also, GSH regenerates AA to produce oxidised glutathione (GSSG). In combination with GSSG, GSH plays a crucial role for maintaining redox balance in the cellular compartments under normal or stress conditions (Gill and Tuteja, 2010; Miller et al., 2010). A high ratio of GSH/GSSG in plants was exhibited in the shoots of frost-tolerant wheat (*T. aestivum*) under cold stress (Kocsy et al., 2002) and leaves of *Myrothamnus flabellifolia* under drought stress, respectively (Kranner et al., 2002).

1.3.7.4. α-Tocopherol

α-tocopherol belongs to a group of vitamin E compounds and acts as a superior scavenger of $^1O_2$ and lipidperoxyl radicals, predominantly in the chloroplast of green plant tissues (Bose et al., 2014). By resonance energy transfer, up to 120 singlet oxygen can be scavenged with one molecule of the α-tocopherol (Gill and Tuteja, 2010). It is only synthesised by photosynthetic organisms such as plants (i.e in plastid envelope) and is then stored in plastoglobuli of the stroma and thylakoid membranes in the chloroplast (Bose et al., 2014; Das and Roychoudhury, 2014). Previous studies revealed an increase of α-tocopherol in tolerant species of plants in response to the environmental stresses such as salinity and heavy metals (i.e. Cu) (Ellouzi et al., 2011; Srivastava et al., 2005; Yu et al., 2011).

1.3.7.5. Flavonoids

Flavonoids are generally found in leaves, floral organs, and pollen grains of most plants. Based on their structures, they are distinguished as flavonols, flavones, isoflavones, and anthocyanins (Das and Roychoudhury, 2014). Flavonoids have multiple roles in providing pigmentation in flowers, fruits, and seeds related to plant fertility and germination of pollen, protecting against plant pathogen and UV light, and acting as signal molecules in interaction of plant-microorganisms (Gill and Tuteja, 2010). Like α-tocopherol, additionally, flavonodis act as antioxidants against ROS such as $^1O_2$ in the chloroplast membrane and also alleviate the damage of the photosynthetic apparatus due to the excess excitation energy (Das and Roychoudhury, 2014; Gill and Tuteja, 2010).

1.3.8. Seed priming for protection of salinity stress

1.3.8.1. History of seed priming

An initial attempt to enhance seed germination backs to the Greek ancient times. Theophrastus (371-287 B.C.) as a philosopher and scientist revealed that using cucumber (*Cucumis sativus* L.) seeds imbibed in water before sowing germinated faster (Paparella et al., 2015). In the Roman, naturalist Gaius Plinius Secunds (A.D. 23-79) investigated the positive effect of pre-soaking cucumber seeds in water and honey on germination. Afterwards, Oliver de Serres (1539-1619) as a French agronomist and botanist reported that the effectiveness of seed soaking in water and drying in the shade prior to sowing on cultivation of grains (including *Triticum*, *Secale,* and *Ordeum* spp.). In the 19th century, Charles Darwin developed an osmo-priming technique using salty sea water, which improved seed germination of cress (*Lepidium sativum* L.) and lettuce. A modern concept of the seed priming was established since 1960s, via some

studies including Ells (1963) and May et al. (1962). Indeed, a large number of studies have shown that pretreatment of both imbibing and drying, which induced seed hardening or advancing, did facilitate seed germination of various crop species under unfavorable conditions (Paparella et al., 2015). The benefits of seed priming under environmental stresses are reviewed by Ibrahim (2016) and Jisha et al. (2013). Currently, many agri-seed companies utilise seed priming as a tool to enhance seed vigour and germination rate as well as try to identify proper priming agents to improve plant tolerance to stress in the field (Jisha et al., 2013; Paparella et al., 2015).

1.3.8.2. Seed priming process

Seed priming is a pre-sowing treatment in a certain solution which involves natural or synthetic compounds (Jisha et al., 2013). This seed treatment potentially influences seed moisture content, which regulates an initial physiological process of seed germination. Thus, seed priming improves speed, rate, and consistency of seed germination. Fig. 1.3 shows a general seed priming process which comprises of three steps (Rajjou et al., 2012). Seed priming firstly involves imbibing seeds in a test solution for a selected time to allow water uptake into the seeds. In this step, for proper seed hydration, it should be prevented from reaching the moisture level which is required for actual germination (Ibrahim, 2016; Rajjou et al., 2012). Secondly, during the lag (or activation) phase, the water status of the primed seeds is maintained stably. At this time, the seeds prepare metabolic activation related to energy metabolism and restore cell level (Ibrahim, 2016; Lutts et al., 2016). Lastly, before radicle protrusion, the primed seeds are removed from the solution and re-dried back to their original moisture content. Drying the seeds is an essential process to maintain the efficiency of seed priming and seed quality (Ibrahim, 2016). The dehydrated seeds are ready to be stored prior to sowing in a field.

Conditions of both drying-back and storage are determinants for longevity of the primed seeds (Butler et al., 2009; Ibrahim, 2016).

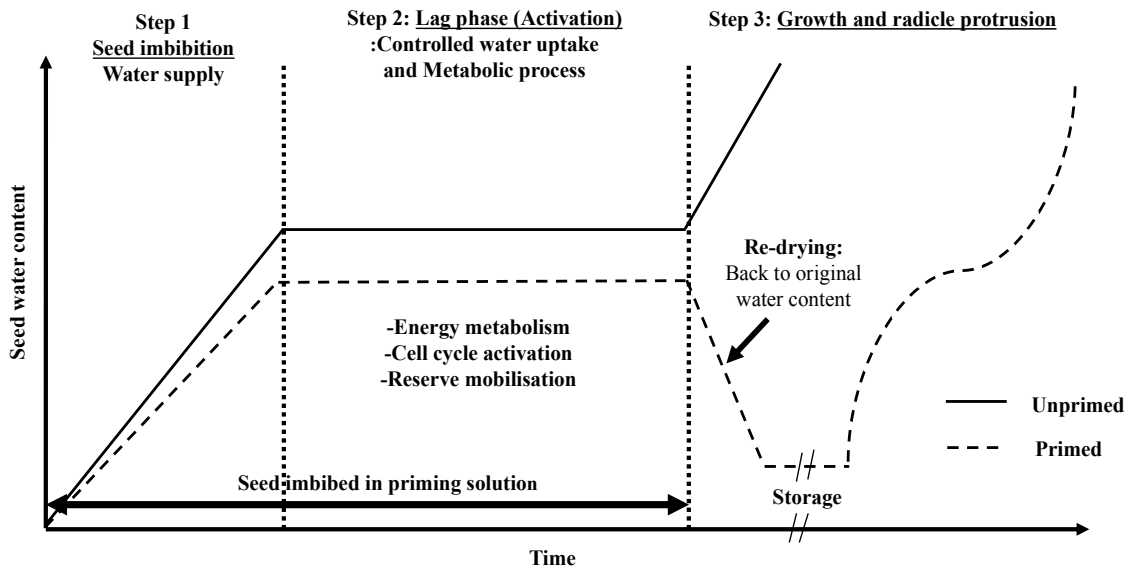


Fig. 1.3. Diagram of general seed priming process, modified from Rajjou et al. (2012).

1.3.8.3. Influence of seed priming on seedling growth under salinity stress

Seed priming technique is applied to a wide range of plant species to enhance tolerance against biotic (i.e. pathogen attack) and abiotic (i.e. cold, heat, drought, and salinity, etc.) stresses during initial seed germination stage (Jisha et al., 2013). Seed germination and early seedling establishment are especially sensitive to salinity (Jamil et al., 2005). Salinity that causes oxidative and osmotic stresses, and ion toxicity would affect growth at these stages, and lead to restraining crop productivity (Ibrahim, 2016). For agricultural production under unfavorable conditions, seed priming has been widely used to promote tolerance to abiotic stress (Jisha et al., 2013; Paparella et al., 2015; Patade et al., 2009). There are many reports showing that seed priming can improve quality and viability of crop seeds under salt stress. For instance, the

following studies have shown the beneficial effects of seed priming in relation to salt stress: sweet pepper (*Capsicum annuumm* L.), okra (*A. esculentus* L.), tomato (*Solanum esculentum* L.), melon (*Cucumis melo* L.), and milk thistle (*S. marianum* L.) (Aloui et al., 2014; Dkhil et al., 2014; Pradhan et al., 2014; Sivritepe et al., 2003; Zavariyan et al., 2015). The primed seeds of these plant species had stronger stress tolerance exhibiting higher seed vigour performance and seedling establishment. Seed priming may improve efficiency for water uptake from growing media as well as energy metabolism and flexible metabolic activity within the embryo of primed seeds, thereby facilitating germination and seedling growth under salinity stress (Elouaer and Hannachi, 2012; Ibrahim, 2016).

1.3.8.4. Seed priming types

There are several seed priming techniques including hydro-priming, osmo-priming, solid matrix priming, bio-priming, chemo-priming, and thermo-priming (Paparella et al., 2015). These priming techniques have widely been applied to improve performance of primed seeds when exposed to a variety of abiotic stresses including salt stress (Table 1.2). Firstly, hydro-priming is that seeds are submerged in water under a range of optimum temperatures (5-20˚C). It relies on water affinity with seeds, which enhances water uptake during hydro-priming (Taylor et al., 1998). In particular, hydro-priming is widely used for growing crop species, which are exposed to adverse climate conditions (i.e. drought) (Paparella et al., 2015). Secondly, osmo-priming is to soak seeds in a low water potential solution that could influence water uptake in seeds (Jisha et al., 2013). This priming method is used for restricting ROS-mediated oxidative damage by delaying water entrance. Since polyethylene glycol (PEG) is of nontoxic nature and large molecular size, it is mostly used as an osmo-priming agent (Lutts et al., 2016; Paparella et al., 2015). However, PEG is relatively expensive and this priming

method limits oxygen entering into seed during priming, compared to other compounds currently used for osmo-priming including inorganic salts of N, K, and Mg and organic molecules of glycerol and mannitol (Paparella et al., 2015). Thirdly, solid matrix priming is developed as an alternative method to osmo-priming because of its high cost and drawbacks (Lutts et al., 2016). It regulates water uptake and improves seed performance. As materials for solid matrix priming, coal, sawdust, vermiculite, calcined clay, and charcoal have been used (Jisha et al., 2013). Fourthly, biopriming is a useful technique to improve plant growth, biochemical process, and tolerance to biotic and abiotic stresses by treating beneficial micro-organisms and bioactive molecules (Lutts et al., 2016; Paparella et al., 2015). Since beneficial bacteria (i.e. plant growth-promoting rhizobacteria (PGPR)) are able to establish endophytic relationship with rhizosphere of plants, seed priming with the bacteria facilitates either germination and plant growth as well as improves biotic and abiotic stress tolerance (Mahmood et al., 2016). Also, phytohormones such as salicylic acid (SA), abscisic acid (ABA), and gibberellic acid (GA), can be added as biopriming agents (Paparella et al., 2015). There are successful examples of biopriming for enhancement of seed germination and seedling growth under salt stress: radish and chickpea seed-primed with PGPR (Kaymak et al., 2009; Mishra et al., 2010) and wheat seed-primed with *Trichoderma harzianum* (Rawat et al., 2011). Fifthly, chemo-priming is used to prevent harmful micro-organism growth/microbial contaminants. There are a variety of materials for chemo-priming: sodium hypochlorite (NaOCl), hydrochloric acids (HCl), fungicides, and pesticides, etc. (Paparella et al., 2015). Additionally, chemo-priming with natural compounds such as essential oils and plant extracts are used for seed disinfection (Van der Wolf et al., 2008). Yang et al. (2014) reported that priming with tebuconazole [(*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-3- (1*H*, 1, 2, 4-triazol-1-ylmethyl) pentan-3-ol] to maize (*Z. mays* L.) seeds resulted in enhancement of seed germination, seedling fresh weight, chlorophyll and carotenoid contents, and phytohormone (i.e. gibberellins) content.

However, due to concern about release of toxic chemicals to the environment, the chemo-priming technique is required to develope as an environment-friendly seed priming method. Sixthly, thermopriming is a technique which is to prime seeds at different temperatures before sowing. The best efficiency of this seed treatment is shown commonly at low temperature (Paparella et al., 2015). High temperature treatment is not widely used, but the treatment is known to improve seed germination, particularly for plant species adaptable to warm climates. Liu et al. (2013) investigated that white spruce (*Picea glauca*) seeds applied with combination of moist-chilling and thermo-priming exhibited improved germination parameters including germination capacity, speed, lag, and dormancy compared to non-primed seeds. In comparison with the relatively large number of reports on the practical beneficial effects of the different seed priming types, there are not enough basic studies to gain a better understanding of the precise physiological and biochemical events linking seed priming and the resulting practical benefits.

Table 1.2. Examples of several seed priming methods for improving seed vigour and seedling growth under salinity stress.

Plant species	Priming method	Reference
Alfalfa (*Medicago sativa* L.)	Osmopriming (mannitol), hydro-priming	Amooaghaie (2011)
Basil (*Ocimum basilicum* L.)	Hydro-priming	Farahani and Maroufi (2011)
Bean (*Vicia faba* L.)	Melatonin (100 and 500 μM)	Dawood and El-Awadi (2015)
Canola (*Brassica napus* L.)	Hydro-priming, KNO_3	Omidi et al. (2009)
Pepper *(Capsicum annuum* L.)	NaCl, KCl, and $CaCl_2$	Aloui et al. (2014)
Fenel (*Foeniculum vulgare* L.)	Salicylic acid (SA)	Farahbakhsh (2012)
Maize (*Zea mays* L.)	Hydro-priming, PEG (6000)	Janmohammadi et al. (2008)
Melon (*Cucumis melo* L.)	NaCl	Sivritepe et al. (2003) Farhoudi et al. (2011)
Mungbean (*Vigna radiata* L.)	β-amino butyric acid	Jisha and Puthur (2016)
Mustard (*Brassica juncea* L.)	Hydro-priming, $CaCl_2$, ABA	Srivastava et al. (2010)
Pumpkin (*Cucurbita pepo* L.)	Ascorbic acid (500 and 1000 μM)	Fazlali et al. (2013)
Okra (*Abelmoschus esculentus* L.)	KCl, Mannitol, and $CaCl_2$	Dkhil et al. 2014
Rape seed (*Brassica napus* L.)	PEG 6000	Kubala et al. (2015)
Sugarance (*Saccharum officinarum* L.)	Halopriming (NaCl)	Patade et al. (2009)
Sunflower (*Helianthus annuus* L.)	Hydro-priming, KNO_3	Kaya et al. (2006)
Wheat (*Triticum aestivum* L.)	Hydro-priming, KCl, KH_2PO_4, and PEG 8000 priming, H_2O_2	Giri and Schillinger (2003), Wahid et al. (2007)

1.3.9. Amino acids in plants

1.3.9.1. Role of amino acids in plant growth

Amino acids (AAs) have highly diverse and play pivotal roles in plant metabolism and growth. The AAs are the building blocks of proteins, and act as precursors for synthesis of various compounds essential to plant development such as nucleotides, chlorophyll, hormones, and secondary metabolites (Pratelli and Pilot, 2014; Tegeder, 2012). In addition, they play as major N transport, N storage substrate, and signaling molecules to environmental stresses for preserving plant cell structures (Hildebrandt et al., 2015; Okumoto et al., 2016). AAs in plants are acquired by a direct uptake from soil or by synthesis of AAs in roots or leaves in which inorganic form of nitrogen (i.e. NO_3^- and NH_4^+) are assimilated to AAs (Tegeder, 2012; Tegeder and Rentsch, 2010). In plastids of the roots or leaves, most of the twenty proteogenic AAs are made. Then, following the synthesis of AAs in cytosol, mitochondria, and peroxisomes, the AAs are used for immediate metabolism or transported to the phloem (Tegeder, 2012).

Amino acids are associated with regulation of seed germination and seedling establishment. During seed germination in the absence of light, degradation of storage proteins provides AAs for biosynthesis of new proteins for seedling growth and development (Hildebrandt et al., 2015). Several metabolites derived from degradation of AAs are used in the citric acid cycles as an energy source (Pratelli and Pilot, 2014). During early seedling growth, plants acquire energy via degradation of fatty acids and starch until the photosynthetic system is established (Hildebrandt et al., 2015). Subsequently, up-regulation of AA biosynthesis occurs in the photosynthetic cells to provide bio-molecules for protein synthesis and growth.

However, these biosynthetic and catabolic processes during plant growth (Fig. 1.4) may be influenced by environmental stresses including salinity. In saline condition, uptake of nitrate as a potential source of the AAs into plant is interfered by high concentration of Cl^-, leading to reduction in plant growth and yield (Mansour, 2000). In addition, more AAs such as proline, alanine, arginine, glycine, leucine, proline, serine, and valine are accumulated in plant organs of both glycophytes and halophytes to protect them from salt stress (Mansour, 2000).

1.3.9.2. Effect of exogenous amino acids on plant resistance to salt stress

Exogenous application of AAs can mitigate inhibition of seed germination and growth due to salinity. Among the 20 AAs, proline was used frequently for exogenous application to various higher plants exposed to salts. The external proline treatments mostly exhibited the enhancement of germination, growth, and photosynthetic pigment contents and induction of tolerance to salt stress as a result of increased scavenging of ROS and activating enzymes including SOD, CAT, and POD as shown in the following studies. Bar-Nun and Poljakoff-Mayber (1977) found that germination rate and radicle growth of pea (*Pisum sativum*) were increased by exogenous proline treatment. In another study, exogenous application of proline (10 mM) to tobacco (*Nicotiana tabacum*) suspension cell culture under salt stress resulted in increased cell growth (Okuma et al., 2000). It is likely that proline plays a role in protecting the cell membranes of tobacco. Similarly, Hoque et al. (2007a, b) found that the exogenous proline added to tobacco cell cultures led to improvement of salt stress tolerance. The authors reported that the addition of proline was associated with increased activities of APX, GR, DHAR, CAT, or POD enzymes in the cells under salt stress. Gadallah (1999) investigated the positive effects of exogenous proline addition on chlorophyll content, K^+ uptake, biomass, and membrane protection in bean (*V. faba*) under salinity stress. Following exogenous application

of proline, reduction in lipid peroxidation and superoxide radical content and increase in chlorophyll content were found in *Mesembryanthemum crystallinum* in response to salt stress (Shevyakova et al., 2009).

There are also beneficial effects of exogenous application with the other AAs in alleviating salt induced-inhibition of seed germination and seedling growth: *C. sativus* treated with glutamate (Chang et al., 2010) and *A. thaliana* treated with glycine, serine, cysteine, and methionine (Cheng et al., 2016). Genisel et al. (2015) also reported that barley (*Hordeum vulgare* L.) seeds treated with exogenously applied cysteine had marked increases in seedling length and APX activity in salt solutions, compared to the control seeds. They also found lowered H_2O_2 content and level of lipid peroxidation (MDA) which are oxidative damage markers.

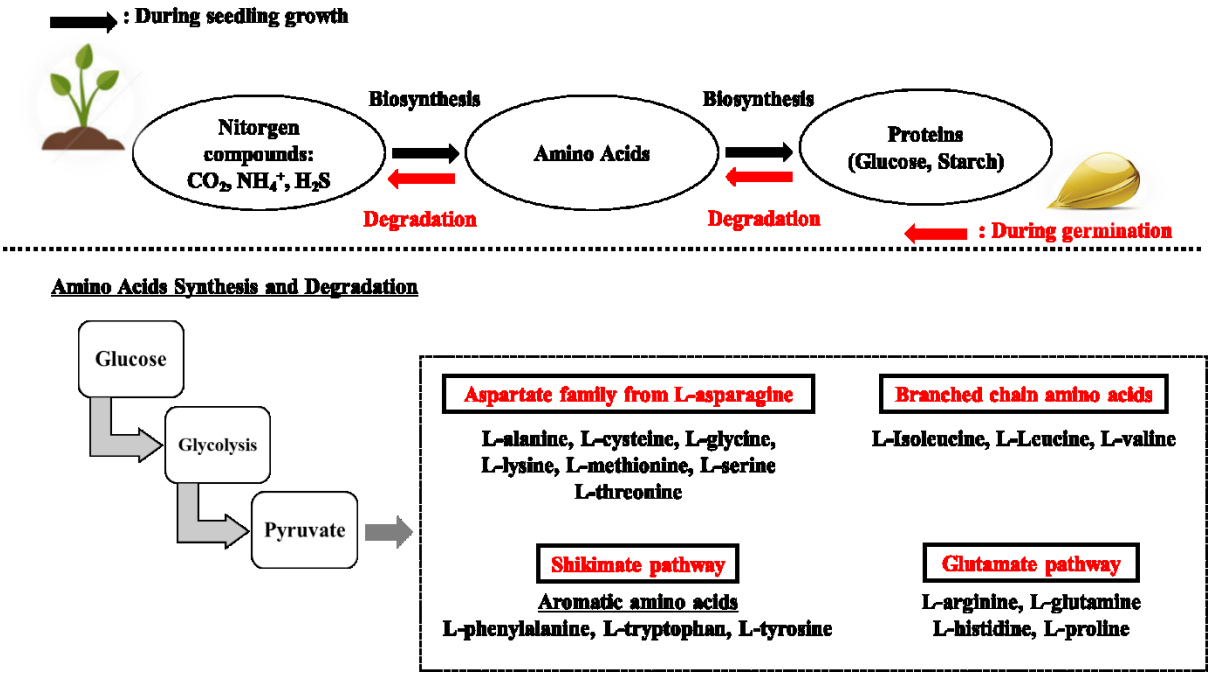


Fig. 1.4. Biosynthesis and degradation of amino acids during seed germination and seedling growth (modified from Hildebrandt et al., 2015).

1.3.9.3. D-amino acids

D- and L-amino acids have similar physical and chemical properties, but only L-AAs can be used for polymerisation and formation of peptides and proteins (Fujii, 2002). In this respect, D-AAs are rarely found in nature, while L-AAs are found abundantly in biological molecules (Vranova et al., 2012). It is well known that D-AAs are found in bacterial cell wall, but there is growing evidence that D-AAs are also found in some living organisms (human, animals, invertebrates, vertebrates, and plants, etc.) as free AAs, peptides, and proteins (Brückner and Fujii, 2010; Fujii, 2002; Vranova et al., 2012). In parts of plants including seeds, fruits, and leaves, D-AAs account for up to approximately 1.5% of the total AA pool (Vranova et al., 2012).

In general, plants may absorb D-AAs directly through roots (Vranova et al., 2012). However, a symbiotic association between plant roots and rhizobacteria (i.e. *Rhizobium* spp. and *Bradyrhizobium* spp.) and mycorrhizal fungi may become an absorption pathway for D-AAs from soil into plants (Brückner and Westhauser, 2003). In addition, D-AA formation is mainly found in prokaryotes, whilst L-AA is incorporated among prokaryotes and eukaryotes (Radkov and Moe, 2014). In particular, plants have a difficulty to metabolise D-AA due to lacking a capacity of oxidative deamination by D-amino acids oxidase (DAAO) (Erikson et al., 2005). D-AAs may have different impacts on plant metabolism depending on the concentration and species, but several reports have shown a potential toxicity of D-AAs to plant growth and development. For example, Erikson et al. (2005) found that application of D-serine (at >3 mM) strongly inhibited the growth of various plant species including tomato (*S. esculentum*), barely (*H. vulgare*), maize (*Z. mays*), poplar (*P. tremuloides*), tobacco (*N. tabacum*), and *A. thaliana*. Also, Forsum et al. (2008) showed that growth of *A. thaliana* was significantly inhibited by

treatment with 10 μM of D-AAs including D-alanine, D-arginine, D-serine, and D-valine. Also, plant roots absorbed these D-AAs at lower rates than the corresponding L-forms.

1.3.10. Effects of casein hydrolysate on plant growth

Casein hydrolysate (CH) has been widely used as an organic supplement in plant tissue culture and somatic embryo production (Daniel et al., 2018; LaMotte and Lersten, 1972; Sun et al., 2016). CH can be a major source of reduced nitrogen, as it contains a large amount of glutamine (George et al., 2008; Molnár et al., 2011). In addition, CH has calcium (Ca^{2+}), phosphate (PO_4^{3-}), several microelements, vitamins, a mixture of up to 18 amino acids including DL-alanine, L-arginine, DL-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-histidine, DL-isoleucine, L-leucine, DL-lysine, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, DL-tryptophan and DL-valine (George et al., 2008; Lauer and Baker, 1977; Molnár et al., 2011; Ziebur et al., 1950). Also, CH may contain some unknown beneficial ingredients for promoting plant growth (Ageel and Elmeer, 2011). Thus, several investigators have suggested that sole addition of CH to culture medium could be more beneficial on cell growth than addition of individual or mixed AAs (Molnár et al., 2011). Daniel al. (2018) found an enhancement of embryo maturation and plantlet production, and regeneration of Okra (*Abelmoschus esculentus* L.) on the media added with CH. Khaleda and Al-Forkan (2006) reported that CH addition to culture medium was required for vigorous callus formation in rice. Abiri et al. (2017) also found that enhanced cell proliferation and embryo regeneration of a Malaysian rice cultivar (MR219), when cultured in the presence of 100 mg L^{-1} CH as well as potassium metasilicate, kinetin, and 2,4-dicholorophenoxy acetic acid. However, another study found no such positive effect on callus induction in the same rice cultivar even when the CH

concentration in the medium was increased up to 1000 mg L^{-1} (Che Mohd. Zain et al., 2012). It is not readily clear why there are different findings in these studies.

1.3.11. Importance of Italian ryegrass

There are a variety of commercial pastoral grass species such as Italian ryegrass (*Lolium multiflorum* L.), perennial ryegrass (*L. perenne* L.), cocksfoot (*Dactylis glomerata* L.), timothy (*Phleum pretense* L.) and fescue grasses (*Festuca* spp.) forproduction of forage, livestock siliage, and dry grass (Scudamore and Livesey, 1998). Of the species, *L. multiflorum* is one of the most abundant species in grassland of temperate regions including Europe (Stanisavljević et al., 2011). It can be rapidly established and vigorously grown especially in winter season (Wang et al., 2015a). The ryegrass species is capable of considerable forage productivity for up to 3 years and is highly nutritious, digestible, and preferable for dairy animals (Charton and Stewart, 1999; Prestidge, 1991; Wang et al., 2015a). Currently, Italian ryegrass has been widely sowed for improvement of forage production in grasslands worldwide including Europe, USA, New Zealand, and elsewhere. However, there is limited information about *L. multiflorum*, particularly compared to perennial ryegrass.

In New Zealand, herbage and seed production of *L. multiflrum* have increased over the last century. Total herbage production of *L. multilfrum* in New Zealand had increased about 1.5% annually between 1975 and 1995 (Easton et al., 2002). In addition, the seed yields of this species had steadily increased about 46 kg per hectare annually from 1992 to 2014 (Chynoweth et al., 2015). The average production in 2011-2013 was 1750 kg per hectare. To improve its productivity and persistence, the hybrid ryegrass with perennial ryegrasses has been bred (Charton and Stewart, 1999).

Italian ryegrass is a crucial pasture species in New Zealand dairy industry since it can produce greater yields of dry matter (DM) than perennial ryegrass during over winter and early spring (DairyNZ, 2016; Thom and Bryant, 1996). Also, herbage produced over this period aids to overcome feed shortage in early lactation (Thom and Bryant, 1996). In general, seeds of this species are usually sown in late summer and early autumn and its growth can last in early summer (Charton and Stewart, 1999). DairyNZ (n.d.) evaluated that *L. multiflorum* annually produces 15 tons DM per hectare, which is of similar or slightly larger amount than productivity of *L. perenne* (14 tons DM per hectare). They also reported that the species yields could be up to 20 tons DM per hectare under irrigation management or wet summer. However, the yields could be significantly reduced by up to 50% (10 tons DM per hectare) in a severely dry summer.

Today, there is a potential concern that the occurrence of salinity stress due to global climate change and intensive irrigation might result in damages in yields of crops including Italian ryegrass in New Zealand. However, there is little information of tolerance of Italian ryegrass to salt stress (i.e. Lee et al., 2104; Marcar, 1987). In the study of Marcar (1987), seed of Italian ryegrass had higher germination rate than that of perennial and wimmera ryegrasses when grown in the Petri dish containing 300 mM NaCl. For sustaining production of the Italian ryegrass under such unfavorable (salinity) condition, therefore, it is strongly needed to improve understanding of physiological response and tolerance mechanism of Italian ryegrass to salt stress, particularly during seed germination and early seedling growth. These growth stages are determinants for its survival and productivity.

1.3.12. Previous studies to induce abiotic stress tolerance in ryegrass species

There are several previous studies showing the enhanced tolerance of ryegrass species against abiotic stresses. Hu et al. (2012a) found that exogenous application of glycine betaine (GB) ameliorated the negative effect of salt stress on perennial ryegrass (*L. perenne* L.) seedlings. The GB treatment led to reduction in lipid peroxidation and improvement in ion homeostasis, biomass, and antioxidant enzyme activities (i.e. SOD, CAT, and APX) under salinity stress (250 mM NaCl). In another study, *L. perenne* seedlings exogenously treated with sodium nitroprusside (SNP, a nitric oxide releasing substance) exhibited Cd tolerance enhancement (Wang et al., 2013b). There were increases in antioxidant enzyme activities including SOD, CAT, POD, and APX, whilst ROS and lipid peroxidation contents were significantly lowered compared to the control plant. Similarly, exogenous treatment of spermidine had a beneficial effect on Kentucky bluegrass (*Poa pratensis* L.) seedlings exposed to salinity stress (200 mM NaCl) (Puyang et al., 2015). Wang et al. (2014) reported that tall fescue (*Festuca arundinacea*) and perennial ryegrass could exhibit enhanced thermo-tolerance after exogenous H_2O_2 treatment. It seems that H_2O_2 signalling contributed to an increase in antioxidant enzyme activities including POD, CAT, APX, GR, and glutathione-dependant peroxidase (GPX) and reduction in MDA and H_2O_2 contents under heat stress (38/30˚C, day/night). However, there has been no prior publication to understand physiological response and tolerance mechanism of *L. multiflorum* plants raised after seed priming against environmental stresses such as salinity.

Chapter 2. General Methodology

2.1. Preparation of materials

2.1.1. Plant seed

Italian ryegrass (*Lolium multiflorum* L.) as one of major pasture species in New Zealand (NZ) dairy industry was selected in this study. Five kilograms of the ryegrass seeds were supplied from a field service center, commercial dairy farmland of Lincoln University, Canterbury, NZ. Of those, visually-healthy seeds were sorted and stored in a fridge at 4 ˚C prior to seed priming application.

2.1.2. Seed priming agents

To conduct seed priming applications, 20 L-amino acids and D-methionine were purchased from Sigma-Aldrich, USA for this study. Also, casein hydrolysate (CH) used for seed priming which contained a complex mixture of 18 amino acids and some vitamins was obtained from Sigma-Aldrich, USA. The component and amount of amino acids in CH chemical powder are shown in Appendix 1. All the amino acids were dissolved in de-ionised (DI) water and used for seed priming in each experimental Chapter outlined as below. In all the Chapters, hydro-priming (HP, seed priming with DI water only) and non-priming (NP) treatments were included for comparisons.

- In Chapter 3: germination and seedling growth assays

  ➔ Twenty of L-amino acids (1 mM)

  : L-alanine, -arginine, -asparagine, -aspartic acid, -cysteine, -glutamic acid, -glutamine, -glycine, -histidine, -isoleucine, -leucine, -lysine, -methionine, -phenylalanine, -proline, -serine, -threonine, -tryptophan, -tyrosine, and -valine.

  ➔ D-methionine (1 mM)

  ➔ Casein hydrolysate (200 mg L^{-1})

- In Chapter 4: salt stress tolerance assays

  ➔ L-methionine (1 mM)

  ➔ D-methionine (1 mM)

  ➔ Casein hydrolysate (200 mg L^{-1})

- In Chapter 5: greenhouse experiment

  ➔ L-methionine (1 mM)

  ➔ Casein hydrolysate (200 mg L^{-1})

  ➔

2.1.3. NaCl solution

One molar stock solution of sodium chloride (NaCl) was made for all experiments in this study; 58.44 g NaCl powder (Merck Millipore, USA) was dissolved in one liter of DI water in a 1-liter volumetric flask. Then, the stock solution was diluted with DI water to prepare different concentrations of salt treatments as below. All NaCl solutions were stored in a fridge at 4 ˚C prior to use.

1) In Chapters 3 & 4: germination tests

➔ 0, 60, and 90 mM NaCl

2) In Chapter 5: pot experiments

➔ 0, 60, 120, and 180 mM NaCl

2.1.4. MES buffer

For adjusting pH levels of either seed priming reagents and NaCl solutions, 2-(N-morpholino) ethanesulfonic acid (MES) ($C_6H_{13}NO_4S$) (Sigma-Aldrich, USA) buffer solutions were prepared for use in Chapter 3. For identifying seed germination and radicle length, firstly, NaCl solustions (60 and 90 mM) were set up at either pH 6.1 and 6.7 using 0.01 M and 0.1 M MES buffer. Secondly, 1 mM of L-proline was added to 0.01 M MES buffer (pH 5.5, 6.1, and 6.7) for use in seed priming, and after seed priming the seeds were incubated in 0, 60, and 90 mM NaCl in the presence of the MES buffer. Thirdly, 1 mM of each of the priming agents including L-methionine, -proline, and -valine was added to 0.01 M MES buffer (pH 6.1), after seed priming the seeds were incubated in 0, 60, and 90 mM NaCl in the presence of the MES buffer. All the seeds in the Petri dishes were incubated for 4 days in a growth room.

2.1.5. Other experimental equipment and materials

In this study, various additional materials and instruments were also prepared. In Chapters 3, 4, & 5: germination tests

➔ Petri dishes, pipettors (1 ml and 5 ml), filter papers (Whatman No. 1), forceps, wrapping, marker pens, and growth room.

- In Chapter 5: pot experiments

➔ Plastic pots, pot-props, pot tray, uniformed-size papers (for blocking holes of

the pot bottom), two hobos (for recording temperature and light intensity), a spade, a sieve (2 mm), a hand-cart, plastic bags (for soil samples), soil, 50 ml of graduated cylinders (for measuring water and NaCl solutions), a balance, weighing plates, forceps, 50 ml centrifuge tubes (for soil samples), 10 ml glass tubes (for leaf samples), DI water, a pipettor (5 ml), scissors, foil, liquid nitrogen and bottles (for collecting leaf samples), 80% acetone, and paper bags (for storing plant samples).

2.2. Experimental treatment

2.2.1. Seed priming

In Chapters 3, 4, & 5 (laboratory and glasshouse assays), all Italian ryegrass seeds were exogenously treated with 20 L-amino acids, D-methionine, or casein hydrolysate for seed priming. Seed priming was progressed as below.

- Visually healthy seeds were selected before soaking in each priming reagent.
- Seeds of *L. multiflorum* were added in a Petri dish (90 mm diameter) with one layer of filter paper (Whatman No 1.).
- Ten ml of de-ionised water for hydro-priming (HP), each of the selected amino acids (1 mM), and/or CH (200 mg L^{-1}) per 40 seeds was added in each Petri dish.
    - ➔ In Chapters 3 & 4, a total 160 seeds were used in each seed priming treatment: Forty seeds were placed in a Petri dish with 10 ml seed priming reagent and there were 4 replicates.
    - ➔ In Chapters 5, a total 455 seeds were used in each seed priming treatment: Sixty-five seeds were placed in a Petri dish with 16.25 ml seed priming reagent and there were 7 replicates.

- After adding the reagents, the Petri dishes were sealed with wrapping plastic tape for maintaining the moisture content.
- Then, the Petri dishes were kept for 24 hours in a growth room at 25 ˚C in the dark.
- The primed seeds were air dried for 24 hours in the same dark growth room.

2.2.2. Salt treatment

As described previously, different concentrations of NaCl were prepared in all experiments.

- In germination tests of Chapters 3 & 4,
    1) Twenty of the primed seeds were placed on a filter paper (Whatman No.1) in a Petri dish.
    2) Ten milliliters of each concentration of NaCl solution (0, 60, and 90 mM) was added to each Petri dish.
    3) The Petri dishes were sealed with wrapping plastic tape and then stored for 4 days in the dark growth room at 25 ˚C.
- In pot experiment of Chapter 5,
    1) Thirty milliliters of each concentration of NaCl solution (0, 60, 120, and 180 mM) was added to the soil surface of a pot.
    2) Since the ryegrass seeds were sowed in pots, the salt treatments were repeated daily.

2.3. Experiment design

2.3.1. Germination test in laboratory

To investigate the effects of seed priming with amino acids and casein hydrolysate on germination, radicle development, and tolerance at the early growth stage of *L. multiflorum* in response to salt stress, the laboratory experiments in either Chapter 3 or 4 were carried out.

1) Seeds primed with DI water (for hydro-priming), 20 different L-amino acids, D-methionine, and casein hydrolysate were prepared. Non-primed seeds were also included as control.
2) Twenty seeds from a priming treatment were placed on a filter paper (Whatman No.1) in a Petri dish (90 mm diameter).
3) Ten milliliters of a different NaCl concentration (0, 60, and 90 mM) were added to each Petri dish. There were 8 replicates in each treatment.
4) All Petri dishes were sealed with a wrapping plastic tape and randomly laid on the table in a growth room.
5) Then, those seeds in the Petri dishes were incubated for 4 days at 25 ˚C in the dark.
6) Germination percentage and radicle length of the ryegrass seeds in each Petri dish were measured after incubation.
7) Antioxidant enzyme activities including peroxidase (POD), superoxidase (SOD), and catalase (CAT) were determined.
8) Hydrogen peroxide (H_2O_2), lipid peroxidation (MDA), and proline content in the radicle of the seeds were determined using spectrophotometric methods.
9) In addition, reactive oxygen species (ROS) such as H_2O_2 and $O_2^{\cdot -}$ in the radicle were detected by staining with 3, 3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT).

2.3.2. Pot experiment in glasshouse

Glasshouse experiments in Chapter 5 were conducted using *L. multiflorum* seeds primed with L-methionine and CH at two different times. Non-primed and hydro-primed seeds were also included in each glass experiment for comparison.

- From 10th April to 15th May in 2016: CH primed, HP, and NP seeds
- From 4th December in 2016 to 7th January in 2017: L-methionine primed, HP, and NP seeds

1) Plastic pots (size: 10.5 cm diameter x 11.5 cm height) were cleaned and blocked those bottom holes using a piece of newspaper to prevent soil loss.
2) Soil substrate was prepared by mixing pumice and topsoil (w/w=1:3) supplied by the University of Canterbury glasshouse.
3) The prepared soil mixture was used to fill each pot, and allowed to get the water holding capacity by soaking the moisture up from the pot tray for up to 24 hours.
4) Sixty-five seeds primed with CH or L-methionine (about 0.25 g) were sown at a depth of 2 cm from soil surface.
5) Thirty milliliters of different NaCl solutions (0, 60, 120, and 180 mM) were added to each pot on a daily basis.
6) Two HOBO (Onset's humidity browser operators) (UA002-64 onset, USA) were installed to record temperature and light intensity in the glasshouse during the pot experiments.
7) Two and five weeks after sowing, ryegrass shoots were sampled for determining chlorophyll and carotenoid contents as well as antioxidant enzyme activities (POD,

SOD, and CAT).

8) After 6 weeks of cultivation, the ryegrass plants were sampled to measure fresh weight and dry weight.
9) Also, root electrolyte leakage (EL) and soil electrical conductivity (EC) were determined.
10) There were 84 pots in each pot experiment: 3 seed priming treatments (NP, HP, CH priming or L-methionine priming) x 7 replicates x 4 different salt concentrations.
11) The Experiment layout was a complete randomised block design (CRD).

2.4. Analysis

2.4.1. Germination percentage

Germination percentage (GP) of the Italian ryegrass seeds was determined following incubation in the growth room or cultivation in a greenhouse.

1) In Chapters 3 & 4, the number of seeds (out of 20 per Petri dish) germinated were counted to determine GP after 4-day incubation in the dark growth room at 25 ˚C.
2) In Chapter 5, the number of seeds (out of 65 per pot) were counted to determine GP after 1-week cultivation in a greenhouse.
3) The germination percentage was calculated as the formula below:

$$\text{Germination percentage (\%)} = \frac{\text{The number of germinated seeds}}{\text{The total number of seeds sown}} X\ 100\%$$

2.4.2. Radicle length

Following GP measurement, radicle length of each germinated seed was recorded using a 300-mm plastic ruler. Among 20 seeds in each Petri dish, 10 seeds were randomly selected for radicle length measurement. The results of radicle length measurement were reported in Chapters 3 and 4.

2.4.3. Biomass

Fresh weight (FW) and dry weight (DW) of the ryegrass plants were measured after pot experiments in Chapter 5.

1) Ryegrass with or without seed priming was divided as roots and shoots.
2) Root samples were cleaned by water washing and removed the moisture by paper tissues.
3) FW was measured using a balance in the glasshouse.
4) After weighing FW, the samples were kept in paper bags and oven-dried for 3 days at 65 ˚C.
5) DW of the samples were measured using a balance in the laboratory.

2.4.4. Photosynthetic pigment content

As mentioned previously, ryegrass leaves were collected 2 and 5 weeks after sowing for determination of total chlorophyll and carotenoid contents in the leaves. The extraction method using 80% acetone (Arnon, 1949) was used in this study.

1) Fresh leaves (0.1 g) were taken from four pots in each seed priming treatment.
2) Leaf discs (less than 100 mm length) sampled were immersed in 10 ml of 80% (v/v)

acetone in a glass vial.

3) The vials were stored for 72 hours at 4 ˚C in the dark.
4) Then, the extracts were measured at 663, 645, and 470 nm wavelengths using a spectrophotometer (Ultrospec 2100 pro UV/Visible Spectrophotometer, England).
5) Total chlorophyll (Chl) and carotenoid (Car) contents were evaluated as equations described in Jiang et al. (2015) and Fambrini et al. (2010), respectively:

$$\text{Chl a (mg g}^{-1}\text{)} = [(12.7 \times A663) - (2.6 \times A645)] \times \text{ml acetone / mg leaf tissue}$$

$$\text{Chl b (mg g}^{-1}\text{)} = [(22.9 \times A645) - (4.68 \times A663)] \times \text{ml acetone / mg leaf tissue}$$

$$\text{Total Chl (mg g}^{-1}\text{)} = \text{Chl a + Chl b or } [(20.21 \times A645) + (8.02 \times A663)] \times \text{ml acetone / mg leaf tissue}$$

$$\text{Total Car (mg g}^{-1}\text{)} = [(1000 \times A470) - (1.90 \times \text{Chl a}) - (63.14 \times \text{Chl b})] / 214$$

2.4.5. Antioxidant enzyme activities

To identify effects of seed priming with amino acids and CH on tolerant responses of *L. multiflorum* to salt stress, antioxidant enzyme activities (POD, SOD, and CAT) in radicles of ryegrass seedlings in the germination assays (laboratory experiments) and leaves of the ryegrass plants in pot experiments.

- In Chapter 3, after the ryegrass seedlings raised from seeds primed with 20 L-amino acids and CH were exposed to salt stress (0, 60, and 90 mM NaCl), POD activity in the radicles of the seedlings was determined.
- In Chapter 4, after the ryegrass seedlings raised from seeds primed with L- and D-methionine, and CH were exposed to salt stress (0, 60, and 90 mM NaCl), POD, SOD, and CAT activities in the radicle of the seedlings were determined.

- In Chapter 5, after the ryegrass seedlings raised from seeds primed with L-methionine and CH were exposed to salt stress (0, 60, and 90 mM NaCl), POD, SOD, and CAT activities in the leaves of the seedlings were determined.

2.4.5.1. Enzyme extraction

- To extract POD from ryegrass seedlings, the protocols of Baque et al. (2010) and Qiu et al. (2014) with modification were used in this study.
    1) 0.1 g of roots or leaves was collected.
    2) The roots or leaves were homogenised to a powder with liquid nitrogen N in a mortar and pestle. The powder was then mixed with 1.2 ml of cold 0.05 M phosphate buffer (pH 6.59).
    3) The homogenates were centrifuged at 10,000 g for 5 min at 4 ˚C.

- To extract SOD and CAT from ryegrass seedlings, the protocols of El-Shabrawi et al. (2010) and Larkindale and Huang (2004) with modification were used in this study.
    1) 0.1 g of root or leaves was collected.
    2) The roots or leaves were homogenised with liquid N in a mortar and pestle to a powder. The powder was then mixed with 1.2 ml of cold 0.05 M phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP).
    3) The homogenates were centrifuged at 12, 000 g for 15 min at 4 ˚C.

- All supernatants were stored at -80 ˚C prior to analyses for POD, SOD, and CAT.
- Total protein content in the supernatant was also measured at 590 nm using a Ultrospec^{TM} 2100 pro uv/visible spectrophotometer (GE Healthcare, USA) as described

in Bradford (1976).

2.4.5.2. Peroxidase

The POD (EC. 1.11.1.7) activity was measured with guaiacol as the substrate in a total volume of 0.95 ml using a slight modification described by Bestwick et al. (1998) and Wang et al. (2015b).

(1) The supernatant (50 µl) was added with a mixture of 893 µl of 0.1 M potassium phosphate buffer (pH 6.59), 2 µl of guaiacol, and 5 µl of 10% H_2O_2 for 5 min at room temperature until the color reaction was developed to orange.

(2) The reactant was measured at a wavelength of 470 nm using a Ultrospec^{TM} 2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA). The POD activity (units•mg^{-1} protein) was estimated by dividing protein content.

2.4.5.3. Superoxide dismutase

The SOD (EC. 1.15.1.1) activity was measured based on its ability in the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) using a slight modification described in Wang et al. (2015b).

1) One milliliter of reaction mixture containing 0.6 ml of 50 mM PBS buffer (pH 7.8), 0.1 mM of 130 mM methionine, 0.1 ml of 1 mM EDTA, 0.1 ml of 750 uM NBT, 0.1 ml of 0.02 mM riboflavin, was reacted with 0.04 ml enzyme extract.
2) After 5 min exposure time to fluorescent light, the absorbance of the reaction mixture was measured at a wavelength 560 nm using a Ultrospec^{TM} 2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA).
3) The SOD activity was determined as the amount of enzyme needed to reach 50%

inhibition of the NBT reduction caused by superoxides generated from the reaction of photo-reduced riboflavin and oxygen. The total SOD activity was expressed in units per protein content (units•mg^{-1} protein).

$$\%\ inhibition\ of\ NBT\ reduction\ by\ SOD = \frac{(Control\ OD - Sample\ OD)}{Control\ OD}\ x\ 100 \div 50 \div protein\ content\ of\ each\ sample$$

2.4.5.4. Catalase

The CAT (EC. 1.11.1.6) activity was determined based on the reduction rate in hydrogen peroxide using a slight modification described by Chance and Maehly (1955) and Lukatkin et al. (2014).

1) The reaction mixture containing 0.95 ml of 50 mM phosphate buffer (pH 7.0) and 0.015 ml of 3% hydrogen peroxide (H_2O_2) was reacted with 0.05 ml of the enzyme supernatant.
2) The decreased amount of H_2O_2 for 1 min was measured at 240 nm of absorbance using a Ultrospec™ 2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA). The CAT activity was expressed in units per protein content (units•mg^{-1} protein).

2.4.6. Reactive oxygen species (ROS)

2.4.6.1. Hydrogen peroxide (H_2O_2)

Total hydrogen peroxide (H_2O_2) content was measured using a slight modification of Sagisaka (1976) and Li et al. (2007).

1) Twenty-three root tips (about 28-30 mg) from 4-day-old seedlings were cut and grinded

with 0.28 ml 10% trichloroacetic acid (TCA), mixed with 0.40 ml 50 mM potassium phosphate buffer (pH 7.5) in a mortar and pestle at 4 ˚C.

2) The homogenate was centrifuged at 12,000 g, 4 ˚C for 10 min. The supernatant was separately moved to new Eppendorf tubes for measuring H_2O_2 content.
3) The supernatant (190 µl) was mixed with 640 µl 50% TCA, 160 µl 10 mM ferrous ammonium sulfate. Lastly, 80 µl of 2.5 M potassium thiocyanate was added. TCA (10%, 190 µl) instead of the supernatant was mixed with the reaction mixture as a control.
4) The absorbance was measured at 480 nm with a uv/visible spectrophotometer (Novaspec III, Amersham Science, USA). The amount of hydrogen peroxide was defined as micromole per root fresh weight (µmol g^{-1} FW).

2.4.6.2. Malondialdehyde (MDA)

The level of malondialdehyde (MDA) was determined using a slight modification of Health and Packer (1968) and Kovács et al. (2009).

1) About 28-30 mg of root tips (twenty-three roots) from 4-day-old seedlings were homogenised with 680 µl of 10% trichloroacetic acid (TCA) in mortar and pestle at 4˚C.
2) The homogenate was centrifuged at 10,000 g, 4˚C, for 10 min.
3) The ratio of the reaction mixture consisted of 1:3 of 250 µl supernatant and 750 µl 20% TCA containing 1% thiobarbituric acid (TBA) in a water bath at 95 ˚C for 30 min.
4) After heating, the reaction mixture was directly cooled for 5 min in the ice box. MDA content was determined at 532 and 600 nm with a uv/visible spectrophotometer (Novaspec III, Amersham Science, USA).

5) The amount of MDA was determined as micromole per root fresh weight (μmol g^{-1} FW) and calculated using the molar-extinction coefficient (155 mM^{-1} cm^{-1}).

$$MDA \text{ level (nmol)} = \frac{\Delta(A\ 532\text{nm} - \text{A}\ 600nm)}{1.56} \times\ 10^5$$

2.4.7. Histochemical staining with 3, 3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) of roots

To detect reactive oxygen species (ROS) including H_2O_2 and $O_2^{\cdot-}$ in Chapter 4, NBT (nitroblue tetrazolium) and DAB (3, 3'-diaminobenzidine) staining were used in this study (Frahry and Schopfer, 2001).

1) Ten 4-day-old roots of uniform sizes were randomly selected from each Petri dish (5 cm diameter).
2) The NBT staining was detected after incubation of the roots in 10 mM potassium citrate (pH 6.0) containing 0.5 mM NBT and DAB staining was detected after incubation of the roots in 10 mM potassium citrate (pH 6.0) containing 2.5 mM DAB and 1mM H_2O_2 for 1 min, respectively (Frahry and Schopfer, 2001).
3) After staining, photos were taken of the roots which were from three replicate experiments.

2.4.8. Proline content

Free proline content in roots was determined in Chapter 4 using slightly modified protocols of Bates et al. (1973) and Kubala et al. (2015).

1) About 28-30 mg (twenty-three) of root tips from 4-day-old seedlings were homogenised with 680 µl 3% sulfosalicylic acid at 4 ˚C.
2) The homogenates were centrifuged at 12,000 g for 10 min at 4 ˚C.
3) The supernatant (200 µl) and 800 µl sulfosalicylic acid were mixed with 1 ml of acid-ninhydrin reagent (prepared 3% (w/v) ninhydrin in 60 ml of 60% (v/v) glacial acetic acid, 40 ml of 6 M phosphoric acid (H_3PO_4), and 1 ml of 96% glacial acetic acid in a water bath at 100 ˚C for 1 hour.
4) Reaction was stopped by the reaction tubes in an ice bath for 5 min. Toluene (2 ml) was finally added to the reaction mixture and vortexed was for 10 seconds.
5) The reaction mixture in the test tubes were kept at room temperature for 20 minutes. The upper phase was gently poured into a glass quartz cuvette for measuring proline content.
6) The absorbance was recorded at 520 nm with a uv/visible spectrophotometer (Novaspec III, Amersham Science, USA).
7) Standard curve of proline concentration was established using by different concentrations of L-proline (0.2 - 2 µg/ml of L-proline in 3% sulfosalicylic acid) and calculated on a micromole per fresh weight (µmol g^{-1} FW). The proline calculation formula is below according to Akram et al. (2012).

$$Proline\ (\mu mol/g\ FW) = \frac{\mu g\ \text{proline ml} - 1\ \times \text{ml of toluene}/115.5}{\text{FW of plant sample (g)}}$$

2.4.9. Electrolyte leakage

Electrolyte leakage (EL) extracted from Italian ryegrass roots was determined after pot experiments in Chapter 5.

1) Fresh root tissues (0.1 g) were randomly selected (n = 3) from each pot in a treatment were cut into 10 mm long pieces.
2) The roots were washed and then placed in 15 ml Falcon tubes filled with 10 ml of de-ionised distilled water and were left for 24 hours in the dark at room temperature.
3) Then, the initial electrolyte leakage (EL_1) of the roots was determined with a conductivity meter (Fisher scientific^{TM} accumet TM AP 85, USA).
4) After measuring EL_1, the roots in the falcon tubes were boiled in a water bath at 95 ˚C for 20 minutes to release all electrolytes
5) After this, the roots were cooled at room temperature for 10 minutes. The final electrolyte leakage (EL_2) was re-measured. Electrolyte leakage was calculated according to the following equation (Shi et al., 2006).

$$Electrolyte\ leakage\ (\%) = \frac{EC_1}{EC_2} \times 100$$

2.4.10. Soil electrical conductivity

Like EL measurement, electrical conductivity (EC) in soils after pot experiments was determined in Chapter 5.

1) After pot experiments, rhizosphere soil of the ryegrass was air-dried in a glasshouse.
2) Five g of the rhizosphere soil were sampled from each pot and kept in a 50 ml centrifuge tube. There were three replicates in each treatment.
3) DI water (25 ml) was added to the tube as a ratio of 1:5 w/v soil suspensions and mixed well.
4) After 1 hour, soil EC was determined using an EC meter (Mettler Toledo Seven Easy).

2.5. Statistical analysis

All the experiments were of completely randomised design with proper replications. One-way analysis of variance (ANOVA) was conducted to investigate the variability and the statistical significance in the means of all data between treatments using Fisher's exact test at the five percent probability ($p< 0.05$) between treatments. Additionally, principal components anylasis (PCA) was conducted to investigate patterns of variation in the datasets, with a focus on the difference degree between seeds grown with and without priming treatments. In this study, a Minita 16 statistical sofrware was used for all statistical analyses.

Chapter 3. Influence of priming Italian ryegrass seeds (*Lolium multiflorum* L.) with amino acids on seedling growth and salt tolerance

3.1. Abstract

Increased salinity in agricultural soils due to climate change and excessive fertilisation have resulted in reduction in crop yields worldwide. Italian ryegrass (*Lolium multiflorum* L.) as a pastoral crop in New Zealand is important for successful dairy industry, but its yields have recently decreased by increase of the environmental alterations in agricultural landscapes. This study aimed to investigate the potential effect of seed priming application with 20 of L-amino acids (AAs) and casein hydrolysate (CH) as a mixture of some L-amino acids on germination, growth, and tolerance mechanism of *L. multiflorum* at the early growth stage under salt stress. Additionally, non-priming (NP) and hydro-priming (HP) seeds were arranged to compare the efficiency of AAs and CH priming on the ryegrass growth. After 4 days incubation in Petri dish, germination percentage (GP) of seed priming with three amino acids including L-glycine, -methionine, and -tryptophan was significantly higher than those of NP ($p<0.05$). When grown in 60 mM NaCl, L-glycine, -lysine, and -serine showed considerably greater radicle length (RL) in comparison with control ($p <0.05$). L-lysine also had a higher values in RL relative to control in 90 mM NaCl. POD activities in roots of seedlings from the seeds primed with various L-AAs including L-arginine, -glutamic acid, -histidine, -methionine, -proline, -tryptophan, and -leucine were significantly higher (up to 57%) than those in from seeds in NP and HP treatments when the seedlings were grown in 60 mM NaCl. Also, priming with L-methionine and -tryptophan increased POD activity in the seedling roots in 90 mM NaCl. Adjustment of pH in NaCl solution and seed-priming reagents using MES (2-(N-morpholino) ethanesulfonic acid) buffer tended to show no or negative effects on seed germination of Italian ryegrass. Of the

seed-priming reagents, L-methionine and CH had the highest scores. These results have not been reported before.

Keywords: Salinity stress, Seed-priming, L-methionine, Casein hydrolysate, *Lolium multiflorum*, Seed development, Antioxidant enzyme activity.

3.2. Introduction

Environmental stresses such as salinity and drought linked to climate change have gradually been problematic for agricultural productivity worldwide (Ahuja et al., 2010; Sivakumar et al., 2005; Webb et al., 2017). Seven percent of total land area throughout the world accounting for 930 million hectares is salt-affected (Munns, 2002). However, a considerable proportion of arable lands worldwide has recently become more salinisation due to land clearance, irrigation, and application of chemical fertilizers (Munns, 2005; Rivero et al., 2003). The increased saline condition in arable lands can lead to more severe salt stress to crops, thereby threatening sustainable agriculture worldwide (Ahuja et al., 2010). As the consequence of increasing salinity, there has been more than fifty percent decline in major crop yields in arid and semi-arid regions (Bybordi, 2010). Thus, a better understanding of resistance mechanism of plants to abiotic stress such as salinity may help to solve the current threats to the security in food production around the world.

Salt stress can adversely influence crop growth by causing osmotic or water stress and ion toxicity as well as imbalanced nutrition in roots and leaves of plants (Farooq et al., 2015; Rivero et al., 2003). In addition, high salinity in soil brings about oxidative stress by generating ROS such as superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) in plants (Fahad et al., 2015), which affects plant physiological processes such as photosynthesis, respiration, and nitrogen fixation (Farooq et al., 2015). As

such, the abiotic stress under saline condition interrupt the entire plant growth, consequently leading to losses of crop production.

Seed germination is the most crucial phase in plant growth cycle. Complete seed germination can lead to vigorous development of early seedling growth, further contributing to plant productivity and propagation (Hasanuzzaman et al., 2013; Rajjou et al., 2012). First of all, testa and endosperm rupture and initial radicle elongation at the early phase of germination require uptake of water into seeds thoroughly, in order to facilitate metabolism during seed germination (Cheng et al., 2016; Rajjou et al., 2012). However, severe environmental stress such as elevated salinity can be deleterious to seed germination and seedling development (Cheng et al., 2016; Yuan et al., 2011). Salt-stress tolerance during seed germination varies depending on plant species (Qu et al., 2008). In particular, seeds of halophyte species can geminate and maintain viability in high salinity with increased capacity of water absorption from the soil substrate (Qu et al., 2008). However, toxicity of sodium ion (Na^+) and osmotic stress in extreme salt concentration could result in over-production of reactive oxygen species in plant seed tissue that can lead to oxidative stress and cellular damages during germination (Gill and Tuteja, 2010; Hameed et al., 2014; Sekmen et al., 2012).

Seeds treated with exogenous amino acids can alleviate inhibition of seed germination by salinity stress (Chang et al., 2010; Cheng et al., 2016). Cheng et al. (2016) investigated that exogenous applications of cysteine, glycine, methionine, and serine in MS medium containing 150 mM NaCl improved seed germination and abiotic stress defence mechanism of *Arabidopsis thaliana*. In another study, exogenous application of proline in salt solution (up to 400 mM NaCl) resulted in increases in seed germination rate, radicle length, and chlorophyll content of Malaysian rice (*Oryza sativa* L.) (Deivanai et al., 2011). Since the amino acids could aid not only to enhance plant physiological process but also to attenuate oxidative stresses under salt stress directly or indirectly, seed priming with amino acids may play significant roles

in enhancement of seed germination and early seedling establishment under salt stress (Teixeira et al., 2017). However, there is little research about seed priming effects with amino acids on plant growth and tolerance mechanism to salt stress.

Pasture productivity including that of Italian ryegrass (*Lolium multiflorum* L.) is primarily important for the national economy in New Zealand as a dairy industry country. There is widespread cultivation of Italian ryegrass in pastoral lands across the South and North Islands of New Zealand (Burgess and Easton, 1986), for production of forage species, livestock silage, and dry grass (Chapman et al., 2017; Wang et al., 2013a). However, this has recently been impacted adversely by environmental stresses including salinity and drought. There are few studies that reported tolerance of the Italian ryegrass to environmental stress such as salinity in soil (Marcar, 1987). Thus, continuous studies on physiological mechanism of the ryegrass to the abiotic stress would enhance its productivity under field conditions. It is hypothesised here that pre-sowing treatments with one or more of the 20 of L-amino acids and casein hydrolysate might increase production of *L. multiflorum* by enhancing resistance to environmental stresses such as salinity. The aims of this study were to identify the effects of seed-priming with L-amino acids and casein hydrolysate on germination and seedling development, and to investigate tolerance mechanism of the seedlings after seed priming to salt stress.

3.3. Materials and Methods

3.3.1. Seed, L-amino acids, and casein hydrolysate

Seeds of Italian ryegrass (*Lolium multiflorum* L.) supplied from a field service center, commercial dairy farmland of Lincoln University was used in the present study. The following

20 L-amino acids were used for seed priming: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. In addition, casein hydrolysate (CH), which is a mixture of 18 L-amino acids and some vitamins, was prepared for seed priming. The component and amount of each amino acid in commercially available CH chemical powder is shown in Table 3.1 (Sigma product information sheet).

Table 3.1. Comparison for each amino acid content in solutions of casein hydrolysate (CH, 200 mg L^{-1}) and individual amino acid (AA, 1mM) used for seed priming in the present study.

	Molecular weight (g mol^{-1})	1 mM AA in 100 ml (mg)	AA content in CH powder (mg g^{-1})	200 mg L^{-1} CH in 100 ml (mg)	Ratio of AA to CH
Alanine	89.09	8.91	30	0.6	15
Arginine	174.2	17.42	31	0.62	28
Aspartic acid	133.1	13.31	67	1.34	10
Cysteine	121.16	12.12	3	0.06	202
Glutamic acid	147.13	14.71	186	3.72	4
Glycine	75.07	7.51	19	0.38	20
Histidine	155.15	15.52	22	0.44	35
Isoleucine	131.17	13.12	44	0.88	15
Leucine	131.17	13.12	75	1.5	9
Lysine	182.65	18.27	68	1.36	13
Methionine	149.21	14.92	27	0.54	28
Phenylalanine	165.19	16.52	40	0.8	21
Proline	115.13	11.51	88	1.76	7
Serine	105.09	10.51	51	1.02	10
Threonine	119.12	11.91	42	0.84	14
Tryptophan	204.23	20.42	10	0.2	102
Tyrosine	181.19	18.12	28	0.56	32
Valine	117.151	11.72	59	1.18	10

3.3.2. Seed priming treatment

For pre-sowing treatment, *L. multiflorum* seeds were soaked in a solution containing 1 mM of one of the twenty L-amino acids (AAs) and 200 mg L^{-1} of CH. Casein hydrolysate has not been used in seed priming before. Previously, it has only been used widely in tissue culture (embryo culture) (i.e. Abiri et al., 2017; Ageel and Elmeer, 2011; Khaleda and Al-Forkan, 2006; Daniel et al., 2018, etc.) or orchid seed germination and seedling growth (Decruse et al., 2013; Parveen et al., 2016, etc.). Although a wide range of concentrations of casein hydrolysate was used in these studies, Sanders and Burkholder (1948) reported its optimal concentrations ranging between 200 and 400 mg L^{-1}. In addition, many studies found positive effect of CH application

when 200 mg L^{-1} CH was applied into the tissue culture medium, together with other plant growth regulator materials (Parveen et al., 2016; Payghamzadeh and Kazemitabar, 2010; Rizzo et al., 1998; Srivastav et al., 2004). However, in case of a study of Che Mohd. Zain et al. (2012), they found that increasing CH concentrations from 300 to 1000 mg L^{-1} did not show difference in the positive effect of CH on rice callus growth. Therefore, based on the results of the literature, 200 mg L^{-1} of casein hydrolysate solution was used in this study. Also, de-ionised (DI) water was used for hydro-priming (HP). Forty individual seeds were placed on a filter paper (Whatman No.1) in a Petri dish and were primed by soaking in 10 ml each AA (1 mM), CH (200 mg L^{-1}), and DI water for 24 hours. The Petri dishes were kept at 25 ˚C in a growth room for 24 hours in the dark. The primed seeds were air-dried for another 24 hours in the same growth room. The reason for air-drying the primed seeds was to allow return to the original moisture content within the seeds. In addition, non-priming (NP) treatment was included for control. For additional details on the seed priming procedure, see the section 2.2.1.

3.3.3. Salt stress treatment

Following seed priming and air-drying, 20 individual seeds were placed on a filter paper (Whatman No. 1) in a Petri dish and 10 ml of 60 or 90 mM sodium chloride (NaCl) or de-ionised water was added. The seeds were incubated at 25 ˚C in a growth room for 4 days in the dark. Germination percentage (GP) and radicle length (RL) of seedlings were measured after 4 days of germination, and physiological responses of the roots like antioxidant enzyme activities to different levels of salt (NaCl) stress were determined. For additional details on salt stress treatment, see the section 2.2.2.

3.3.4. Adjusting pH of NaCl solution

It was questioned throughout this study that pH variation between NaCl concentrations used for seed priming may influence seed germination and seedling growth of *L. multiflorum*. Therefore, there were three steps which were conducted to investigate pH effect on seedling growth as well as efficiency of seed-priming. Firstly, decreased pH level in NaCl solutions (60 and 90 mM) was set up at either pH 6.1 and 6.7 using 0.01 M and 0. 1 M MES (2-(N-morpholino) ethanesulfonic acid) buffer solutions, and the seeds were incubated for 4 days in a growth chamber room. Secondly, L-proline dissolved in 0.01M MES buffers (pH 5.5, 6.1, and 6.7) were prepared for seed priming, and then the seeds were incubated in 0, 60, and 90 mM NaCl (see 3.3.2). Lastly, priming reagents including methionine, proline, and valine were prepared with 0.01 M MES (pH 6.1) buffer to investigate their effects on the seed germination in 0, 60, and 90 mM NaCl and in the presence of the MES buffer.

3.3.5. Plant analyses

3.3.5.1. Germination percentage and radicle length

The number of germinated seeds of *L. multiflorum* in each Petri dish was counted after 4-day-incubation. Germination percentage (GP) of the seeds was calculated as a formula below. To measure radicle length (RL), in each Petri-dish ten seedlings with radicles more than 2 mm extruded from the seed coat were randomly selected. The seed incubation trial was performed in duplicate, and in each treatment, there were four replications.

Germination percentage (%) = (the number of germinated seeds/total number of seeds) x 100

3.3.5.2. Antioxidant enzyme activities

a. Enzyme extraction and protein content

To assay activities of peroxidase (POD), Italian ryegrass seeds from different priming treatments in response to salt stress, 0.1 g of seedling roots was harvested after 4 days of incubation in 0-90 mM NaCl. For POD analysis, the roots were homogenised with liquid N in a mortar and pestle and extracted with 1.2 ml of 0.1 M potassium phosphate buffer (pH 6.59). The homogenates were centrifuged at 10,000 g for 5 min at 4 ˚C (Baque et al., 2010). Supernatants were stored at -80 ˚C, prior to analyses of POD activity. Total protein content in the extracts was also measured at a wavelength of 590 nm using an Ultrospec^{TM} 2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA) as described in Bradford (1976).

b. Peroxidase activity (POD (EC. 1.11.1.7))

After extraction, the supernatant (50 ul) was added to a mixture of 893 µl of 0.1 M potassium phosphate buffer (pH 6.59), 2 µl of guaiacol, and 5 µl of 10% H_2O_2 for 5 min at room temperature until the color reaction is developed to orange. The absorbance of the reaction mixture was then measured spectroscopically at a wavelength of 470 nm using an Ultrospec^{TM} 2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA). The POD activity (units•mg^{-1} protein) was calculated.

3.3.6. Statistical analysis

This experiment was laid out in a completely randomised design (CRD) with two times and four replications. One-way analysis of variance (ANOVA) was conducted with all the data for confirming the variability of data. Fisher's exact test ($p< 0.05$) was performed to determine significant difference between treatments using a Minitab 16.

3.4. Results and Discussion

3.4.1. Identification of the potential of L-amino acids and casein hydrolysate for seed priming

3.4.1.1. Effect on seed germination

Twenty L-amino acids and casein hydrolysate were used for seed priming in the present study. The effects of the seed priming treatments on GP of Italian ryegrass seeds incubated in 0, 60, and 90 mM NaCl were analysed and shown in Table 3.2a. There was no significant difference ($p > 0.05$) in GP on distilled water observed in the seeds of the NP, and the other seed priming treatments. After 4 days of incubation, NaCl toxicity appeared to reduce GP of seeds in the NP treatment. GP of seeds in the HP treatment was significantly (about 15%) higher than that in the NP treatment, particularly when the seeds were incubated in 60 mM NaCl ($p < 0.05$). Among the 20 L-amino acids, tyrosine and phenylalanine primed seeds showed the highest GP up to about 94 and 93%, respectively, when the seeds were incubated in water (Table 3.2b). In 60 mM NaCl, GP of the seeds primed with the following three L-amino acids and CH was significantly higher than NP seeds ($p < 0.05$) (Table 3.2b): tryptophan (90%), methionine (87%), and glycine (86%) and CH (87%) and NP (71%). However, the GP of seeds primed with these three amino acids and CH was not significantly different from that of HP seeds ($p > 0.05$). In 90 mM NaCl, fewer L-amino acids, for example, lysine and leucine applied for seed priming resulted in significantly higher GP than NP and/or HP ($p < 0.05$) (Table 3.2a).

In this study, salinity stress inhibited seed germination of *L. multiflorum* probably by impeding water uptake into the seed (Farooq et al., 2015). However, the seeds primed with L-amino acids including tryptophan, methionine, and glycine and CH appeared to result in higher GP, particularly when the Italian ryegrass seeds were germinated in 60 mM NaCl. Although there is no similar case study about seed priming effect with AAs so far, some researchers

found beneficial effect on GP when seeds were incubated in a solution containing both AAs and NaCl. For example, Cheng et al. (2016) found that seeds of *Arabidopsis thaliana* exogenously applied with glycine, cysteine, serine, and methionine in 150 mM NaCl increased germination percentages, by 41, 73, 55, and 57%, respectively, while there was no effect of external application of the other L-amino acids including alanine, asparagine, and glutamine on seed germination even in absence of NaCl treatment.

L-methionine produces S-adenosylmethionine which is a precursor of several metabolites such as glycinebetaine, methylated polyols, polyamines, and ethylene in higher plants (Ogawa and Mitsuya, 2011). Subsequent production of L-methionine and the metabolites increase salt stress tolerance by regulating osmotic balance, cell proliferation, enzyme activities in plants (Ogawa and Mitsuya, 2011). Tryptophan is associated with auxin biosynthesis including phytoalexin camalexin, phenyylpropanoids, other natural products in plants (El-Awadi and Hassan, 2010) and supply high food nutritional quality with the other essential amino acids such as lysine, methionine, and threonine (Tzin and Galili, 2010; Wakasa and Ishihara, 2009). In fennel (*Foeniculum vulgare* L.) seedlings, seed priming with L-methionine and -tryptophan showed positive effects on plant height and fresh and dry weights (El-Awadi and Hassan, 2010). In this study, the germination enhancement of Italian ryegrass seeds was observed after priming with L-methionine and -tyrptophan.

Table 3.2a. Differences in germination percentages (GP) of seeds primed with L-amino acids priming (AAP) and casein hydrolysate priming (CHP) compared to non-priming (NP) and hydro-priming (HP). After seed priming, the seeds were incubated in 0, 60, and 90 mM NaCl. Values present the mean of difference rate of GP between priming treatments (n=8). The actual germination percentages in the different treatments are given in the accompanying Table 3.2b.

Amino acids	Score	0 mM NaCl (pH 6.1)		60 mM NaCl (pH 5.3)		90 mM NaCl (pH 5.4)	
		vs NP	*vs* HP	*vs* NP	*vs* HP	*vs* NP	*vs* HP
		(%)					
Arginine[1]	0	3 [ns]	3 [ns]	10 [ns]	-4 [ns]	- 14 [ns]	- 10 [ns]
Asparagine[1]	2	7 [ns]	7 [ns]	19 [a]	4 [ns]	- 16 [ns]	- 13 [ns]
Aspartic acid[1]	2	-13 [ns]	-13 [b]	17 [a]	2 [ns]	- 20 [ns]	- 18 [b]
Cysteine[1]	0	5 [ns]	5 [ns]	12 [ns]	-2 [ns]	-3 [ns]	1 [ns]
Glutamic acid[1]	2	-13 [ns]	-13 [ns]	17 [a]	2 [ns]	- 20 [ns]	- 18 [b]
Glutamine[1]	2	-1 [ns]	-1 [ns]	19 [a]	4 [ns]	4 [ns]	8 [ns]
Histidine[1]	0	5 [ns]	5 [ns]	5 [ns]	-8 [ns]	-10 [ns]	-6 [ns]
Lysine[1]	5	7 [ns]	7 [ns]	19 [a]	4 [ns]	14 [ns]	18 [a]
Serine[1]	0	7 [ns]	7 [ns]	14 [ns]	-1 [ns]	-1 [ns]	3 [ns]
Threonine[1]	0	7 [ns]	7 [ns]	14 [ns]	-1 [ns]	-11 [ns]	-8 [ns]
Alanine[2]	2	-1 [ns]	-1 [ns]	18 [a]	2 [ns]	2 [ns]	6 [ns]
Glycine[2]	2	-2 [ns]	-2 [ns]	21 [a]	5 [ns]	-3 [ns]	1 [ns]
Leucine[2]	6	9 [ns]	9 [ns]	15 [ns]	0 [ns]	25 [a]	30 [a]
Methionine[2]	2	-1 [ns]	-1 [ns]	22 [a]	6 [ns]	2 [ns]	6 [ns]
Proline[2]	0	7 [ns]	7 [ns]	8 [ns]	-6 [ns]	7 [ns]	11 [ns]
Phenylalanine[2]	0	10 [ns]	10 [ns]	12 [ns]	-2 [ns]	-15 [ns]	- 12 [ns]
Tryptophan[2]	2	7 [ns]	7 [ns]	26 [a]	10 [ns]	2 [ns]	6 [ns]
Tyrosine[2]	3	11 [ns]	11 [ns]	16 [a]	1 [ns]	- 18 [ns]	- 15 [ns]
Valine[2]	0	7 [ns]	7 [ns]	8 [ns]	-6 [ns]	7 [ns]	11 [ns]
Isoleucine[2]	0	7 [ns]	7 [ns]	14 [ns]	-1 [ns]	-6 [ns]	-3 [ns]
Casein hydrolysate[4]	2	1 [ns]	1 [ns]	22 [a]	6 [ns]	2 [ns]	6 [ns]

[1] Polar
[2] Non-polar
[ns] no significance in GP between AAP and CHP *vs* either NP and HP
[a] significantly higher GP in AAP and CHP than in either NP and HP
[b] significantly lower GP in AAP than in either NP and HP

Score criteria: If AAs priming in GP has more effect in comparison with Control (0 mM NaCl), the score is "1". If AAs priming in GP has more effect in comparison with 60 mM NaCl, the score is "2". If AAs priming in GP has more effect in comparison with 90 mM NaCl, the score is "3". Then, all the scores are totalled up.

Table 3.2b. Actual germination percentages (%) in the different seed priming treatments. Data presented are mean±SE (n=8). Within each NaCl concentration, different letters indicate significant difference by Fisher's range test at $p<0.05$.

Priming treatment	---------- NaCl treatement ----------		
	0 mM	60 mM	90 mM
NP	84.37±3.00 ^{abcd}	71.25±4.27 ^{d}	73.33±2.50 ^{bcdef}
HP	84.45±1.33 ^{b}	81.82±2.91 ^{abc}	70.73±1.24 ^{cdef}
Arginine	86.67±1.67 ^{ab}	78.33±6.67 ^{abcd}	63.33±3.33 ^{defgh}
Asparagine	90.00±0.00 ^{ab}	85.00±5.77 ^{abc}	61.67±6.01 ^{efgh}
Aspartic-acid	73.33±11.67 ^{d}	83.33±1.67 ^{abc}	58.33±1.67 ^{h}
Cysteine	88.75±5.20 ^{ab}	80.00±3.54 ^{abcd}	71.25±2.39 ^{bcdefgh}
Glutamic acid	73.33±11.67 ^{cd}	83.33±1.67 ^{abc}	58.33±1.67 ^{gh}
Glutamine	83.75±3.15 ^{abcd}	85.00±6.12 ^{abc}	76.25±3.75 ^{bcd}
Histidine	88.75±4.27 ^{ab}	75.00±2.04 ^{cd}	66.25±5.54 ^{cdefgh}
Lysine	90.00±2.89 ^{ab}	85.00±0.00 ^{abc}	83.33±6.67 ^{ab}
Serine	90.00±3.54 ^{ab}	81.25±3.15 ^{abcd}	72.50±3.23 ^{bcdef}
Threonine	90.00±2.04 ^{ab}	81.25±3.75 ^{abcd}	65.00±5.40 ^{cdefgh}
Alanine	83.75±4.73 ^{abcd}	83.75±3.75 ^{abc}	75.00±2.04 ^{bcde}
Glycine	82.50±3.23 ^{bcd}	86.25±2.39 ^{ab}	71.25±8.26 ^{bcdefgh}
Leucine	91.67±1.67 ^{ab}	81.67±8.33 ^{abcd}	91.67±1.67 ^{a}
Methionine	83.33±4.41 ^{abcd}	86.67±1.67 ^{abc}	75.00±7.64 ^{bcde}
Proline	90.00±5.00 ^{ab}	76.67±3.33 ^{bcd}	78.33±9.28 ^{abc}
Phenylalanine	92.50±3.23 ^{ab}	80.00±5.00 ^{abcd}	62.50±4.79 ^{efgh}
Tryptophan	90.00±0.00 ^{ab}	90.00±2.89 ^{a}	75.00±5.77 ^{bcde}
Tyrosine	93.75±2.39 ^{a}	82.50±4.33 ^{abc}	60.00±2.04 ^{fgh}
Valine	90.00±5.00 ^{ab}	76.67±3.33 ^{bcd}	78.33±9.28 ^{abc}
Iso-leucine	90.00±4.08 ^{ab}	81.25±5.54 ^{abcd}	68.75±3.15 ^{cdefgh}
Casein hydrolysate	85.00±2.89 ^{abcd}	86.67±6.01 ^{abc}	75.00±7.64 ^{bcde}

3.4.1.2. Effects on seedling radicle elongation

Similar to the effects found in the germination trial above, seed priming with some L-amino acids (AAs) and CH seemed to also benefit the early stage of seedling development under salt stress (Tables 3.3a and 3.3b). Radicle length (RL) of NP seedlings of *L. multiflorum* was gradually reduced with increasing NaCl concentrations: 24.4 mm (at 0 mM), 9.3 mm (at 60 mM), and 4.2 mm (at 90 mM). Although HP tended to increase RL more than NP in 0 and 60 mM NaCl treatments, more significant increases in RL were found in seedlings from seeds following priming with some AAs and CH ($p< 0.05$) (Table 3.3b). Moreover, influence of seed priming on the RL of *L. multiflorum* seedlings differed depending on the L-amino acids used for seed priming (Table 3.3a). Among the polar L-AAs used for seed priming, glutamine, lysine, and arginine were of particular interest. Seedlings from seeds primed with these three amino acids exhibited significantly greater RL than that of seeds of the NP and HP treatments when the seedlings were grown in 0 mM NaCl ($p< 0.05$). When grown in 60 mM NaCl, RL of the seedlings from seeds primed with glutamine, lysine, and serine was increased up to 66, 66, and 38%, respectively, which were significantly greater than that of NP seeds ($p< 0.05$) (Table 3.3a). When grown in 90 mM NaCl, seedlings from seeds primed with L-lysine showed significantly greater RL (about 250%) than that of NP seeds ($p<0.05$). Lysine, arginine, and glutamine are widely used for nitrogen storage and transport in plants (Miflin and Habash, 2002; Skinner and Street 1954). Inorganic nitrogen including nitrate (NO_3^-) and ammonium (NH_4^+) absorbed by roots is used for synthesising other amino acids and nitrogenous compounds and is incorporated into glutamine and glutamate as primary N assimilation (Skinner and Street, 1954; Okumoto and Pilot, 2011). Skinner and Street (1954) found an increase in root elongation and lateral root development of *Syringa vulgaris* seedings incubated in medium containing lysine and arginine, respectively. It might be that lysine is a greatly

effective seed priming reagent as a sole nitrogen source (Skinner and Street, 1954). Lysine is biosynthesised from aspartate as a precursor, which is under the regulation of a complex pathway (Azevedo et al., 2006). Particularly, dihydrodipicolinate synthase enzyme is required for lysine biosynthesis. For example, root cultures of transgenic potato plant (*Solanum tuberosum* L.) exhibited a higher level of dihydrodipicolinate synthase enzyme activity which is related to the accumulation of lysine (Perl et al., 1992). As such, lysine used for seed priming in this study might enhance radicle length of *L.mutiflroum* seedlings. Kan et al. (2015) observed an increase in root length of rice seedlings treated with 0.1 and 0.5 mM of glutamine compared to control (DI water). The increase in radicle length in this result suggests that the amino acids were potential nitrogen source in the roots of *L. multiflorum*. When grown in 90 mM NaCl, seedlings from seeds primed with L-methionine and CH, there was a significantly greater RL (about 481 and 378%, respectively) than that of NP seeds ($p<0.05$). In contrast, L-glutamic acid priming appeared to have a negative effect on RL development of ryegrass compared to NP, particularly when the seedlings were grown in 90 mM NaCl. Non-polar L-AAs showed more effectiveness in seed priming in terms of radicle growth of seedlings grown in NaCl treatments than polar L-AAs (Table 3.3a). Of the non-polar AAs, valine, tryptophan, and methionine appeared to be of interest for beneficial seed priming as far as radicle development in 60 and 90 mM NaCl is concerned. When the seedlings were grown in 90 mM NaCl, priming seeds with L-methionine resulted in seedlings with significantly (about 6 times) greater RL than that those of NP ($p< 0.05$). In contrast, seedlings from seeds primed with L-proline, -tyrosine, and -isoleucine exhibited significantly reduced RL compared to those from NP when the seedlings were incubated in 90 mM NaCl ($p< 0.05$). In addition, the RL of the seedlings from seed priming with L-aspartic acid with a charged acidic side chain was not different from that of the seedlings in NP and HP. CH priming application showed a very significant impact on the RL development in seedlings grown in NaCl treatments, compared to NP and HP (Table

3.3a). Particularly, the RL of seedlings primed with CH was about 2.6 and 4.8 times greater than that with NP in 60 and 90 mM NaCl, respectively.

Throughout the Petri dish experiments, a considerable decrease in RL of *L. multiflorum* seedlings had been seen with increasing NaCl concentrations (Table 3.3a). It can be inferred that the increased salt stress on the roots inhibited primary root elongation and lateral root formation of the seedlings by suppressing root meristem activity including cell division, expansion, and differentiation during a week of incubation (Liu et al., 2015; West et al., 2004). However, in the present study, priming with L-methionine and CH appeared to markedly diminish the adverse effect of salinity stress on the radicle elongation of the Italian ryegrass seedlings. Application with these amino acids to seeds prior to sowing might play a positive role in resistance induction to the abiotic stress via enhancement of nutrient status (i.e. nitrogen), metabolic activity, and antioxidant system within the seeds during germination and early seedling growth (Nasibi et al., 2016; Teixeira et al., 2017).

Table 3.3a. Differences in radicle length (RL) of *L. multiflorum* seedlings primed with L-amino acids priming (AAP) and casein hydrolysate priming (CHP) compared to non-priming (NP) and hydro-priming (HP). After seed priming, the seeds were incubated in 0, 60, and 90 mM NaCl. Values present the mean of difference of RL (in percentages) between priming treatments (AAP including CHP *vs* NP and HP) (n=8). The actual RL (in mm) in the different treatments are given in Table 3.3b.

Amino acids	Score	0 mM NaCl (pH 6.1)		60 mM NaCl (pH 5.3)		90 mM NaCl (pH 5.4)	
		vs NP	*vs* HP	*vs* NP	*vs* HP	*vs* NP	*vs* HP
		(%)					
Arginine[1]	1	45 [a]	0 [ns]	40 [ns]	30 [ns]	-25 [b]	19 [ns]
Asparagine[1]	0	20 [ns]	-17 [ns]	-1 [ns]	-9 [ns]	13 [ns]	37 [ns]
Aspartic acid[1]	0	-12 [ns]	-39 [b]	9 [ns]	1 [ns]	-37 [b]	0 [ns]
Cysteine[1]	0	17 [ns]	-19 [ns]	18 [ns]	9 [ns]	-7 [ns]	48 [ns]
Glutamic acid[1]	0	-4 [ns]	-34 [ns]	29 [ns]	20 [ns]	- 35 [ns]	3 [ns]
Glutamine[1]	5	47 [a]	2 [ns]	68 [a]	55 [a]	-14 [ns]	35 [ns]
Histidine[1]	1	26 [a]	-13 [ns]	17 [ns]	8 [ns]	-31 [ns]	9 [ns]
Lysine[1]	12	56 [a]	8 [a]	66 [a]	54 [a]	249 [a]	454 [a]
Serine[1]	0	17 [ns]	-19 [ns]	41 [ns]	31 [ns]	-25 [ns]	19 [ns]
Threonine[1]	0	14 [ns]	- 22 [ns]	22 [ns]	14 [ns]	-9 [ns]	45 [ns]
Alanine[2]	0	17 [ns]	-19 [ns]	46 [ns]	36 [ns]	-3 [ns]	54 [ns]
Glycine[2]	0	13 [ns]	-22 [ns]	45 [ns]	35 [ns]	-7 [ns]	47 [ns]
Leucine[2]	1	27 [a]	-12 [ns]	5 [ns]	-3 [ns]	7 [ns]	69 [ns]
Methionine[2]	12	75 [a]	21 [a]	241 [a]	216[a]	481 [a]	821 [a]
Proline[2]	0	12 [ns]	-23 [ns]	42 [ns]	32 [ns]	-33[ns]	6 [ns]
Phenylalanine[2]	1	29 [a]	-11 [ns]	25 [ns]	16 [ns]	-14 [ns]	37 [ns]
Tryptophan[2]	5	44 [a]	0 [ns]	139 [a]	122 [a]	23 [ns]	95 [ns]
Tyrosine[2]	4	12 [ns]	-22 [ns]	56 [a]	45 [a]	-29 [ns]	12 [ns]
Valine[2]	4	45 [a]	0 [ns]	27 [ns]	18 [ns]	33 [ns]	111 [a]
Isoleucine[2]	1	23 [a]	-15 [ns]	24 [ns]	15 [ns]	-25 [ns]	19 [ns]
Casein hydrolysate[4]	12	62 [a]	12 [a]	145 [a]	128 [a]	378 [a]	657 [a]

[1] Polarity
[2] Non-polarity
[ns] no significance in RL between AAP and CHP *vs* either NP and HP
[a] significantly longer RL in AAP and CHP than in either NP and HP
[b] significantly shorter RL in AAP and CHP than in either NP and HP

Score criteria: If AAs priming in RL has more effect in comparison with Control (0 mM NaCl), the score is "1". If AAs priming in RL has more effect comparison with 60 mM NaCl, the score is "2". If AAs priming in RL has more effect in comparison with 90 mM NaCl, the score is "3". Then, all the scores are totalled up.

Table 3.3b. Actual radical length (in mm) in the different seed priming treatments. Data presented are mean±SE (n=8). Within each NaCl concentration, different letters indicate significant difference by Fisher's range test at $p<0.05$.

Priming treatment	NaCl treatment		
	0 mM	60 mM	90 mM
NP	24.40±0.41 ^{gh}	9.27±1.22 ^{e}	4.15±0.32 ^{de}
HP	31.30±2.17 ^{cdef}	10.00±1.23 ^{e}	2.62±0.15 ^{e}
Arginine	35.37±2.02 ^{bcd}	12.97±1.33 ^{cde}	3.12±0.06 ^{de}
Asparagine	29.20±2.78 ^{efg}	9.15±0.47 ^{e}	3.60±0.47 ^{de}
Aspartic-acid	21.59±1.24 ^{h}	10.06±0.52 ^{e}	2.61±0.13 ^{e}
Cysteine	28.58±1.43 ^{fg}	10.92±1.34 ^{de}	3.88±0.31 ^{de}
Glutamic acid	23.34±3.65 ^{gh}	11.97±1.70 ^{cde}	2.71±0.34 ^{de}
Glutamine	35.95±2.92 ^{bc}	15.53±2.05 ^{c}	3.55±0.21 ^{de}
Histidine	30.70±1.48 ^{cdef}	10.80±0.75 ^{de}	2.85±0.23 ^{de}
Lysine	38.13±0.78 ^{ab}	15.37±2.08 ^{cd}	14.50±5.30 ^{c}
Serine	28.49±0.74 ^{fg}	13.06±0.45 ^{cde}	3.13±0.39 ^{de}
Threonine	27.74±1.30 ^{fg}	11.35±0.93 ^{cde}	3.79±0.29 ^{de}
Alanine	28.63±1.57 ^{fg}	13.56±0.54 ^{cde}	4.04±0.20 ^{de}
Glycine	27.52±2.56 ^{fg}	13.48±0.28 ^{cde}	3.86±0.71 ^{de}
Leucine	31.05±1.44 ^{cdef}	9.73±0.69 ^{e}	4.44±0.13 ^{de}
Methionine	42.63±1.63 ^{a}	31.62±2.39 ^{a}	24.13±2.45 ^{a}
Proline	27.21±2.32 ^{fgh}	13.17±1.28 ^{cde}	2.79±0.38 ^{de}
Phenylalanine	31.48±1.67 ^{cdef}	11.63±0.17 ^{cde}	3.58±0.30 ^{de}
Tryptophan	35.23±0.95 ^{bcde}	22.18±6.23 ^{b}	5.12±0.56 ^{de}
Tyrosine	27.43± 0.78 ^{fg}	14.50±0.33 ^{cd}	2.94±0.16 ^{de}
Valine	35.26±0.83 ^{bcde}	11.78±0.93 ^{cde}	5.53±0.63 ^{d}
Iso-leucine	30.08±4.12 ^{def}	11.47±0.88 ^{cde}	3.11±0.17 ^{de}
Casein hydrolysate	39.46±1.22 ^{ab}	22.75±2.90 ^{b}	19.84±0.47 ^{b}

3.4.1.3. Effects of amino acid priming on peroxidase enzyme activity

The effects of twenty L-amino acids and casein hydrolysate used for seed priming in this study on peroxidase (POD) activity in roots of 4-day-old *L. multiflorum* seedlings are shown in Tables 3.4a and 3.4b. POD activities in roots of seedlings from the seeds primed with various L-AAs including arginine, glutamic acid, histidine, methionine, proline, tryptophan, and leucine were significantly higher (up to 57%) than those in from seeds in the NP and HP treatments when the seedlings were grown in 60 mM NaCl ($p< 0.05$). In contrast, priming with alanine, asparagine, glutamine, glycine, isoleucine, and tyrosine did not significantly increase POD activity in the roots of the seedlings grown in 60 mM NaCl, compared to either NP and HP ($p> 0.05$). L-AAs such as methionine and tryptophan appeared to be effective in terms of inducing POD activity in the roots of the seedlings grown in 90 mM NaCl. These amino acids are considered as physiological precursors of plant hormones involved in regulation of root and shoot growth (El-Awadi and Hassan, 2010; Naveed et al., 2015; Quiroz-Villareal et al., 2012). El-Awadi and Hassan (2010) found that pre-sowing with methionine and tryptophan to seed of *Foeniculum vulgare* led to significant increases in plant growth. Additionally, both amino acids could act as antioxidants scavenging ROS toxicity in plants (Namiki, 1990). In this respect, it seems that methionine and tryptophan might contribute to the increase in POD enzyme activity of the *L. multiflorum* roots under salinity stress in this study. In addition, roots of the seedlings from seeds primed with CH showed considerably higher POD activity than those of seedlings from NP and HP treatments ($p< 0.05$), particularly when the seedlings were grown in 90 mM NaCl. These results suggest that methionine, tryptophan, and casein hydrolysate were probably taken up within the seeds during priming. Then these amino acids could increase POD activity, which would scavenge salinity-induced reactive oxygen species in the seedling roots. Teixeira et al. (2017) also found that priming soybean seeds (*Glycine max* L.) with a combination of

glutamate, cysteine, phenylalanine, and glycine increased POD activity in leaves of the plant. In this study, the soil was not amended with added NaCl.

Table 3.4a. Differences in peroxidase (POD) enzyme activity in roots of *L. multiflorum* seedlings primed with L-amino acids priming (AAP) and casein hydrolysate priming (CHP) compared to non-priming (NP) and hydro-priming (HP). After seed priming, the seeds were incubated in 0, 60, and 90 mM NaCl. Values present the mean of difference in POD activities between priming treatments (AAP including CHP *vs* NP and HP) (n=8). The actual enzyme activity measurements are given in Table 3.4 b.

Amino acids	Score	0 mM NaCl (pH 6.1)		60 mM NaCl (pH 5.3)		90 mM NaCl (pH 5.4)	
		vs NP	*vs* HP	*vs* NP	vs HP	vs NP	*vs* HP
		(%)					
Arginine[1]	12	36 [a]	52 [a]	57 [a]	31 [a]	31 [a]	36 [a]
Asparagine[1]	2	23 [a]	37 [a]	7 [ns]	-11 [ns]	18 [ns]	22 [ns]
Aspartic acid[1]	4	32 [a]	48 [a]	32 [a]	11 [ns]	-20 [ns]	-17 [ns]
Cysteine[1]	4	29 [a]	44 [a]	24 [a]	4 [ns]	5 [ns]	9 [ns]
Glutamic acid[1]	4	10 [ns]	23 [ns]	46 [a]	22 [a]	-13 [ns]	-9 [ns]
Glutamine[1]	2	36 [a]	52 [a]	15 [ns]	-4 [ns]	-1 [ns]	3 [ns]
Histidine[1]	6	34 [a]	50 [a]	46 [a]	22 [a]	16 [ns]	21 [ns]
Lysine[1]	9	18 [ns]	32 [a]	31 [a]	9 [ns]	36 [a]	42 [a]
Serine[1]	4	46 [a]	63 [a]	42 [a]	18 [ns]	7 [ns]	12 [ns]
Threonine[1]	3	13 [ns]	26 [a]	40 [a]	17 [ns]	10 [ns]	15 [ns]
Alanine[2]	0	1 [ns]	12 [ns]	-4 [ns]	-20 [ns]	-5 [ns]	-1 [ns]
Glycine[2]	0	-6 [ns]	5 [ns]	19 [ns]	0 [ns]	11 [ns]	16 [ns]
Leucine[2]	6	26 [a]	41 [a]	45 [a]	21 [a]	17 [ns]	22 [ns]
Methionine[2]	12	27 [a]	42 [a]	47 [a]	23 [a]	41 [a]	47 [a]
Proline[2]	12	37 [a]	53 [a]	57 [a]	32 [a]	26 [a]	31 [a]
Phenylalanine[2]	2	8 [ns]	20 [ns]	43 [a]	19 [ns]	-32 [ns]	-29 [ns]
Tryptophan[2]	12	30 [a]	45 [a]	47 [a]	23 [a]	37 [a]	43 [a]
Tyrosine[2]	2	22 [a]	36 [a]	16 [ns]	-3 [ns]	9 [ns]	13 [ns]
Valine[2]	4	27 [a]	42 [a]	36 [a]	14 [ns]	-32 [ns]	-29 [ns]
Isoleucine[2]	0	-5 [ns]	6 [ns]	19 [ns]	-1 [ns]	-2 [ns]	2 [ns]
Casein hydrolysate[4]	12	34 [a]	50 [a]	51 [a]	26 [a]	35 [a]	41 [a]

[1] Polarity
[2] Non-polarity
[ns] no significance in POD between AAP *vs* either NP and HP
[a] significantly higher PODin AAP than in either NP and HP
[b] significantly lower PODin AAP than in either NP and HP

Score criteria: If AAs priming in POD has more effect in comparison with Control (0 mM NaCl), the score is "1". If AAs priming in POD has more effect in comparison with 60 mM NaCl, the score is "2". If AAs priming in POD has more effect in comparison with 90 mM NaCl, score is "3". Then, all the scores are totalled up.

Table 3.4b. Actual POD activity (unit/mg protein min) measurements in the different treatments. Data presented are mean±SE (n=8). Within each NaCl concentration, different letters indicate significant difference by Fisher's range test at $p<0.05$.

Priming treatment	NaCl treatment		
	0 mM	60 mM	90 mM
NP	1.82±0.18 ^{fgh}	1.59±0.21 ^{f}	1.76±0.06 ^{efg}
HP	1.63±0.11 ^{h}	1.90±0.06 ^{cdef}	1.69±0.07 ^{efg}
Arginine	2.47±0.00 ^{ab}	2.49±0.00 ^{a}	2.30±0.13 ^{abc}
Asparagine	2.24±0.22 ^{bcde}	1.70±0.14 ^{ef}	2.07±0.25 ^{bcde}
Aspartic-acid	2.41±0.19 ^{abc}	2.10±0.17 ^{abcd}	1.40±0.19 ^{g}
Cysteine	2.35±0.13 ^{abcd}	1.97±0.09 ^{bcde}	1.85±0.03 ^{def}
Glutamic acid	2.01±0.29 ^{cdefgh}	2.32±0.07 ^{ab}	1.53±0.09 ^{fg}
Glutamine	2.47±0.01 ^{ab}	1.83±0.16 ^{def}	1.74±0.18 ^{efg}
Histidine	2.44±0.00 ^{ab}	2.32± 0.06 ^{ab}	2.04±0.13 ^{bcde}
Lysine	2.15±0.10 ^{bcdef}	2.08±0.16 ^{abcde}	2.40±0.08 ^{ab}
Serine	2.66±0.20 ^{a}	2.25±0.21 ^{abc}	1.89±0.15 ^{def}
Threonine	2.06±0.17 ^{cdefg}	2.22±0.14 ^{abcd}	1.94±0.23 ^{cde}
Alanine	1.83± 0.09 ^{efgh}	1.52±0.40 ^{f}	1.67±0.08 ^{efg}
Glycine	1.71±0.20 ^{gh}	1.90±0.24 ^{cdef}	1.96±0.35 ^{cde}
Leucine	2.30±0.13 ^{abcd}	2.30±0.19 ^{ab}	2.06±0.23 ^{bcde}
Methionine	2.31±0.12 ^{abcd}	2.33±0.15 ^{ab}	2.49±0.01 ^{a}
Proline	2.50±0.00 ^{ab}	2.50±0.00 ^{a}	2.21±0.11 ^{abcd}
Phenylalanine	1.96±0.08 ^{defgh}	2.27±0.22 ^{abc}	1.20±0.16 ^{cde}
Tryptophan	2.37±0.11 ^{abc}	2.34±0.15 ^{ab}	2.41±0.07 ^{ab}
Tyrosine	2.22±0.07 ^{bcde}	1.85±0.06 ^{def}	1.91±0.10 ^{cdef}
Valine	2.31±0.16 ^{abcd}	2.16±0.15 ^{abcd}	1.20±0.15 ^{cde}
Iso-leucine	1.73±0.01 ^{gh}	1.89±0.03 ^{cdef}	1.72±0.04 ^{efg}
Casein hydrolysate	2.44±0.06 ^{ab}	2.40±0.18 ^{a}	2.38±0.10 ^{ab}

3.4.1.4. Beneficial amino acids for seed priming

Overall, seed priming with several L-amino acids such as lysine, methionine, and tryptophan and also casein hydrolysate showed significant benefits for germination and seedling growth under salt stress. Therefore, these seed-priming agents in the present study appeared to enhance salt tolerance of Italian ryegrass seedlings. Based on the calculations that these three AAs and CH had over 20 scores, they can be classified as beneficial agents for seed priming in this study. In terms of root growth and antioxidant enzyme activity under salt stress via a scoring evaluation of each amino acid and casein hydrolysate (Table 3.5), in particular, seedlings from seeds primed with L-methionine and casein hydrolysate showed relatively improved radicle growth and POD enzyme activity than the seedlings primed with other AAs. In addition, seed priming with L-methionine and casein hydrolysate effectively provented the reduction in seed germination percentage caused by salt stress (i.e. in 60 mM NaCl). Thus, further studies were performed to have a closer examination of these results as well as an investigation of resistance mechanism to salt stress by seed priming with L-methionine and CH in Chapter 4.

Table 3.5. Sum of scores of L-amino acids (AAs) and casein hydrolysate (CH) primed seeds assigned based on measurements of germination percentage (GP), radicle length (RL), and peroxidase (POD) enzyme activity following statistical comparisons with non-primed (NP) and hydro-primed (HP) seeds. If each AA or CH priming significantly increased GP, RL, and POD activity compared to either NP and HP ($p < 0.05$), it is total-up from GP, RL, and POD, from Table 3.2a, 3.3a, and 3.4a, respectively.

Amino acids	Total score	Measurement		
		Germination percentage[a]	Radicle length[b]	Peroxidase activity[c]
Arginine[1]	13	0	1	12
Asparagine[1]	4	2	0	2
Aspartic acid[1]	4	0	0	4
Cysteine[1]	7	0	3	4
Glutamic acid[1]	6	2	0	4
Glutamine[1]	12	2	8	2
Histidine[1]	7	0	1	6
Lysine[1]	26	5	12	9
Serine[1]	9	2	3	4
Threonine[1]	8	2	3	3
Alanine[2]	9	2	7	0
Glycine[2]	6	2	4	0
Leucine[2]	16	6	4	6
Methionine[2]	26	2	12	12
Proline[2]	12	0	0	12
Phenylalanine[2]	9	0	4	5
Tryptophan[2]	22	2	8	12
Tyrosine[2]	8	2	4	2
Valine[2]	11	0	7	4
Isoleucine[2]	0	0	0	0
Casein hydrolysate[4]	26	2	12	12

[1] Polarity
[2] Non-polarity
[a] From the scores shown in Table 3.2a
[b] From the scores shown in Table 3.3a
[c] From the scores shown in Table 3.4a

3.4.2. Influence of pH variability on seed germination

3.4.2.1. Cause of pH reduction

Like temperature, light, and soil moisture, pH of the solution surrounding seeds is another environmental factor that could influence seed germination (Chachalis and Reddy, 2000; Nandula et al., 2006). In addition, development of a young seedling root consisting of root tip,

meristematic region, transition, and elongation zones is sensitive to many external factors such as pH (Kagenishi et al., 2016). Italian ryegrass (*L. multiflorum*) has been grown in weak acidic field soil at around pH 5.0, but its best growth is shown on soil ranging between pH 6 and 7, with pH 8 as maximum (CABI, n.d.). In the present study, however, addition of sodium chloride to DI water appeared to reduce pH of the solution used for germination and seedling growth as the following: 0 mM NaCl (pH 6.7), 60 mM NaCl (pH 5.3) and 90 mM NaCl (pH 5.4). Also, the pH of all the salt solutions in our lab gradually decreased in the 4-day incubation without seed sowing (Fig. 3.1). Acidification of the NaCl solutions might be related to dissolved CO_2 from ambient into water during preparation of the salt solutions, mainly settling at about pH 5.65 (Reddi, 2013). Moreover, the pH of DI water used in the current study occasionally dropped to pH 6.1 by unknown causes. Therefore, we did expect that such causes for pH change may influence seed physiology of the Italian ryegrass throughout this study. Since the same source of DI and the NaCl solutions were prepared in the same way for use in all the experiments, the influence of pH, if any, on Italian ryegrass root growth would not be biased for a particular seed priming treatment of interest here. Although the primary focus of this study was to find a effective seed priming treatment for Italian ryegrass, it might be worthwhile to examine more closely about the effect of pH variation in NaCl solutions on root growth as this was hardly investigated before.

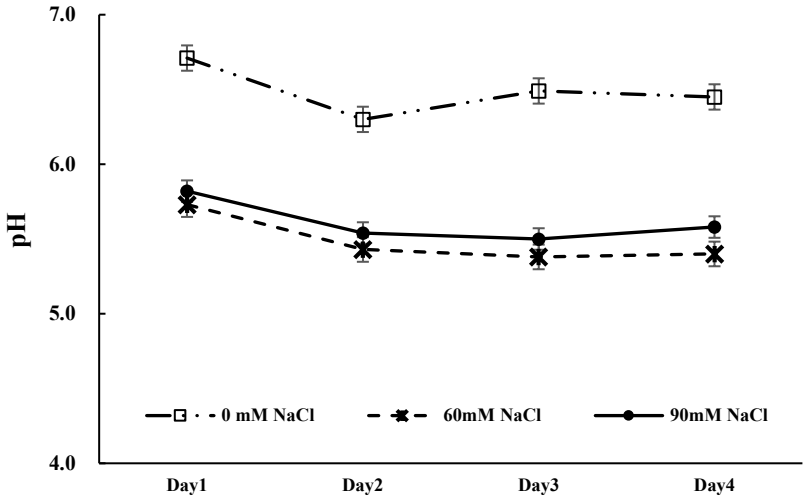


Fig. 3.1. pH variation of control (0 mM NaCl) and salt solutions (60 and 90 mM NaCl) for 4 days.

3.4.2.2. Potential of MES buffer for pH adjustment

Due to pH variation as mentioned in 3.4.2.1, MES (2-(N-morpholino) ethanesulfonic acid) buffer was used to adjust pH in preparing NaCl solutions. The MES buffer has been known as highly water-soluble and non-toxic material to living organisms (Kagenishi et al., 2016). Appropriate concentration of MES can lead to positive or no effect on plant growth and nutrient uptake (Imsande and Ralston, 1981; Schuttler, 1987), but the proper concentration range may vary depending on plant species and target pH value. High concentrations of the MES have been shown to have negative effects on plant growth by inhibiting uptake of nutrients (Stahl et al., 1999) as well as nitrogen fixation of legume plants (Rys and Phung, 1985). Also, in a soybean growth study, Nicholas and Harper (1993) reported that MES did not maintain pH of nutrient solution for a long period as it dropped from pH 6.5 to 4 within 5 days. However, since there was no prior study on use of MES for Italian ryegrass seedling growth and there was no

complex nutrients involved in the present study, it was decided to investigate the possible use of MES for buffering different NaCl solutions.

In the present study, 0.1 M and 0.01 M MES buffers (2 and 0.2%, respectively) were used to adjust pH level of a NaCl solution for seed germination experiments. However, there was no significant difference in GP of Italian ryegrass seeds incubated in 0 and 60 mM NaCl prepared in DI or 0.01 M MES buffer (pH 6.1 and 6.7), but GP was significantly decreased in 90 mM NaCl prepared in 0.01 M MES (pH 6.1 and 6.7) compared to that prepared in DI ($p<0.05$) (Table 3.6). There were significant differences in GP and RL when the seeds were incubated in 0.1 M and 0.01 M MES buffer (pH 6.1 and pH 6.7) in the absence or presence of NaCl ($p< 0.05$). Particularly, the NaCl solutions prepared in 0.1 M MES buffer (pH 6.7) could severely suppress seed germination and seedling growth. Interestingly, Kagenishi et al. (2016) found that low concentrations of MES buffer (0.01 to 0.1% w/v) improved root growth of seedling of *Arabidopsis thaliana* seedlings, while 1% MES buffer was inhibitory. It might be worthwhile to investigate the effect of other buffer systems on Italian ryegrass seed germination and root growth in future, but the primary focus of the present study was to investigate seed priming.

Table 3.6. Germination percentage and radicle length of *L. multiflorum* (4-day-old seedlings) at pH 6.1 and 6.7 in NaCl solutions (0, 60, and 90 mM) prepared in 0.1 M and 0.01 M MES buffers. Data indicate the means of each treatment, and same letters in each row mean no significantly difference (n=20, $p<0.05$, LSD).

	NaCl concentration	Control (DI water)	---------- MES treatment ----------			
			0.1M MES		0.01M MES	
			pH 6.1	pH 6.7	pH 6.1	pH 6.7
Germination percentage (%)	0 mM	86 ^{a}	57 ^{b}	7 ^{c}	82 ^{a}	85 ^{a}
	60 mM	71 ^{a}	10 ^{b}	0 ^{c}	75 ^{a}	67 ^{a}
	90 mM	74 ^{a}	0 ^{c}	0 ^{c}	50 ^{b}	50 ^{b}
Radicle length (mm)	0 mM	24 ^{a}	5.1 ^{c}	0 ^{d}	22 ^{a}	17.4 ^{b}
	60 mM	9.3 ^{a}	2.0 ^{bc}	0 ^{c}	4.2 ^{b}	3.6 ^{b}
	90 mM	4.2 ^{a}	0 ^{c}	0 ^{c}	2.3 ^{b}	2.4 ^{b}

Based on the results above, 0.01 M MES buffer was used to investigate its effects on GP and RL of hydro- and L-proline primed *L. multiflorum* seeds (Table 3.7). The GP and RL of non-primed seeds were lower than hydroprimed seeds (ie. the priming solution was just DI or 0.01 M MES, pH 5.5). However, after hydro-priming at pH 6.1 (in 0.01 M MES), generally there was a significant decrease in GP and RL of the seedlings grown in the absence or presence of NaCl compared to HP in de-ionised water ($p< 0.05$). In the presence of 60 or 90 mM NaCl, there was no difference in RL of the seedlings from seeds primed with proline prepared in DI or in 0.01M MES at pH 5.5. However, RL was significantly lower in seedlings from seeds primed with proline prepared in 0.01M MES at pH 6.1. than that of seeds primed with proline prepared in de-ionised water ($p< 0.05$). Similarly, seed priming with L-methionine and L-valine at pH 6.1 prepared in 0.01 M MES buffer had a negative effect on GP and RL of the Italian ryegrass seeds across all NaCl concentrations used (Table 3.8). Therefore, these results suggest that the effect of MES buffer addition outweighed the effect of adjusting pH of seed priming and germination solutions in this study. The pH variation in seed-priming solutions

probably influences priming efficiency like seed germination as well as further seedling growth. However, the pH values of almost L-amino acids priming (AAP) were between 5 and 7 (Table 3.9). Particularly, the pH of L-amino acids including lysine, methionine, and tryptophan and casein hydrolysate identified as the potential seed priming agents for improving seedling growth (see Table 3.5) was within the range from 5.3 and 6.4 (Table 3.9). These findings suggest that pH of these amino acids at the seed priming step might not be critical for their effectiveness for protection of the seedlings from the primed seeds under salt stress.

Table 3.7. Influence of pH adjustment in hydro- and L-proline priming treatment with 0.01 M MES buffer on germination percentage and radicle length of 4-day-old *L. multiflorum* seedlings. The pH levels were set up as 5.5, 6.1, and 6.5. Data are presented as treatments mean. Data followed by same letter are not significantly different by Fisher's individual test at $p< 0.05$ (n= 20).

	NaCl concen-tration	Non-priming seeds	Hydro-priming seed				Proline-priming seed			
			MES absence	MES presence			MES absence	MES presence		
				pH 5.5	pH 6.1	pH 6.5		pH 5.5	pH 6.1	pH 6.5
Germination percentage (%)	0 mM	78 ^{c}	92 ^{a}	93 ^{a}	78 ^{bc}	80 ^{abc}	90 ^{abc}	91 ^{abc}	83 ^{abc}	80 ^{abc}
	60 mM	71 ^{b}	87 ^{a}	83 ^{ab}	72 ^{b}	80 ^{ab}	77 ^{ab}	80 ^{ab}	82 ^{ab}	80 ^{ab}
	90 mM	74 ^{b}	88 ^{a}	85 ^{ab}	69 ^{ab}	80 ^{ab}	75 ^{ab}	78 ^{ab}	80 ^{ab}	80 ^{ab}
Radicle length (mm)	0 mM	24.4 ^{d}	33.4 ^{ab}	32.1 ^{b}	17.9 ^{e}	30.5 ^{bc}	36.4 ^{a}	29.3 ^{bc}	17.7 ^{e}	27.0 ^{cd}
	60 mM	9.3 ^{e}	11.2 ^{de}	15.6 ^{b}	10.5 ^{de}	14.5 ^{bc}	21.8 ^{a}	19.4 ^{a}	12.5 ^{cd}	14.5 ^{bc}
	90 mM	4.2 ^{d}	13.3 ^{b}	14.0 ^{b}	10.1 ^{c}	4.2 ^{d}	17.7 ^{a}	15.2 ^{ab}	10.5 ^{c}	4.2 ^{d}

Table 3.8. Germination percentages of *L. multiflorum* seeds after priming with L-methionine, -proline, and -valine in 0.01M of MES (pH 6.1) and then being incubated in different concentrations (0-90 mM) of NaCl. Data are presented as treatments mean. Data followed by same letter are not significantly different by Fisher's individual test at $p<0.05$.

Treatment		Non-priming	Absence of MES buffer				0.01M MES (pH 6.1)			
			HP	Methi-onine	Proline	Valine	HP	Methi-onine	Proline	Valine
Germination percentage (%)	0 mM	78 ^{b}	92 ^{a}	83 ^{ab}	90 ^{ab}	90 ^{ab}	78 ^{ab}	85 ^{ab}	83 ^{ab}	83 ^{ab}
	60 mM	71 ^{c}	87 ^{a}	87 ^{ab}	77 ^{abc}	77 ^{a}	72 ^{c}	73 ^{c}	82 ^{abc}	75 ^{bc}
	90 mM	74 ^{ab}	88 ^{a}	75 ^{ab}	75 ^{ab}	78 ^{a}	69 ^{bc}	52 ^{c}	80 ^{ab}	75 ^{ab}
Radicle length (mm)	0 mM	14.6 ^{f}	29.5 ^{c}	42.6 ^{a}	27.2 ^{c}	35.3 ^{b}	20.2 ^{de}	22.0 ^{d}	17.7 ^{ef}	21.8 ^{d}
	60 mM	6.5 ^{e}	10.3 ^{cd}	31.6 ^{a}	13.3 ^{b}	12.0 ^{bcd}	12.2 ^{de}	5.1 ^{e}	11.4 ^{bcd}	12.6 ^{bc}
	90 mM	3.4 ^{d}	3.3 ^{d}	24.1 ^{a}	11.5 ^{b}	11.8 ^{b}	8.3 ^{c}	2.3 ^{d}	10.5 ^{b}	11.4 ^{b}

Table 3.9. Variations in pH of 20 L-amino acids (1 mM prepared in de-ionised water), casein hydrolysate (200 mg· L^{-1} dissolved in de-ionised water), and de-ionised (DI) water alone used for seed priming in this study.

Amino acids	pH	Amino acids	pH
Alanine	6.92	Leucine	5.82
Arginine	9.83	Lysine	5.32
Aspartic acid	3.31	Methionine	6.08
Asparagine	5.58	Phenylalanine	5.93
Cysteine	7.49	Proline	6.48
Glutamic acid	3.65	Serine	5.61
Glutamine	5.69	Threonine	6.02
Glycine	5.81	Tryptophan	6.26
Histidine	7.56	Tyrosine	5.67
Isoleucine	6.28	Valine	6.38
Casein hydrolysate	5.98	DI water	6.71

3.4.2.3. No effect of pH of priming solutions on seed germination and growth

Seed germination and seedling growth are affected by pH in addition to temperature, water potential, and nitrogen source, etc. However, by comparing the total scores associated with the Italian ryegrass growth in priming solutions of similar pH level, it was found here that there was no effect of pH of priming solutions with different amino acids on seed germination and seedling establishment (Tables 3.5 and 3.9). For example, among isoleucine (at pH 6.28), proline (at pH 6.48), and valine (at pH 6.38), the growth scores of them were 0, 12, and 11, respectively. Also, between glycine (at pH 5.81) and leucine (at pH 5.82), their scores were 6 and 16, respectively. Additionally, among methionine (at pH 6.08), threonine (at pH 6.02), and phenylalanine (at pH 5.93), the growth scores were 26, 8, and 9, respectively. These results suggest that the pH of the priming solution may have little or no relationship to the seed priming test results. In addition, according to Pérez-Fernández et al. (2006), rather than absolute pH values, the amount and form of nitrogen exogenously applied in the germination media were more related to seed germination rates of several species: *Medicago arabica* L., *Epilobium*

hirsutum L., *Cynosurus cristatus* L., *Dactylis glomerata* L., *Foeniculum vulgare* Miller, *Daucus carota* L., *Thapsia villosa* L., and *Rumex crispus* L. Thus, the effect of priming on seed germination and radicle growth of Italian ryegrass in the present study is unlikely to be dependent on the pH of the priming solutions containing amino acids.

3.4.4. Overall effect of seed priming

This study explained that application of amino acids for seed priming had significant effects on Italian ryegrass's seed germination, seedling growth, and tolerant mechanism to salt stress, but the variables were influenced differentially. According to Teixeira et al (2017), application of a mixture of 4 amino acids including glutamine, cysteine, phenylalanine, and glycine to soybean seeds triggered negative or no effect on plant physiology relative to application of single amino acid. Soybean (*Glycine max* L.) leaves of plants from seed primed with single amino acids had greater antioxidant enzyme activities (CAT and SOD) and lower lipid peroxidation than those from seeds primed with the mixture of amino acids used. Thus, it was suggested that seed priming using the mixture amino acids might be less effective than priming with a single amino acid. In addition, the concentration (1mM) of each amino acid used in this study was much higher when compared to the respective amino acid content in a solution of casein hydrolysate (200 mg L^{-1}) (Table 3.1). As such, seed priming with some individual amino acid may have a better or an equal effectiveness for seed priming compared to the treatment with CH containing up to 18 amino acids.

However, in the results of this study, priming with casein hydrolysate showed better efficacy in all the plant growth indexes under salt stress condition than priming with a single amino acid. It seems to have produced better results by other components of CH (i.e. calcium, phosphate, several microelements, and several vitamins) beyond the effect of amino acid only (George et al., 2008; Molnár et al., 2011). Similarly, Sanders and Burkholder (1948) revealed that adding single and in combination of several amino acids into the medium showed much less effect on or even inhibited embryo culture of *Datura* than casein hydrolysate addition. Addition of single or several amino acids may possibly lead to: (1) counteracting effects among the few selected amino acids used (Washburn and Niven, 1948), and (2) changes in the internal

balance of amino acids interfering with amino acid metabolism for plant morphology and growth (Steinberg, 1949; Steinberg et al., 1950). In this regard, casein hydrolysate containing almost all of the amino acids normally required for protein synthesis might be a more balanced mixture of amino acids to help plant growth without causing such competing amino acid interactions, in addition to by providing plant nutrient sources.

3.5. Conclusion

Throughout this chapter, positive effects of seed priming on germination and seedling development were found. Hydro-priming appeared to result in better germination percentage and radicle growth of *L. multiflorum* for four days of incubation. Additional increases in GP and RL were found following seed priming with varying L-amino acids as well as casein hydrolysate. Of the twenty L-amino acids used for seed priming, lysine, methionine, and tryptophan seemed to have a greater potential for improving seedling growth and tolerance mechanisms to salt stress in this study. Also, L-amino acids seemed to be better than D-amino acids for seed priming. Moreover, casein hydrolysate as a mixture of L-amino acids could be a better seed priming agent rather than the three L-amino acids applied individually with respect to efficiency as well as economy. Use of MES buffer to adjust pH in NaCl solution and seed priming reagent was not effective or tended to interrupt seed germination and radicle growth of the Italian ryegrass in this study. Further studies would be required to have a better understanding of tolerance mechanism of *L. multiflorum* seedlings after seed priming with L-methionine and casein hydrolysate. In addition, a cultivation experiment of the Italian ryegrass in saline soil is needed to ascertain the practical aspect of seed priming in the cropping field.

Chapter 4. Effects of seed priming with L-methionine and casein hydrolysate on Italian ryegrass (*Lolium multiflorum*) seedlings under salt stress: hydrogen peroxide, lipid peroxidation, and proline levels

4.1. Abstract

Salinity is one of the environmental stresses which can restrict plant growth, particularly at the early phase of plant development like germination and seedling establishment. Thus, salt stress can limit further crop production. In an earlier study in Chapter 3, L-methionine and casein hydrolysate (CH) was found to have the potential for seed priming to improve salt tolerance of 4-day-old *L. multiflorum* seedlings. The aim of the experiments in this Chapter 4 was to investigate the enhanced salt stress tolerance mechanism against reactive oxygen species (ROS) accumulation in the Italian ryegrass seedlings. In this study, analysis was carried out to determine seed germination, radicle length, antioxidant enzymes (CAT, POD, and SOD), the contents of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and proline in the roots of 4-days-old *L. multiflorum* seedlings grown under salt stress (0, 60, and 90 mM NaCl). The seedlings were raised from seeds primed with L- and D-methionine and CH. Of the seed-priming reagents, L-methionine and CH significantly increased seedling radicle elongation and antioxidant enzyme activities such as SOD, CAT, and POD in 60 and 90 mM NaCl, while D-methionine tended to cause negative effects on these enzyme activities. This suggests that the L-amino acid rather than the D-amino acid was useful as a seed priming agent. Particularly, CH priming was most likely to suppress or eliminate accumulation of ROS such as superoxide (O_2^-) and H_2O_2 in the seedling roots of *L. multiflorum* compared to NP, HP, and L-methionine priming. The roots of Italian ryegrass in CH priming treatment exhibited less H_2O_2 and superoxide in histochemical staining with diaminobenzidine (DAB) and nitroblue tetrazolium

(NBT), respectively. These results may indicate that seed-priming with L-methionine as well as casein hydrolysate could ameliorate adverse effects of salt stress on the growth of Italian ryegrass, particularly at the early seedling growth stage by reducing oxidative stress. These results had not been reported elsewhere before. The H_2O_2 concentrations in the roots of seedlings in the CH priming treatment were 24, 25, and 31% lower than those in the control (NP) when the seedlings were grown in 0, 60, and 90 mM NaCl, respectively. The roots of the seedlings raised from seeds primed with L-methionine also exhibited significantly lower H_2O_2 content than NP when the seedlings were grown in 90 mM NaCl, while the roots in the D-methionine priming treatment had the highest H_2O_2 content. Moreover, the lowest level of malondialdehyde (MDA), as products of lipid peroxidation, was found in the roots of the seedlings raised from seeds primed with CH when the seedlings were grown in the different concentrations of NaCl. The MDA contents in the roots of the seedlings in the CH priming treatment were 55, 56 and 41% lower than those in the control (NP) treatment when the seedlings were grown in 0, 60, and 90 mM NaCl, respectively. In terms of proline production under salt stress, the roots of the seedlings in the CH priming treatment had up to 40% more proline than NP when the seedlings were grown in 60 mM NaCl. However, in the L- and D-methionine priming, the roots of the seedlings exhibited significantly lower proline contents than NP when the seedlings were grown at the different NaCl levels. These results suggest that CH priming of seeds could potentially improve salt stress tolerance and oxidative stress damages in *L. multiflorum* seedlings by lowering ROS accumulation such as H_2O_2 and lipid peroxidation, and by accumulating more proline in the roots. These beneficial mechanisms from CH priming against salt stress would be expected to enhance plant growth and productivity.

Keywords: seed priming, salt stress tolerance, casein hydrolysate, *Lolium multiflorum*, hydrogen peroxide, malondialdehyde, proline.

4.2. Introduction

Salinity in soil is one of the environmental stresses that causes ion toxicity, mineral nutrient deficiency, osmotic stress, and degradation of photosynthesis in plants (AbdElgawad et al., 2016; Rangani et al., 2016). Such negative effects generate reactive oxygen species resulting in plant growth retardation and cell death (Cheng et al., 2016; Genisel et al., 2015). Particularly, highly saline soil leads to over-production of ROS comprising superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) in plant cells. Thus, ROS products impair protein, nucleic acids, and lipid membrane, resulting in decrease in crop yields (AbdElgawad et al., 2016; Das and Roychoudhury, 2014; Gill and Tuteja, 2010). ROS are generally generated under normal and abiotic stress conditions in various cell compartments including the apoplast, chloroplast, mitochondria, and peroxisome (Das and Roychoudhury, 2014; Trchounian et al., 2016). However, low ROS levels also play a role as a signaling molecule in the plant cell, which beneficially affect plant growth and development. High ROS levels induced under stress and they are toxic and can damage cells (Bose et al., 2014; Mittler et al., 2004; Sharma et al., 2012).

The extent of oxidative stress in plants can be estimated through determining both H_2O_2 and MDA contents. H_2O_2 as one of the ROS is converted from $O_2^{\bullet-}$ by SOD, which is significantly accumulated in the peroxisome under normal or abiotic stress as well as biotic stress such as pathogen attack (Das and Roychoudhury, 2014; Miller et al., 2010; Sharma et al., 2012). High concentrations of H_2O_2 in the cell can oxidise cysteine (-SH) and methionine ($-SCH_3$) as well as deactivate Calvin cycle enzymes and SOD enzyme by oxidising their thiol

groups (Das and Roychoudhury, 2014). Malondialdehyde (MDA) is generated by polyunsaturated fatty acids, as one of the final decomposition products in plasma membrane, under salinity stress (Chen et al., 2011; Meloni et al., 2003). For instance, Bandeoğlu et al. (2004) found significant increases in both H_2O_2 and MDA contents in leaves and roots of lentil (*Lens culinaris*) accompanied by cell membrane damage exposed to salt stress.

To withstand severe effects of increased ROS levels, plants have scavenging mechanisms and enhanced tolerance to the oxidative stress by exhibiting enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), monodehydroasorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and guaiacol peroxidase (GPX). In addition, enhanced tolerance to stress might also be associated with non-enzymatic antioxidant metabolites such as ascorbic acid (AA), glutathione, α-tocopherol, carotenoids, flavonoids, and proline (Das and Roychoudhury, 2014; Gill and Tuteja, 2010). The activities of antioxidant enzymes usually enhance stress tolerance by detoxifying ROS such as $^1O_2$, $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ and protecting plant cells from oxidative damages (Gill and Tuteja, 2010; Mittler et el., 2004; Sharma et al., 2012).

Of the non-enzymatic antioxidant metabolites, proline is accumulated in plants under drought and salinity, which plays a role as an osmoprotectant for stabilising protein structures, controlling cellular redox homeostasis, and scavenging free radical (Székely et al., 2008). It is also responsible for stabilising mitochondrial respiration, reducing cell acidity, preventing cell death, and maintaining plant water potential as well as storing nitrogen source under adverse environment (Bose et al., 2014; Misra and Gupta, 2005; Szabados and Savouré, 2010). As reported by various researchers, salt tolerant plant species exhibited increased proline content in the roots of seedlings including wheat (*Triticum durum* Desf.) (Bouthour et al., 2015), 5-day-old seedlings of foxtail millet (*Setaria italic* L.) (Veeranagamallaiah et al., 2007), and

shoots of green gram (*Phaseolus aureus*) (Misra and Gupta, 2005). However, there is little information about induction and its functionality of abiotic stress tolerance mechanism associated with proline regulation within plant cell tissues (Szabados and Savouré, 2010).

Priming treatments of seeds with chemicals such as amino acids, plant hormone, and nutrients have been applied to enhance seed quality and germination uniformity as well as seedling growth of crops (Jisha et al., 2013; Nasibi et al., 2016). As shown in Chapter 3, seed priming with L-methionine and casein hydrolysate (CH) as a mixture of some amino acids had shown significantly reduced accumulation of ROS including superoxide (O_2^-) and H_2O_2 via staining assays with 3, 3'-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), and also markedly increased activities of SOD, POD, and CAT. To better understand about the effects of L-methionine and CH as seed priming agents on improved plant physiological mechanisms and tolerance to salt stress, the present study was performed to determine quantities of H_2O_2, MDA, and proline contents in the roots of *L. multiflorum* seedlings in different seed priming treatment when grown under the same levels of NaCl.

4.3. Materials and Methods

4.3.1. Seed priming and NaCl treatments

Seeds of Italian ryegrass (*Lolium multiflorum*) were soaked in casein hydrolysate (200 mg L^{-1}), 1 mM L-methionine, and 1 mM D-methionine singly for seed priming. Hydro-priming (HP) and non-priming (NP) treatments were prepared for comparison in this study as described in Chapter 2 (section 2.2.1. seed priming). To set up salt stress to *L. multiflorum* seeds, three different concentrations of NaCl including 0, 60, and 90 mM were arranged as described in Chapter 2 (section 2.2.2. salt treatment). After 4 days of incubation, twenty-three (30 mg) roots

with four replications of *L. multiflorum* seedlings were randomly collected from each Petri dish in a treatment.

4.3.2. Antioxidant enzyme activities

a. Enzyme extraction and protein content

To assay activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), Italian ryegrass seeds from different priming treatments in response to salt stress, 0.1 g of seedling roots was harvested after 4 days of incubation in 0-90 mM NaCl. For POD analysis, the roots were homogenised with liquid N in a mortar and pestle and extracted with 1.2 ml of 0.1 M potassium phosphate buffer (pH 6.59). The homogenates were centrifuged at 10,000 g for 5 min at 4 ˚C (Baque et al., 2010). To extract SOD and CAT, the roots were ground to powder with liquid N in a mortar and pestle and extracted with 1.2 ml of cooled 0.05 M phosphate buffer (pH 7.0) including 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The extracts were centrifuged at 12,000 g for 15 min at 4 ˚C (El-Shabrawi et al., 2010; Larkindale and Huang, 2004). All supernatants were stored at - 80 ˚C, prior to analyses of POD, SOD, and CAT activities. Total protein content in the extracts was also measured at a wavelength of 590 nm using an Ultrospec^{TM} 2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA) as described in Bradford (1976).

b. Peroxidase activity (POD (EC. 1.11.1.7))

After extraction, the supernatant (50 ul) was added to a mixture of 893 µl of 0.1 M potassium phosphate buffer (pH 6.59), 2 µl of guaiacol, and 5 µl of 10% H_2O_2 for 5 min at room temperature until the color reaction is developed to orange. The absorbance of the reaction mixture was then measured spectroscopically at a wavelength of 470 nm using an Ultrospec^{TM}

2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA). The POD activity (units•mg^{-1} protein) was calculated.

c. Superoxide dismutase (SOD (EC. 1.15.1.1))

Total SOD activity was estimated based on the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) using a modified method as described in Wang et al. (2015b). One milliliter of reaction mixture containing 0.6 ml of 50 mM phosphate buffer (PBS at pH 7.8), 0.1 mM of 130 mM methionine, 0.1 ml of 1 mM EDTA, 0.1 ml of 750 uM NBT, 0.1 ml of 0.02 mM riboflavin was reacted with 0.04 ml enzyme extract. After 5 min of exposure time to fluorescent light, the absorbance was measured at a wavelength 560 nm using a Ultrospec^{TM} 2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA). SOD activity was determined as 50% inhibition of the NBT reduction caused by superoxides generated from the reaction of photo-reduced riboflavin and oxygen. SOD activity was expressed in units per protein content (units•mg^{-1} protein).

d. Catalase (CAT (1.11. 1.6))

CAT activity was determined based on the reduction rate in hydrogen peroxide using a modified method of Chance and Maehly (1955). The reaction mixture containing 2.5 ml of 50 mM phosphate buffer (pH 7.0) and 0.035 ml of 3% hydrogen peroxide (H_2O_2) was reacted with 0.05 ml of the enzyme extract. The decreased amount of H_2O_2 for 1 min was measured at 240 nm of absorbance using a Ultrospec^{TM} 2100 Pro UV/Visible spectrophotometer (GE Healthcare, USA). CAT activity was expressed in units per protein content (units•mg^{-1} protein). Additional information on enzyme extraction and assays are found in the section 2.4.5.

4.3.3. Histochemical detection of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)

Reactive oxygen species (ROS) such as H_2O_2 and $O_2^{\bullet-}$ in roots of 4-day-old *L. multiflorum* seedlings were detected using staining protocols for 3, 3'-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively (Frahry and Schopfer, 2001). Ten roots from four-day-old seedlings were randomly selected from each Petri dish, and uniformly cut prior to staining the roots. To detect $O_2^{\bullet-}$, the roots were soaked for 1 min in 10 mM potassium citrate (pH 6.0) containing 0.5 mM NBT. For detection of H_2O_2, the roots were soaked for 1 min in 10 mM potassium citrate (pH 6.0) containing 2.5 mM DAB and 1mM H_2O_2. After staining, photographs of the roots were taken using a digital camera. The staining experiments were repeated three times.

4.3.4. Biochemical analyses

4.3.2.1. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide content determination was carried out as described in 2.4.6.1 (see Chapter 2).

4.3.2.2. Lipid peroxidation

Lipid peroxidation content determination was carried out as described in 2.4.6.2 (see Chapter 2).

4.3.2.3. Proline content

Proline content determination was carried out as described in 2.4.8 (see Chapter 2).

4.3.5. Statistical analysis

For biochemical analyses of H_2O_2, MDA, and proline contents, four Petri dishes of Italian ryegrass seeds were set up for each treatment. All the experiments were repeated for reproducibility. One-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) post-hoc test ($p< 0.05$) was conducted with all the data for confirming the variance which was performed to determine significant difference between treatments using a Minitab 16.

4.4. Results and Discussion

4.4.1. Capacity of seed germination

In the 60 mM NaCl treatment, GP of unprimed seeds was significantly lower compared with the seeds in the different seed priming treatments (hydro-priming, CH-priming, L- and D-methionine priming) ($p< 0.05$) (Table 4.1). However, the GP of all the primed seeds were reduced to the same level as the unprimed seeds under 90 mM NaCl stress. It seemed that these seed priming treatments might improve the capacity of seed germination in the presence of 60 mM NaCl, but not in the presence of a higher concentration of NaCl (90 mM). Similarly, Lee et al. (2014) and Marcar (1987) investigated that increasing levels of salt stress inhibited seed germination of *L. multiflorum*. Poor germination under severe salinity stress could often be occurred due to reduced water uptake potential, ion toxicity of Na^+ and Cl^- into the embryo, or limiting protein synthesis (Farooq et al., 2015; Srivastava et al., 2010). Additionally, high level of salinity cannot only cause a decrease in germination capacity but also influence metabolism in a plant (Filho et al., 1983).

Table 4.1. Germination percentage (%) of *L. multiflorum* seeds primed with de-ionised (DI) water (hydro-priming; HP), D-methionine, L-methionine, and casein hydrolysate. Following seed priming, the seeds were incubated in 0, 60, and 90 mM NaCl (n=8, $p<0.05$).

Priming source	NaCl concentration		
	0 mM	60 mM	90 mM
Non-priming (control)	86 [a]	71 [b]	73 [a]
DI water (HP)	84 [a]	82 [a]	71 [a]
D-methionine	88 [a]	86 [a]	75 [a]
L-methionine	83 [a]	87 [a]	75 [a]
Casein hydrolysate	85 [a]	87 [a]	75 [a]

4.4.2. Developmental of seedling roots

The radicle length (RL) of the seedlings in the unprimed seed treatment was decreased in response to increasing concentrations of NaCl (Fig. 4.1). The RL of the seedlings in the hydropriming and D-methionine priming treatments was the same as those in the unprimed seed treatment in response to 60 mM NaCl. In contrast, seed priming with L-methionine and casein hydrolysate resulted in significantly better seedling growth (longer roots) than the other seed priming treatments when the seedlings were incubated in 60 mM NaCl ($p< 0.05$). In response to 90 mM NaCl, seed priming with L-methionine resulted in the best protection against salt stress on RL of the seedlings. Interestingly, in the absence of salt stress, all the seed priming treatments resulted in better seedling growth in relation to RL than the unprimed seed treatment. It seems that the reduction in RL of the seedlings in response to increased salt concentrations was strongly associated with a decrease in seed germination percentage (Amooaghaie, 2011). Indeed, the root elongation phase of *L. multiflorum* tended to be more influenced by increasing NaCl concentrations than the germination phase in this study. It might

be due to decreased water potential and inhibited metabolism in the roots under salt stress condition (Aroca et al., 2012; Filho et al., 1983).

Nevertheless, L-methionine and CH priming considerably enhanced radicle elongation of the Italian ryegrass seedlings compared to the seedlings from NP when the seedlings were incubated in NaCl solutions. In contrast, D-methionine priming did not have the same effect (Fig. 4.1). In nature, both L- and D-amino acids in soil are generally originated from living organisms including plants, animals, and microbiota, but L-amino acids are more abundant than D-amino acids (Vranova et al., 2012). D-amino acids including methionine are slowly minerlised in soil rather than L-amino aicds, and low capacity of plants to metabolise some of D-amino acids may lead to inhibition of plant growth (Aldag and Young, 1970; Erikson et al., 2005; Vranova et al., 2012). Forsum (2016) revealed that the roots of *Arabidopsis thaliana* did uptake more L-amino acids including serine (25%), valine (60%), isoleucine (60%), and arginine (75%) than the corresponding D-amino acids. It is known that methionine could play roles as key component in protein synthesis and other metabolic reactions as well as antioxidant (Levine et al., 2000). In this respect, L-methionine used for seed priming appeared to show better function than D-methionine in the present study.

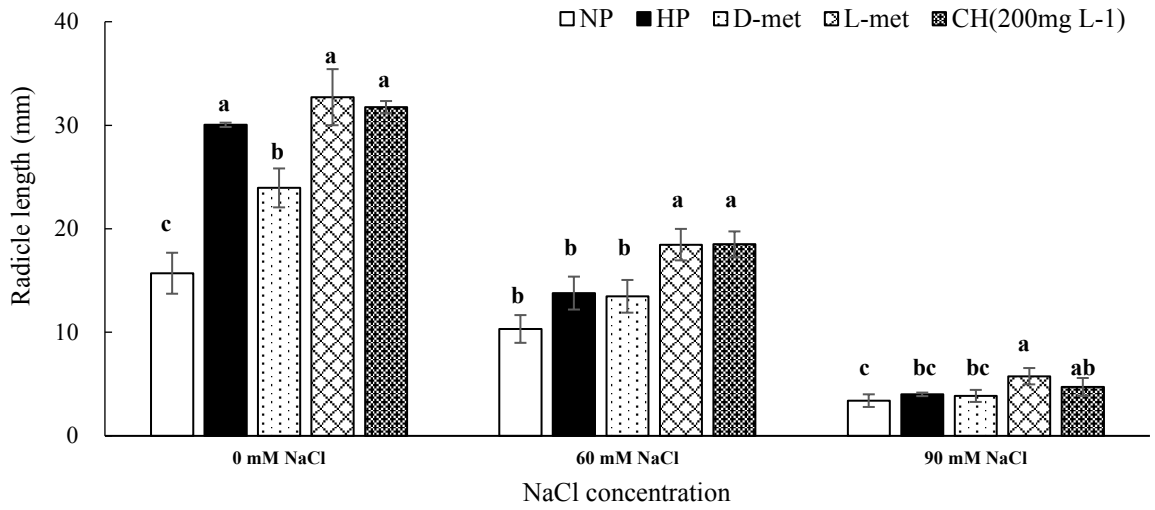


Fig. 4.1. Influence of seed-priming with de-ionised water (hydro-priming; HP), D-methionine, L-methionine, and casein hydrolysate (CH) on radicle length (mm) of 4-day-old *L. multiflorum* seedlings. After seed priming, the seeds were incubated in 0, 60, and 90 mM NaCl. Data are presented as treatments mean ±SE. Data followed by same letter are not significantly different by Fisher's individual test at $p < 0.05$.

4.4.3. Antioxidant defence activities

Activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) in seedling roots of *L. multiflorum* were different under salt stress following selected seed priming treatments (Fig. 4.2). Compared to NP (control), HP, and L-methionine-priming, CHP significantly increased SOD activity in the Italian ryegrass roots when the seedlings were incubated in the different concentrations of NaCl ($p < 0.05$). When the seedlings were incubated in 0, 60, and 90 mM NaCl, the roots of the seedlings primed with CH had 70, 115, and 175% higher SOD activities, respectively, than NP seedling roots. Additionally, the effects of CHP on SOD activity in the roots of the seedlings incubated in the different NaCl concentrations were significantly greater than the other priming treatments ($p < 0.05$). However, compared to NP, D-methionine priming considerably decreased SOD activity in the roots of

the seedlings incubated in 60 and 90 mM NaCl ($p< 0.05$). Superoxide dismutase plays a critical role in initial antioxidant defence mechanism that catalyses the formation of superoxide anion radical ($O_2^{\bullet-}$) which is then converted to hydrogen peroxide (H_2O_2) (Bhaskaran and Panneerselvam, 2013). The present study showed that L-methionine and CH priming treatments significantly enhanced SOD activity in the Italian ryegrass seedling roots, and would thereby lead to a reduction in harmful reactive oxygen. Similarly, priming of Indian mustard (*Brassica juncea* L.) seeds with calcium chloride ($CaCl_2$) and abscisic acid (ABA) significantly increased SOD activity in the seedlings grown under salt stress (150 mM NaCl) compared to the unprimed seeds (Srivastava et al., 2010).

Like the SOD activity, CAT activity in seedling roots of *L. multiflorum* was markedly increased by seed priming with L-methionine and CH, compared to NP (control) ($p< 0.05$) (Fig. 4.2). Particularly, the roots of the seedlings from CH-primed seeds had the highest level of CAT in each NaCl treatment. The CAT activities in the roots of seedlings from CH-primed seeds incubated in the presence of 0, 60, and 90 mM NaCl were about 6.1, 3.4, and 4.8 times greater than those in the NP seedling roots, respectively. Seed priming with L-methionine and CH also significantly increased POD activity in the Italian ryegrass roots compared to NP, HP, and D-methionine when the seedlings were incubated in the different concentrations of NaCl ($p< 0.05$). The POD activity in the roots of seedlings from seeds primed with CH was 77, 58, and 65% higher than that in NP when the seedlings were incubated in 0, 60, and 90 mM NaCl, respectively. However, seed priming with D-methionine significantly decreased POD activity in the seedling roots, similar to its effect on SOD activity, compared to NP when the seedlings were incubated in 60 mM NaCl ($p< 0.05$). These results suggest that the increases in SOD, CAT, and POD activities with L-methionine and CH priming treatments effectively protected oxidative damages in Italian ryegrass roots by reducing reactive oxygen species (ROS) (Jabeen and Ahmad, 2013).

ROS caused by unfavorable environmental condition such as salt stress can interfere with cellular redox homeostasis in plant organelles including chloroplasts, mitochondria, and peroxisomes, which is strongly associated with adaptation of plants to the stress environment (Miller et al., 2010). In general, plants have efficient antioxidant defence mechanisms to protect cellular structures and metabolism against ROS produced under the abiotic stress (Amooaghaie, 2011; Gill and Tuteja, 2010). The results above suggest that seed priming with L-methionine and CH in the current study may induce better or elevated levels protective enzyme activities including SOD, CAT, and POD in the roots against salt stress, whereby salt stress-induced ROS could be converted to less toxic substances than oxygen radicals (Amooaghaie, 2011; Bhaskaran and Panneerselvam, 2013; Gill and Tuteja, 2010; Miller, 2012; Seckin et al., 2009). Similar results following application of seed priming with amino acids were reported by Teixeira et al. (2017).

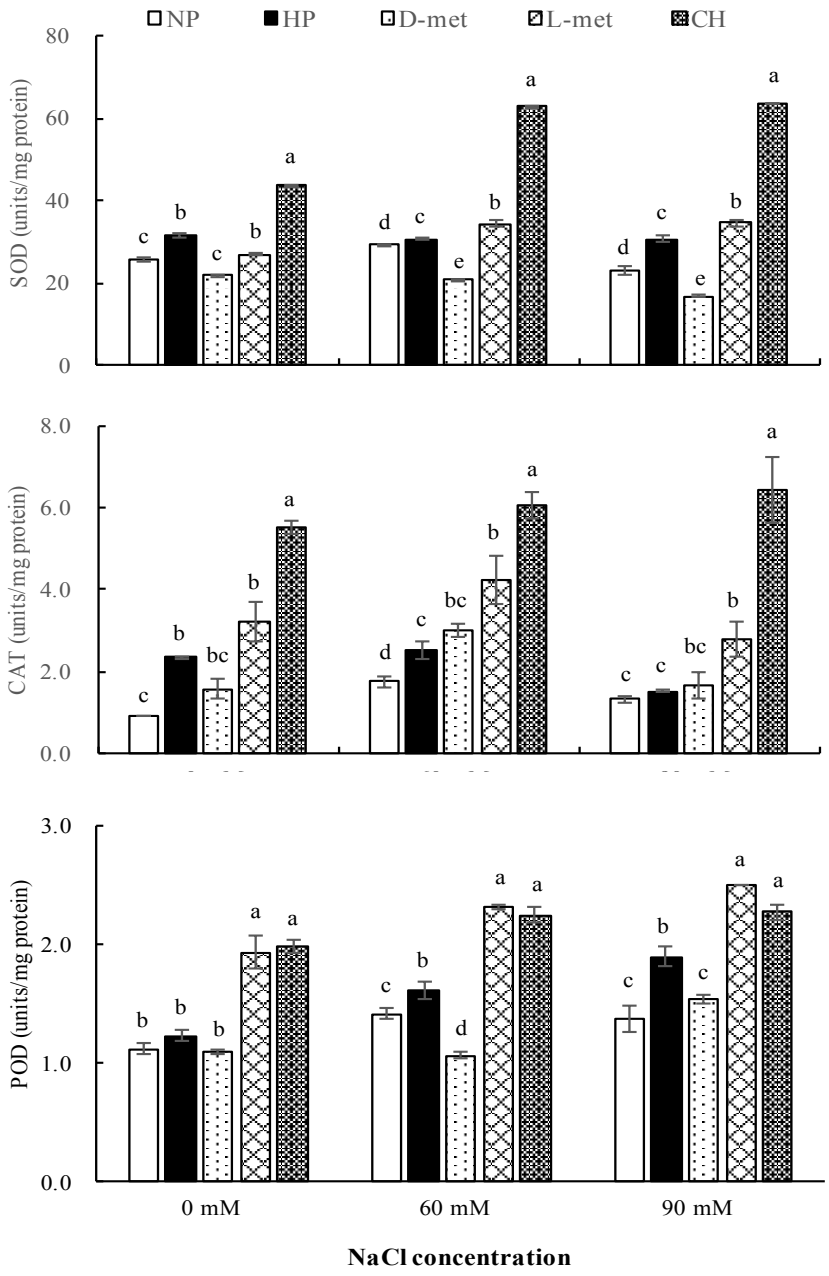


Fig. 4.2. Influence of seed-priming with deionised water (hydro-priming; HP), D-methionine, L-methionine, and casein hydrolysate (CH) on antioxidant enzymes of 4-day-old *L. multiflorum* seedlings. After seed priming, the seeds were incubated in 0, 60, and 90 mM NaCl. Data are presented as treatments mean ± SE. Data followed by same letter are not significantly different by LSD Fisher's individual test at $p < 0.05$.

4.4.4. Production of reactive oxygen species (ROS)

From the results of nitroblue tetrazolium (NBT) (Fig. 4.3) and 3,3-diaminobenzidine (DAB) staining (Fig. 4.4), the detected accumulation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) differed in the roots of 4-day-old *L. multiflorum* seedlings depending on seed priming treatments: control (NP, no priming), priming with de-ionised water (hydro-priming, HP), D-methionine, L-methionine, and casein hydrolysate (CH). The NBT staining revealed that more superoxide ($O_2^{\bullet-}$) were detected around the root tips of the NP seedlings in the different NaCl treatments which adversely affected seed germination and seedling growth (Fig. 4.3). Interestingly, reduced intensity of purple color was exhibited at the root tips of the seedlings from seeds primed with CH compared to the other seed priming treatments. This suggests that the enhanced SOD activity in roots of the seedlings following CH seed priming (see Fig. 4.2) led to considerably reduced accumulation of $O_2^{\bullet-}$ (de Azevedo Neto et al., 2006; Seckin et al., 2009), particularly when the seedlings were incubated in 60 and 90 mM NaCl. Following H_2O_2 detection based on DAB staining, there were no clear differences in staining (brown color) of the roots of Italian ryegrass seedlings among the different seed priming treatments except for CHP (Fig. 4.4). The roots of the seedlings following CH seed priming exhibited the brightest DAB staining (dark brown colour) in comparison with the other seed priming treatments. In addition, the roots of seedlings following L-methionine priming showed less dark brown staining than those following NP, HP, and D-methionine priming. These results suggest that enhanced CAT and POD activities by CH and L-methionine priming (see Fig. 4.2) might help to reduce H_2O_2 accumulation in the roots of *L. multiflorum* under salt stress (de Azevedo Neto et al., 2006; Seckin et al., 2009). However, NaCl did not make a significant difference in the DAB staining of the seedling roots in each priming treatment. This may be due to relatively short duration of the experiment so

that small differences in accumulation of H_2O_2 might not be detectable. Also, under salt stress, hydrogen peroxide may accumulate more in leaves than in roots. For instance, root tips of 35-day-old *Lotus japonicas* had less H_2O_2 than the leaves under abiotic stress (Signorelli et al., 2013).

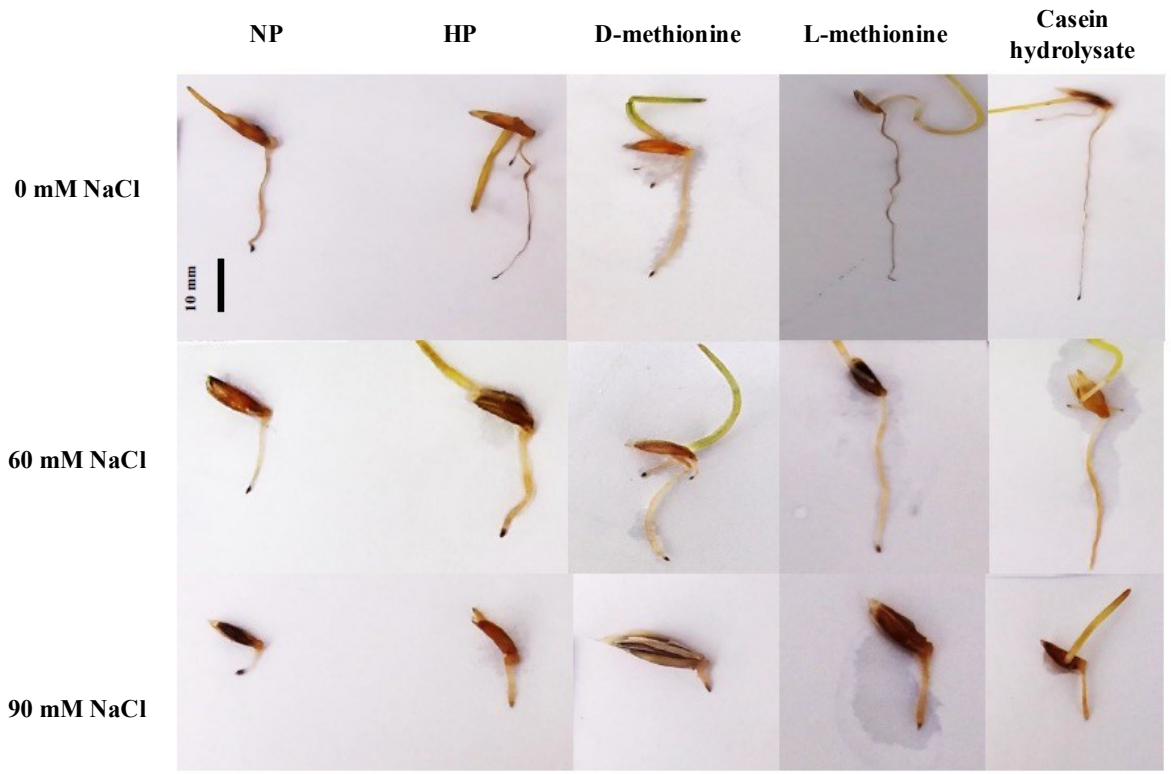


Fig. 4.3. NBT (nitroblue tetrazolium) stained roots of Italian ryegrass seedling (*L. multiflorum*) after 4 days of incubation in 0, 60, 90 mM NaCl (NP: Control, HP: DI water).

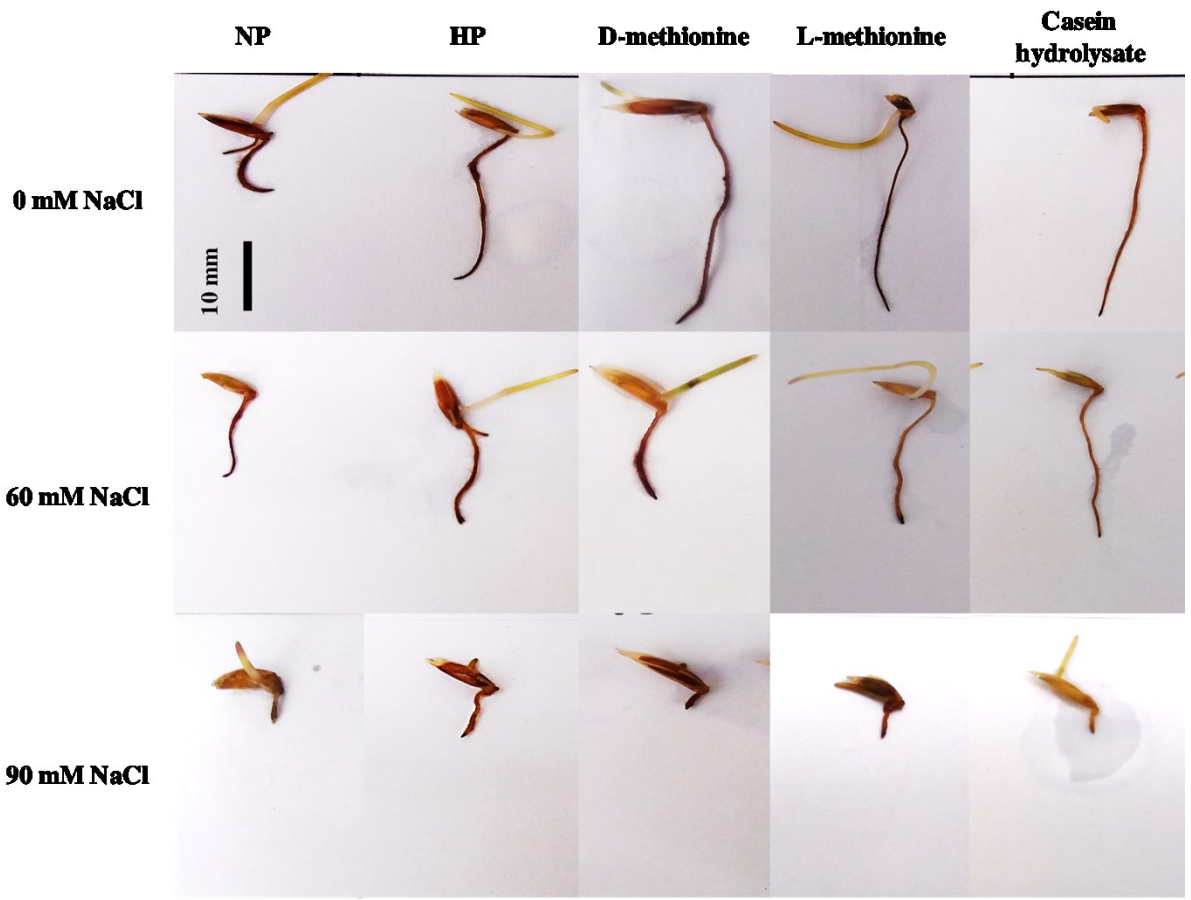


Fig. 4.4. DAB (3,3-diaminobenzidine) stained roots of Italian ryegrass seedlings (*L. multiflorum*) after 4 days of incubation in 0, 60, 90 mM NaCl (NP: Control, HP: DI water).

4.4.5. Effect on H_2O_2 content

With increasing NaCl concentrations, hydrogen peroxide (H_2O_2) content in the roots of *L. multiflorum* seedlings from unprimed seeds (NP, control) tended to decrease steadily (Fig. 4.5). There was a significant decrease in the H_2O_2 content in the roots of the seedlings from NP treatment when the seedlings were incubated in 90 mM NaCl. When the seedlings from the seeds in different seed priming treatments were grown in 60 mM NaCl, there was a significant reduction in H_2O_2 concentration only in the roots of the seedlings in casein hydrolysate priming treatment ($p< 0.05$) (Fig. 4.5). When the seedlings were grown in 90 mM NaCl, hydro-, L-methionine, and CH priming treatments there was a significantly lowered H_2O_2 content compared to the non-priming control ($p< 0.05$). In particular, the roots of the seedlings in the CH priming treatment had the lowest levels of H_2O_2. In contrast, the roots of the seedlings from seeds primed with D-methionine had a notable increase in H_2O_2 content compared to those in the other seed priming treatments ($p<0.05$). These results suggest that the CH and L-methionine priming could improve salt tolerance by minimising H_2O_2 level in *L. multiflorum* roots.

It is generally known that salt stress leads to accumulation of ROS such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radical ($OH^{\bullet}$) in plant cell organelles such as chloroplasts and mitochondria (Nasibi et al., 2016; Sekmen et al., 2012). Of the reactive oxygen species, H_2O_2 accumulation under abiotic stress can be increased by superoxide dismutase (SOD) activity contributing to conversion of $O_2^{\bullet-}$ to H_2O_2 (Bose et al., 2014; Carvalho et al., 2011). However, there was no change in SOD activity with an increase in NaCl concentration as shown in Chapter 4 (see Fig. 4.2). In addition, H_2O_2 content in roots of Italian ryegrass seedlings tended to decrease with increase of NaCl concentrations. In particular, significant decreases in H_2O_2 content were found in the seedling roots from seeds primed with L-methionine and CH when grown in 90 NaCl treatment. This is that the reduction

in the H_2O_2 content under salt stress may be closely associated with increased activities of the other antioxidant enzymes including CAT and POD in the Italian ryegrass seedlings (see Fig. 4.2; Bose et al., 2014). Mittova et al. (2004) found that excessive H_2O_2 level produced in the mitochondria and peroxisomes of root cells of tomato (*Lycopersicon pennellii*) species exposed to salt stress was scavenged by APX and CAT, resulting in less oxidative stress damage. Also, similar effects on wheat (*Triticum aestivum* L.) and barely (*Hordeum vulgare* L.) treated exogenously with jasmonic acid and cysteine, respectively, were found by Qiu et al. (2014) and Genisel et al. (2015), when the plants were grown under salt stress.

There are few studies on the effect of seed priming using amino acids on H_2O_2 content and antioxidant enzyme activities in plants under salt stress. Nasibi et al. (2016) revealed that roots and shoots of 14-day-old wheat (*T. aestivum* L.) seedlings following seed priming with cysteine had reduction in H_2O_2 content compared to non-priming control when the seedlings were grown under salt stress. The researchers suggested that the effect of cysteine pre-application to the seeds induced detoxification and tolerance to salt stress by enhancing ROS-scavenging mechanism like antioxidant enzymes activities including APX, CAT, and POD. However, Carvalho et al. (2011) did not find such positive effect of seed priming with water, chloroethylphosphonic acid (CEPA), and salicylic acid (SA) on alleviation of H_2O_2 accumulation under salt stress (30 mM NaCl) in 5-day-old seedlings of maize (*Zea mays* L.).

Particularly, a convincing evidence of the decline in H_2O_2 content in the roots of Italian ryegrass seedlings from seeds primed with CH was previously obtained through the 3,3-diaminobenzidine (DAB) staining assay in Chapter 4 (see Fig. 4.4). The DAB staining intensity in the root tips of the seedlings from seeds primed with L-methionine and D-methionine was similar to those in NP and HP treatments incubated under salt stress. In contrast, the roots of the seedlings from seeds primed with CH exhibited less dense DAB staining. CH-priming may produce activation of oxidative stress signaling in the plant cell, as well as antioxidant enzyme

activities, that could neutralise ROS and free radicals including H_2O_2 and $O_2^{\bullet -}$ under salt stress. Therefore, such benefits of CH-priming regulating H_2O_2 level under salt stress could be part of the enhanced antioxidant enzymatic activities contributing to maintenance of the membrane integrity.

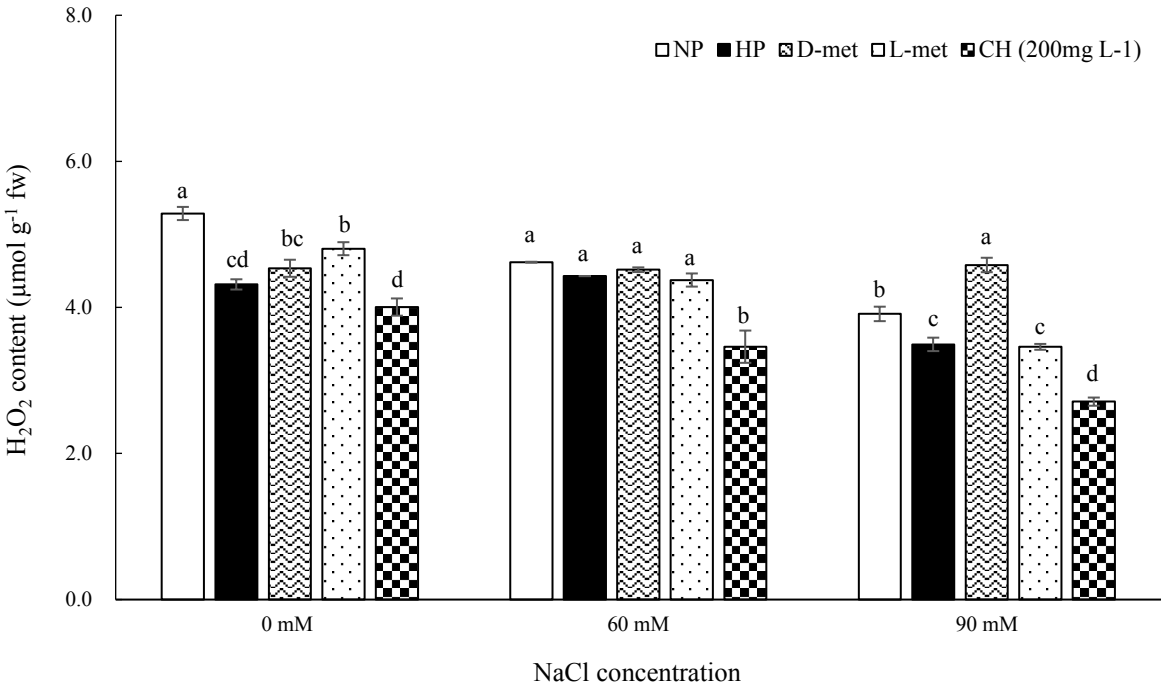


Fig. 4.5. Levels of hydrogen peroxide (H_2O_2) in the roots of 4-day-old seedlings of *L. multiflorum* in different seed priming treatments: with distilled water (Hydro-priming; HP), casein hydrolysate (CH), L-methionine, and D-methionine and control (Non-priming; NP). The seedlings were incubated under different concentrations of NaCl (0, 60, and 90 mM). Data represent the mean of each treatment with standard errors (SE) (n=8). Same letters indicate no difference in each NaCl concentration (LSD, $p< 0.05$).

4.4.6. Effect on lipid peroxidation

The roots of the seedlings in the CH priming treatment exhibited the lowest level of malondialdehyde (MDA) compared to the other priming treatments whether the seedlings were grown in the absence or presence of 60 or 90 mM NaCl ($p< 0.05$) (Fig. 4.6). In contrast, those in the D-methionine treatment seemed to have the highest MDA levels when the seedlings were grown in the different levels of NaCl.

Alteration in MDA level is associated with products of lipid peroxidation in plant tissues under salt stress (Gunes et al., 2007; Sekmen et al., 2012). Therefore, the extent of lipid peroxidation in plant tissues can reflect plant sensitivity and tolerance to salt stress (Pérez-López et al., 2009). In the present study, significant decrease in MDA concentration in the seedling roots in the CH-priming treatment under salt stress suggested lowered lipid peroxidation. This may lead to less oxidative damages to the peroxisomes of *L. multiflorum*. In particular, the MDA content in the roots of CH-priming caused a 56% decrease compared to the non-priming control when the seedlings were exposed to salinity (60 mM NaCl). Similarly, there was a significant decrease in MDA levels of wheat (*T. aestivum* L.) seedlings from seeds primed with cysteine (Nasibi et al., 2016). The researchers demonstrated that increased antioxidant enzyme activities via cysteine seed-priming treatment may contribute to the MDA reduction, thereby alleviating oxidative damages to the structural integrity cell membranes. In addition, exogenous application of salicylic acid, proline, and cysteine significantly reduced MDA levels in seedlings of *H. vulgare, Torreya grandis,* and *Arabidopsis thaliana*, respectively, compared to seedlings in the control when the seedlings were grown under salt stress (Genisel et al., 2015; Li et al., 2014; Slama et al., 2014).

In contrast, various researchers reported negative effects of seed priming on seedlings of some plant species in terms of lipid peroxidation under salt stress including Indian mustard (*Brassica juncea* L.) seedlings from $CaCl_2$ seed priming (Srivastava et al., 2010) and maize

(*Zea mays* L.) seedlings from chloroethylphosphonic acid (CEPA) seed priming (Carvalho et al., 2011). In the present study, D-methionine priming caused a comparatively significant increase, relative to the other seed priming treatments, in MDA concentration in *L. multiflorum* seedling roots, suggesting that it may impose more oxidative damages in cell membranes due to increased lipid peroxidation. While L-amino acids are widely distributed in all living organisms including higher plants (Brückner and Westhauser, 2003; Shahjee et al., 2002), D-amino acids are usually in some animals, insects, and microorganisms. The D-amino acids play a role in activating metabolism of the organisms (Brückner and Westhauser, 2003; Shahjee et al., 2002). Indeed, it was reported that plants contain much less D-amino acids rainging from 0.2 to 8% relative to the corresponding L-amino acids (Brückner and Westhauser, 2003). Similarly to this study, Shahjee et al. (2002) found that application of different types of L- and D-amino acids had different effects on growth of *Escherichia coli* under salt stress. D-proline, D-lysine, D-serine, and D-alanine significantly inhibited cell growth of *E. coli* under salt stress compared to L-amino acids. The researchers suggested that the L-amino acid such as L-proline may benefit cellular metabolism of the bacteria by playing a role as an osmoprotectant under salt stress. Thus, it seems that D-amino acids applied for seed priming negatively affected osmotic balance as well as antioxidant mechanism of the Italian seedling roots in the present study, compared to L-amino acids.

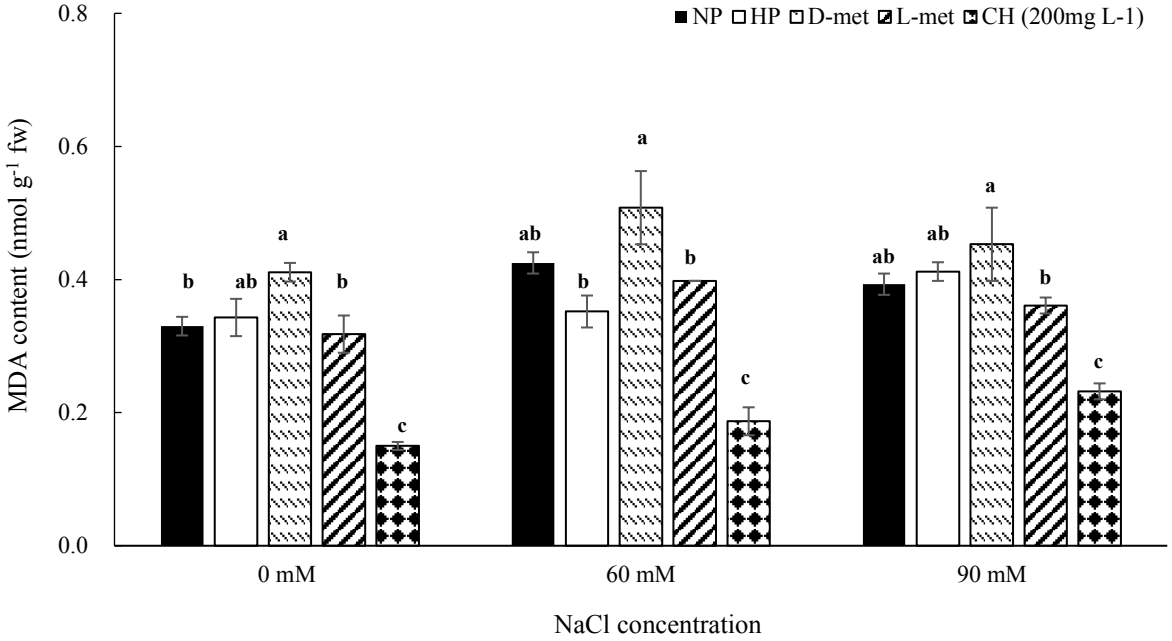


Fig. 4.6. Malondialdehyde (MDA) content (µmol g^{-1} FW) in roots of 4-day-old seedlings of *L. multiflorum* in different seed priming treatments: priming with distilled water (Hydro-priming; HP), casein hydrolysate (CH), L-methionine, and D-methionine and control (Non-priming; NP). The seedlings were grown in different concentrations of NaCl (0, 60, and 90 mM). Data represent the mean of each treatment with standard errors (SE) (n=8). Same letters indicate no difference in each NaCl concentration ($p< 0.05$).

4.4.7. Increase in proline content

Proline content in the roots of *L. multiflorum* seedlings increased gradually with increasing NaCl concentrations in the different treatments in this study (Fig. 4.7). Among the different seed priming treatments, CH priming significantly increased proline contents in the roots exposed to different levels of NaCl compared to NP ($p < 0.05$). In particular, in 60 mM NaCl, the proline concentration in the roots of the CH seed priming treatment was 40% higher than that in the NP. The increase in proline level can contribute to salt stress tolerance by acting as an osmoprotectant for protecting damage in cellular organelles, maintaining water content, and reducing ROS including $^1O_2$ and $OH^{\bullet}$ (Jiménez-bremont et al., 2006; Li et al., 2014; Misra and Gupta, 2005; Semida and Rady, 2014). There are no previous studies that showed the effect of seed priming, particularly with amino acids, on proline content under salt stress. However, similar tendency in proline content of plant roots to the current study were found through exogenous treatments. Misra and Saxena (2009) found a higher concentration of proline in the roots of lentil (*Lens esculenta*) seedlings by exogenous treatment with salicylic acids than the control plant in response to NaCl treatment. Jiménez-Bremont et al. (2006) also found that seedling roots of *Phaseolus vulgaris* L. from seeds exogenously treated with a mixture of polyamines involving putrescine, spermidine, and spermine contained increased proline content when incubated in 150 mM NaCl. The other exogenous treatments with plant hormone (24-epibrassinolide) (Rady, 2011) and spermidine (Duan et al., 2008) markedly increased proline content in roots of bean (*P. vulgaris* L.) and cucumber (*Cucumis sativus* L.) seedlings, respectively, when the seedlings were grown under salt stress.

In contrast, L- and D-methionine priming treatments compared to NP showed a very significant reduction in proline content in the Italian ryegrass roots ($p < 0.05$). The proline concentration in the roots from seeds primed with both types of amino acids was approximately

80% lower than that in the NP roots in 0 and 60 mM NaCl. Particularly when the seedlings were grown in 90 mM NaCl, in the D-methionine priming treatment the most significant reduction (by 72%) in proline concentration in the roots compared to NP. This may suggest that D-methionine influenced osmotic adjustment negatively, resulting in insufficient radicle development after 4 days of incubation. On the other hand, considerably increased radicle length of *L. multiflorum* seedlings by L-methionine priming in the present study may be related to the enhanced capacity of mitigating salt stress for osmoregulation to some extent in this case, independent of proline content in the plant tissue. Similar tendency of reverse relationship between plant growth and proline concentration in the roots of wheat seedlings (*T. aestivum* L.) from seeds primed with cysteine was previously reported by Nasibi et al. (2016).

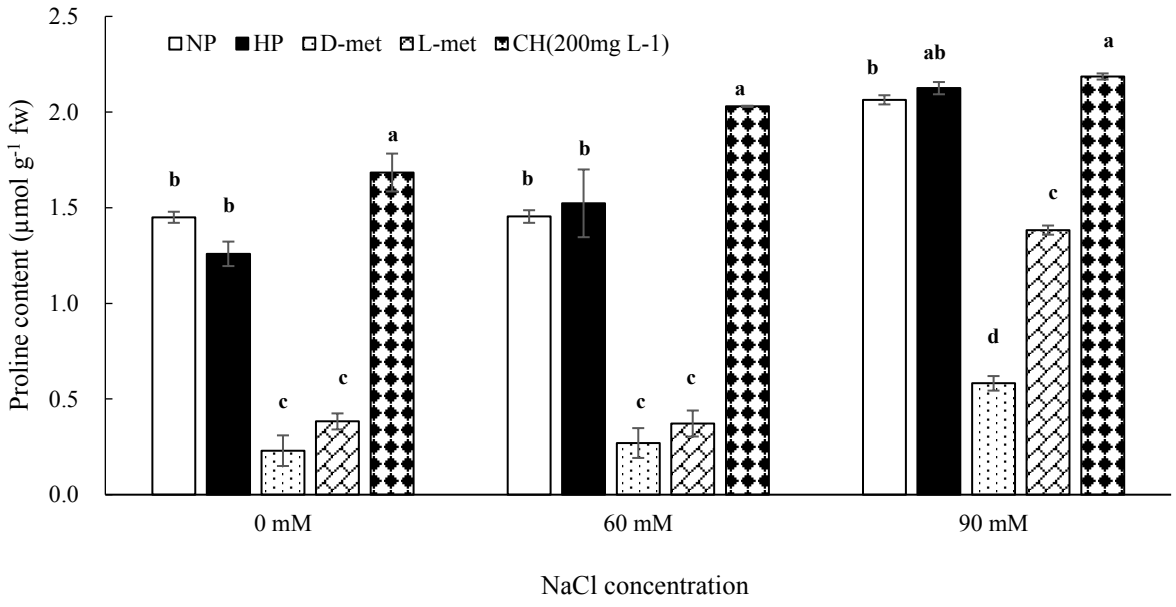


Fig. 4.7. Proline content (µmol g^{-1} FW) in roots of 4-day-old *L. multiflorum* seedlings from seeds primed with distilled water (Hydro-priming; HP), casein hydrolysate (CH), L-methionine, and D-methionine and control (Non-priming; NP). The seedlings were grown in different concentrations of NaCl (0, 60, and 90 mM). Data represent the mean of each treatment with standard errors (SE) (n=8). Same letters indicate no difference in each NaCl concentration (LSD, $p< 0.05$).

4.4.8. Overall effect of seed priming in laboratory

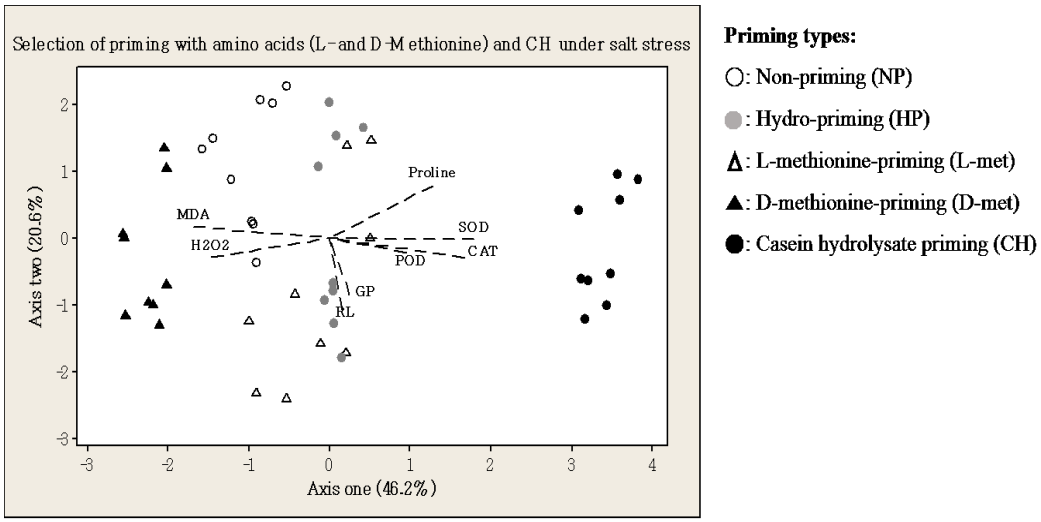
Multivariable analysis was conducted to interpret the overall effects of seed priming applications of L- and D-met, and CH under salt stress tolerance of *L. multiflorum* using a Principle Component Analysis (PCA) (Fig. 4.8). Data for germination percentage (GP), radicle length (RL), antioxidant enzymes (POD, SOD, and CAT) activities, oxdidative stress markers (H_2O_2 and MDA), proline content collected from various laboratory experiments were used for PCA. This analysis clearly showed that different seed priming treatment varied the physiological responses of the ryegrass at this earlier time of exposure against salt stress.

There were different trends among L- and D-met, and CH seed priming assays. Along with Axis 1, CH seed priming treatment was markedly separated from other priming treatments, with significant positive associateion with SOD, CAT, and POD enzyme activities and proline content and negative association with MDA and H_2O_2 levels. On the other hand, D-methionine priming treatment showed the opposite trend and the remaining priming treatments had less effects on those variables.

In addition, the combined datasets apparently separated the salt treatments of different concentrations (0, 60, and 90 mM NaCl) along with Axis 2. Based on the PCA results, increasing NaCl concentrations caused decreases in GP and RL but an increase in proline content of the 4-day-old ryegrass seedlings.

The increase in proline content might suggest that osmo-protectant capacity in the roots of Italian ryegrass seedlings was enhanced in CH-priming when grown in severe salt stress (90 mM NaCl) in this study. Therefore, the PCA results may suggest that casein hydrolysate containing 18 amino acid compounds, some vitamins, and nutrients is more beneficial for seed priming than L-met. This might be related to an enhancement in metabolic and biosynthetic

pathways associated with salt stress tolerance, particularly at the early post-germinative growth stage of Italian ryegrass.



 # Coefficients of each variable for PC1 and PC2 through the PCA.

Variable	GP	RL	SOD	CAT	POD	H_2O_2	MDA	Proline
PC1	0.068	0.042	0.485	0.454	0.258	-0.397	-0.456	0.347
PC2	-0.513	-0.650	-0.020	-0.178	-0.132	-0.173	0.102	0.474

Fig. 4.8. Principal Component Analysis (PCA) of growth and physiological responses of *L. multiflorum* to salt stress in L- and D-methionine, and CH-priming assay

4.5. Conclusion

This study shows that the use of L-methionine and CH priming on Italian ryegrass seeds could have positive effects on the germination and radicle growth under salt stress. Particularly, CH priming best enhanced the ROS scavenging mechanisms in seedlings of *L. multiflorum* under salt stress by promoting SOD, CAT, and POD activities and by reducing H_2O_2 and MDA (lipid peroxidation) accumulation. L-methionine priming also seemed to enhance salt tolerance of the ryegrass, but D-methionine priming did not. In addition, increased

proline in the roots of the Italian ryegrass seedlings following seed priming with CH may improve cellular redox potential, protect subcellular, and stabilise antioxidant enzymes against salt stress (Kubala et al., 2015; Szabados and Savouré, 2010). These increased antioxidant enzymatic activities seemed to contribute to reduction of ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) or protection of plant organelles from damages caused by the ROS at the early phase of seedling establishment. These results indicate that the seed priming treatment with CH can reinforce salt stress tolerance of *L. multiflorum* at least during germination and at the early seedling growth. Therefore, in relation to cropping in saline soil, the seed priming technology with CH may be an innovative way to reduce salt-stressed sensitivity of crops and enhance their productivity. Further practical works are needed to evaluate the effect of CH priming on the physiology and yield of the Italian ryegrass in saline soil in a greenhouse experiment.

Chapter 5. Effects of seed priming with casein hydrolysate and L-methionine on physiological responses and growth of Italian ryegrass (*Lolium multiflorum* L.) in saline soil: a glasshouse study

5.1. Abstract

Italian ryegrass (*Lolium multiflorum*) is one of the major pasture species in New Zealand, but its productivity has been threatened due to increasing salinity in agricultural soils across both North and South Islands. In earlier studies of Chapters 3 and 4, seed priming with casein hydrolysate (CH) and L-methionine (L-met) showed the potential for improving seedling growth and salt stress tolerance of the ryegrass under controlled laboratory conditions. The objective of the present study was to investigate effects of the CH and L-met priming treatments of the ryegrass seeds on the productivity and physiological responses of the plants in saline soils through pot experiments under greenhouse conditions. Seeds of non-priming (NP) and hydro-priming (HP) as well as those primed with CH and L-met were sowed in pots, and solutions of different NaCl concentrations (0, 60, 120, and 180 mM) were added daily for 6 weeks. During the experiment, germination percentage (GP), electrolyte leakage (EL) of roots, chlorophyll and carotenoid contents, antioxidant enzyme activities including SOD, CAT, and POD, and biomass in roots and leaves were measured. In all the pots, increasing NaCl concentrations gradually increased electrical conductivity (EC) in soil and EL in root, which appeared to decrease GP and growth of all the ryegrass plants. CH and L-met priming showed benefits for germination, growth, and physiological response of the Italian ryegrass in soils added with salt. In 60 and 120 mM NaCl, CH priming seemed to be more effective in relation to maintaining higher GP as well as production of photosynthetic pigments (chlorophyll and carotenoid). In particular, increase in plant biomass was greater in the CH priming treatment

than in the L-met priming treatment. Activities of antioxidant enzymes (SOD, CAT, and POD) in leaves in the CH priming treatment were also considerably different than those of NP and HP at the second week. Of these enzymes, POD activity was significantly increased by both CH and L-met seed priming, while CAT activity was significantly enhanced only by the CH seed priming when the seedlings were grown in salt-added soils. Multivariate analysis showed that the effects of CH seed priming distinctly outweighed the effects of L-met seed priming, in terms of enhancement of tolerance mechanism to salt stress during growth phases, particularly from germination to seedling growth. These results suggest that CH (from Sigma) seed priming containing various amino acids and possibly undefined levels of other salts and vitamins has better potential for enhancing yields of this pasture species in the agricultural soils with increased salinity level.

Keywords: Casein hydrolysate, L-methionine, Saline soil, Antioxidant enzymes, Chlorophyll content, Productivity

5.2. Introduction

Soil salinity has threatened crop productivity of agricultural lands in New Zealand and elsewhere. Globally, it is estimated that about 20% of agricultural lands and 33% of irrigated arable lands has been influenced by high salinity (Shrivastava and Kumar, 2015). Nowadays, the area of saline soils is increasing at a rate of 10% per annum due to climate changes leading to low precipitation and high evaporation in land surface and human activities including irrigation and intensive land management. Therefore, crops yields could be reduced and give rise to eco-environmental problems in the surrounding water environment (Chen et al., 2018;

Shrivastava and Kumar, 2015). Jamil et al. (2011) speculated that over 50% of total agricultural lands would be salinised in the next 30 years.

The metabolism of roots in salinised soil would be interrupted, and thereby crop productivity and quality could be suppressed (Farooq et al., 2015; Shrivastava and Kumar, 2015). In particular, high salinity in soil may cause inhibition of growth phases from seed germination to seedling establishment as the ROS level linked to damages of lipid membranes, proteins, and nucleic acids could be elevated (AbdElgawad et al., 2016; Akbarimoghaddam et al., 2011). To alleviate the salinity damage, plants protect themselves for stress tolerance through various defence mechanisms. Under abiotic stress, plants can activate higher activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) (Puyang et al., 2015). The antioxidative enzymes play a crucial role in protecting salt stress-induced cellular membrane damages by scavenging deleterious reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) (Gill and Tuteja, 2010). In addition, significant salt stress-induced damages in the organelles such as chloroplasts, cytosol, mitochondria, and vacuole can be alleviated by secondary metabolites such as tocopherols, phenolics, flavonoids, ascorbic acid, glutathione, carotenoids, and proline, etc. (Das and Roychoudhury, 2014; Trchounian et al., 2016).

To increase productivity of various crops cultivated in saline soils, various researchers have tried to study experimental ways to trigger the mechanisms associated with salinity tolerance. In particular, exogenous application of amino acids such as proline and salicylic acid to seeds, roots, or leaves during plant growth (i.e. not seed priming as after application of amino acids, drying of the plant material was not carried out) showed considerable benefits in the context of morphology, physiology, and yields of the plants (Abd El-Samad et al., 2011; Hasanuzzaman et al., 2014; Hoque et al., 2007a, b; Misra and Saxena, 2009). Moreover, it has

been investigated in several studies (e.g. Chang et al., 2010; Cheng et al., 2016; Genisel et al., 2015; Zeid, 2009) that exogenous application of amino acids enhanced production of antioxidant enzymes and secondary metabolites in plants, contributing to increases in germination and growth when the plants were exposed to salt stress. Teixeira et al. (2017) noted that following treatment of soybean (*Glycine max* L.) seeds with amino acids including cysteine, glutamate, glycine, and phenylalanine, singly, and a mixture of them, there was a higher CAT activity in the amino acid-treated soybean plants compared to those without treatment with amino acids in a greenhouse trial. Additionally, the same seed treatments with amino acids improved nitrogen metabolism and productivity of the soybean in a field experiment (Teixeira et al., 2018). In these experiments, the soils were not amended with NaCl.

Italian ryegrass (*L. multiflorum*) is one of the major pastoral species in New Zealand and its forage productivity is critical to the yields of dairy farm production (Charton and Stewart, 1999; Thom and Bryant, 1996). However, low rainfall, high evaporation, and increasing salinisation in arable soil due to intensive management of farmlands may result in simultaneous occurrence of drought and salt stresses (Hu and Schmidhalter, 2005; IPCC, 2007). The increased abiotic stresses will cause concern about decline in the current NZ agricultural production. In earlier chapters, applications of CH and some amino acids for priming of seeds of *L. multiflorum* had shown significant benefits for salt stress tolerance in relation to GP and radicle growth, as well as proline content and enhanced enzymatic activities of SOD, CAT, and POD compared to unprimed control. In addition, ROS and lipid peroxidation in the roots of Italian ryegrass seedlings were considerably reduced in the CH priming treatment. However, the earlier studies had limitations in the laboratory environment and were of a short duration (4 days of early post-germinative seedling growth). Therefore, this study aimed to investigate efficiency and potential of seed priming with CH and L-met for improving salt stress tolerance and productivity of *L. multiflorum* in soils amended with salt for several weeks under less

controlled environment in the glasshouse. In particular, through periodical measurements of photosynthetic pigments and antioxidant enzyme activities, influences of CH and L-met priming applications on physiological and biochemical responses against salinity stress were studied during the growth of Italian ryegrass plants in pot trials.

5.3. Materials and Methods

5.3.1. Greenhouse experiment

Seeds of Italian ryegrass (*Lolium multiflorum*) were soaked in casein hydrolysate (200 mg L^{-1}) and 1 mM L-methionine singly for seed priming. Hydro-priming (HP) and non-priming (NP) treatments were prepared for comparison in this study as described in Chapter 2 (section 2.2.1. seed priming).

Pot experiments were carried out in a greenhouse of the University of Canterbury, Christchurch, New Zealand. Each plastic pot (Ø: 10.5 cm and height: 11.5 cm) was filled with a mixture of pumice and soil (v/v=1:3). Each pot was laid on a plant tray containing tap water to control moisture content in the soil mixture properly. Then, all pots were left for one day on a table (without the plant tray) for draining before sowing. Seventy seeds of the Italian ryegrass (about 0.25 g) of NP, HP, and CH priming or L-met priming were sowed in 2 cm depth of topsoil in each pot. Different concentrations of NaCl (0, 60, 120, and 180 mM) were prepared and stored in a fridge at 4 ˚C prior to salt stress treatment. Following sowing, 30 ml of each concentration of NaCl was supplied to each pot on a daily basis. A total of 84 pots were laid on tables randomly, and there were seven replicates in each treatment.

The present mesocosm experiments were conducted at two different times; the Italian ryegrass seeds primed with CH and L-met were sowed in April and December (2016), respectively. Controls of either NP and HP seeds were included in each greenhouse assay. After

sowing, the seeds were germinated in a week and cultivated for 5 weeks after sprouting. Environmental conditions in the greenhouse during the cultivation period were recorded using a HOBO data logger (UA-002-64 onset, USA) and then the data were extracted.

5.3.2. Analyses of plant and soil

5.3.2.1. Germination rate and biomass

Germination percentage (GP) of the seeds of *L. multiflorum* was recorded weekly during the cultivation period; only seedlings exhibiting more than 2 mm of hypocotyl length were included in this study. The GP was calculated using the following formula: germination percentage (%) = (number of germinated seeds/total number of seeds sown) x 100. When harvesting, shoots and roots of the Italian ryegrass were separated and their fresh weight (FW) were determined using a balance (Sartorius TE412, Germany). Before weighing, the roots were rinsed with tap water and then surface dried with tissue papers. For determination of dry weight (DW), the shoots and roots were stored in paper bags and oven-dried for three days at 65 ˚C.

5.3.2.2. Electrolyte leakage

To measure electrolyte leakage (EL), 0.1 g of fresh roots of *L. multiflorum* were randomly sampled from three of seven pots in each treatment after the determination of root FW. The samples were placed in a 15 ml Falcon tubes filled with 10 ml of de-ionised (DI) water and stored for 24 h at room temperature in the dark. Electrical conductivity (EC1) of the soaking solution was first determined using a potable conductivity meter (Fisher scientific^{TM} accumet TM AP 85, USA). Then the Falcon tubes were heated for 2 min in a water bath at 95 ˚C. After

cooling down for 10 min at room temperature, the second electrical conductivity (EC2) was determined. EL was calculated as a ratio of EC1/EC2 (Shi et al., 2006).

5.3.2.3. Chlorophyll and carotenoid contents

Determination of chlorophyll and carotenoid contents determination was carried out as described in 2.4.4 (see Chapter 2).

5.3.2.4. Electrical conductivity in soil

Following plant sampling, soils were air-dried in the greenhouse and sieved using a 2 mm steel sieve. The dried soils were soaked for 1 h in DI water (soil: DI water = 1:5 w/v). The soil EC was measured using an EC meter (SevenEasy, Mettler Toledo, Switzerland).

5.3.3. Antioxidant enzyme extraction and assays

Enzyme extraction was carried out as described in 2.4.5.1 (see Chapter 2). About 0.1 g of the Italian ryegrass leaves were randomly collected from pots in each treatment for activity assays (section 2.4.5.2 to 2.4.5.4) of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) at 2 and 5 weeks after sowing.

5.3.4. Statistical analysis

All the data were statistically analysed using a Minitab 16 (Minitab Inc.) in the present study. Variations in GP, total chlorophyll and carotenoid contents, antioxidant enzyme activities (CAT, SOD, and POD), EL in roots, and FW and DW of roots and shoots in response to seed priming treatments were assessed separately at a NaCl concentration with one-way analysis of

variance (ANOVA) using Fisher's LSD test (n=7; $p < 0.05$). Principal Components Analysis (PCA) was conducted to investigate physiological influence of seed priming with CH and L-met compared to NP and HP in salt-treated soil. Relative dissimilarities between seed priming treatments in ordination space were observed along on Axis 1 and 2 of PCA.

5.4. Results and discussion

5.4.1. Variations in germination and growth

Seed priming with casein hydrolysate (in the first pot trial) and L-methionine (in the second pot trial) resulted in different germination percentages of *L. multiflorum* seeds (Table 5.1). In the two pot trials to investigate the effect of CH and L-met, GP of non-primed Italian ryegrass seeds were decreased with increasing NaCl concentrations added to soil (Table 5.1). There appeared to be no considerable differences in GP among NP and the two seed priming treatments when seeds were germinated in 180 mM NaCl ($p > 0.05$). In the first pot experiment, the seeds primed with CH or de-ionised water (HP) exhibited significantly higher GP compared to NP when incubated in 60 and 120 mM NaCl ($p < 0.05$). In the second pot trial, seeds primed with L-met exhibited significantly higher GP than NP ($p < 0.05$), when the seeds were germinated in 120 mM NaCl.

Table 5.1. Germination percentages of *L. multiflorum* seeds without priming (NP), primed with de-ionised water (hydro-priming or HP), or casein hydrolysate (CH), or L-methionine (L-Met). The seeds were germinated in different NaCl concentrations (0, 60, 120, and 180 mM). Data presented are means ± standard errors (n= 7). Within each NaCl treatment in each pot experiment, same letters indicate no significant differences by Fisher's range test at $p< 0.05$.

NaCl concentration	---------- 1st pot experiment ----------			---------- 2nd pot experiment ----------		
	NP	**HP**	**CH**	**NP**	**HP**	**L-Met**
0 mM	77.7±2.3 ^{a}	78.5±2.3 ^{a}	85.6±1.5 ^{a}	71.4±2.4 ^{b}	77.4±2.5 ^{b}	91.4±2.5 ^{a}
60 mM	62.3±3.1 ^{b}	81.6±2.5 ^{a}	91.9±0.8 ^{a}	74.5±2.2 ^{ab}	69.7±2.4 ^{b}	81.1±1.3 ^{a}
120 mM	51.4±4.4 ^{c}	71.9±1.8 ^{b}	85.5±1.2 ^{a}	53.0±1.4 ^{b}	59.1±1.8 ^{ab}	63.5±2.4 ^{a}
180 mM	58.5±2.1 ^{a}	57.8±1.2 ^{a}	65.9±3.0 ^{a}	25.7±3.6 ^{a}	36.7±3.2 ^{a}	32.1±3.6 ^{a}

At the end of the two pot trials (6 weeks each), FW and DW of both the roots and shoots of the Italian ryegrass seedlings in all the treatments were gradually reduced with increasing salt concentrations added to soil (Table 5.2). In particular, the greatest reductions in the FW and DW were observed in higher NaCl concentrations (120 and 180 mM) in the first pot trial for investigating the effect of CH seed priming. In response to 60 mM NaCl, the fresh weights of the shoots of the seedlings raised from hydro-primed and CH-primed ryegrass seeds did not seem to be affected, but those of the seedlings from unprimed seeds were lowered. Meanwhile, the fresh weights of the roots of the seedlings raised from hydro-primed seeds were the highest compared to those of seedlings from unprimed and casein hydrolysate-primed seeds. The fresh weights of the roots of the seedlings from NP-seeds were the lowest. When the ryegrass seedlings were exposed to 120 mM NaCl for 6 weeks under the glasshouse conditions, CH seed priming appeared to be the most beneficial for the fresh weights of the shoots compared to NP and HP. The fresh weights of the roots of the seedlings in the NP treatment were significantly lower than HP and CH ($p<0.05$). In the absence of added NaCl, the dry weights

of the shoots were highest in the seedlings in the CH seed priming treatment compared to the NP and HP treatments, while there was no difference in the dry weights of the roots among the three seed priming treatments (Table 5.2). In response to 60 mM NaCl, the seedlings in NP exhibited the lowest dry weights of the shoots and roots among the seed priming treatments. In response to 120 mM NaCl, the dry weights of shoots and roots in the CH seed priming treatment were the highest compared to NP and HP and were 2.1 and 2.7 times higher than those of NP ($p< 0.05$).

In the second pot trial (Table 5.2), the fresh and dry weights of the shoots of Italian ryegrass seedlings in the three seed priming treatments were not different when grown under different added NaCl concentrations (0-120 mM). In response to 60 mM NaCl, the fresh weights and dry weights of the roots of the seedlings in the L-met seed priming treatment were significantly higher than those in the NP ($p< 0.05$).

Table 5.2. Effect of CH (for first pot experiment) and L-methionine (for second pot experiment) seed priming treatments on fresh and dry weights (FW and DW) of *L. multiflorum* seedlings grown with different added NaCl concentrations (0, 60, 120, and 180 mM). Data presented are means ± standard errors (n= 7). Within each NaCl treatment, the means with same letters indicate no significant differences among priming treatments by Fisher's range test at $p< 0.05$.

NaCl concentration	Seed priming treatment	---------- 1st pot experiment ----------				Seed priming treatment	---------- 2nd pot experiment ----------			
		FW (g)		DW (g)			FW (g)		DW (g)	
		Shoot	Root	Shoot	Root		Shoot	Root	Shoot	Root
0 mM	NP	8.29±0.30 ^{a}	4.63±0.47 ^{a}	0.81±0.04 ^{b}	0.68±0.05 ^{a}	NP	3.18±0.16 ^{a}	5.00±0.41 ^{ab}	0.81±0.03 ^{a}	1.13±0.20 ^{a}
	HP	8.63±0.58 ^{a}	4.81±0.26 ^{a}	0.86±0.05 ^{b}	0.51±0.06 ^{a}	HP	3.18±0.15 ^{a}	4.64±0.35 ^{b}	0.82±0.04 ^{a}	0.62±0.05 ^{a}
	CH	7.78±0.49 ^{a}	4.22±0.20 ^{a}	1.10±0.01 ^{a}	0.62±0.09 ^{a}	L-met	3.38±0.20 ^{a}	6.26±0.52 ^{a}	0.78±0.05 ^{a}	1.41±0.14 ^{a}
60 mM	NP	7.21±0.38 ^{b}	4.27±0.13 ^{c}	0.80±0.05 ^{b}	0.42±0.04 ^{b}	NP	3.25±0.13 ^{a}	2.92±0.09 ^{b}	0.77±0.03 ^{a}	0.40±0.08 ^{b}
	HP	8.02±0.21 ^{a}	6.37±0.11 ^{a}	0.98±0.03 ^{a}	0.63±0.07 ^{a}	HP	3.35±0.23 ^{a}	3.18±0.04 ^{b}	0.82±0.04 ^{a}	0.62±0.05 ^{b}
	CH	8.10±0.14 ^{a}	5.10±0.17 ^{b}	0.94±0.02 ^{a}	0.56±0.09 ^{ab}	L-met	3.26±0.23 ^{a}	3.98±0.24 ^{a}	0.84±0.06 ^{a}	0.97±0.14 ^{a}
120 mM	NP	2.62±0.11 ^{c}	1.35±0.06 ^{b}	0.34±0.04 ^{c}	0.15±0.02 ^{c}	NP	3.00±0.15 ^{a}	1.66±0.19 ^{b}	0.60±0.03 ^{a}	0.37±0.04 ^{a}
	HP	4.37±0.12 ^{b}	2.24±0.12 ^{a}	0.61±0.03 ^{b}	0.29±0.04 ^{b}	HP	3.26±0.15 ^{a}	2.27±0.14 ^{a}	0.66±0.03 ^{a}	0.39±0.03 ^{a}
	CH	4.79±0.07 ^{a}	2.76±0.06 ^{a}	0.71±0.03 ^{a}	0.41±0.04 ^{a}	L-met	3.17±0.14 ^{a}	2.33±0.16 ^{a}	0.68±0.03 ^{a}	0.46±0.04 ^{a}
180 mM	NP	1.66±0.17 ^{a}	0.96±0.09 ^{a}	0.26±0.02 ^{a}	0.13±0.02 ^{a}	NP	0.81±0.11 ^{c}	0.53±0.07 ^{b}	0.18±0.02 ^{b}	0.15±0.01 ^{a}
	HP	1.99±0.17 ^{a}	1.02±0.09 ^{a}	0.29±0.03 ^{a}	0.12±0.01 ^{a}	HP	1.42±0.05 ^{a}	1.01±0.06 ^{a}	0.26±0.03 ^{a}	0.19±0.02 ^{a}
	CH	1.78±0.17 ^{a}	1.00±0.13 ^{a}	0.27±0.04 ^{a}	0.16±0.03 ^{a}	L-met	1.13±0.09 ^{b}	0.81±0.06 ^{a}	0.23±0.02 ^{ab}	0.17±0.03 ^{a}

Soil salinity inhibits seed germination and also early seedlig growth that would consequently reduce crop productivity (Ashraf et al., 2008; Ashraf and Foolad, 2005). In particular, salinity stress causes metabolic changes in hydrolysis, utilisation of seed (food) reserves, enzyme activity, plant hormones, and N metabolism during seed germination (Ashraf and Foolad, 2005). The metabolic activities could also be affected at the early phase of plant growth. Indeed, accumulation of toxic Na^+ ion delays plant growth and development under salinity, in particular at the stages of seed germination and early seedling growth, which is highly sensitive to salt stress (Farooq et al., 2015). In this study, however, seed priming mitigates effects of salt stress on plant growth such as root length and fresh and dry weight of shoots and roots (Ashraf and Foolad, 2005). In particular, CH seed priming of *L. multiflorum* was found to show the most potential to protect the seedlings against salt stress compared to the other two seed priming treatments (NP and HP), particularly when the seedlings were grown with added 120 mM NaCl. This suggests that the increase in GP of the seeds primed with CH compared to NP might be contributed by a higher efficiency for water uptake from the soil substrate and metabolic activity during germination (Bajehbaj, 2010). In addition to rapid and uniform seed germination, this beneficial effect of CH seed priming could enhance seedling establishment and crop productivity under salt stress (Ashraf and Foolad, 2005; Ibrahim, 2016).

Germination and growth of the Italian ryegrass might also be affected by variations in seasonal factors such as temperature and sunlight intensity during the pot experiments (Fig. 5.1). Indeed, these external factors in summer (second pot trial involving L-met seed priming) that could lead to higher rates of evaporation than in autumn (first pot trial involving CH seed priming) in pot soil might increase salt stress to the Italian ryegrass seedlings (Allbed et al., 2014; Gorai et al., 2014). The precise influence, if any, these external factors that affected the

performance of the primed seeds under glasshouse conditions remained to be investigated further.

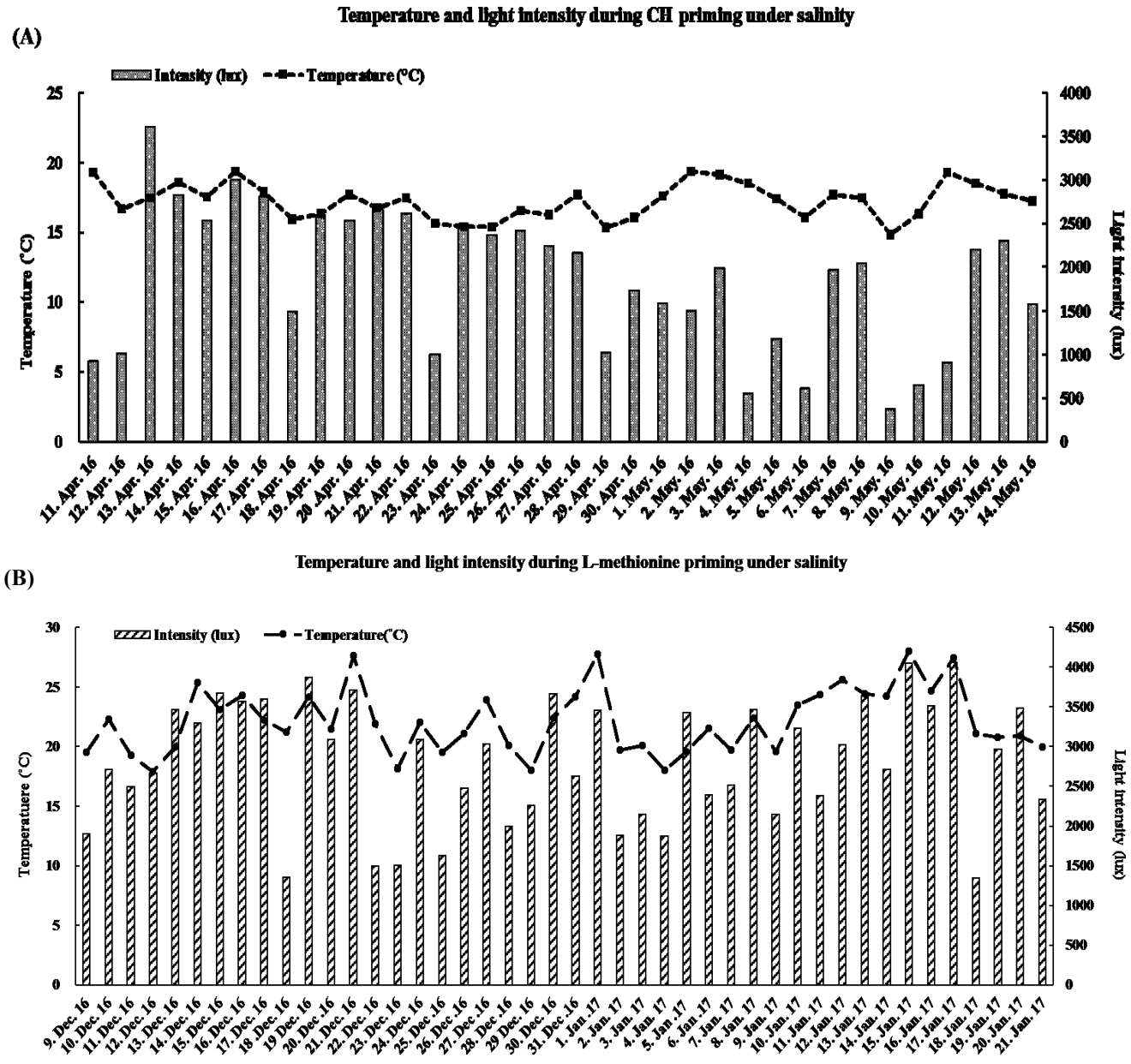


Fig. 5.1. Variations in sunlight intensity and temperature during the pot experiments of casein hydrolysate seed priming (A) and L-methionine seed priming (B).

5.4.2. Evidence of salt accumulation in soil and root

Soil electrical conductivity (EC) in the pots with different concentrations of NaCl was different after 6 weeks of plant growth. Significant increases in EC values in the soil added with increasing NaCl concentrations in the autumn of 2016 (the first pot trial on CH seed priming) and summer of 2017 (the second pot trial with L-met seed priming) were detected ($p< 0.05$). Additionally, values of soil EC were higher in summer than in autumn except for control treatment (Table 5.3) ($p<0.05$). The difference in soil EC between two seasons may be due to dry weather condition in summer causing more frequent water evaporation from soil and plant's water use, which in turn influence water retention and subsequently drainage volume of salt solution used during the rygrass cultivation. Soil EC can be considered as an indicator of salt accumulation in the soil (Allbed et al., 2014; Aydin et al., 2012). Increased soil EC in the different treatments with increasing NaCl levels seemed to influence soil water potential (Allbed et al., 2014; Aydin et al., 2012). Therefore, water availability to the Italian ryegrass plants were affected negatively, and the dry weights of the plants were reduced (Table 5.3). The EL values extracted from the roots of seedlings grown in the presence of 120 and 180 mM NaCl were similar, but higher than those from lower salinity levels (Fig. 5.2). This suggests that increasing salinity caused increases in EL levels of the seedling roots. Thus, the increased EL of roots might be associated with salinity-induced oxidative stress or accumulation of ROS in the root and reduced root growh (Liu et al., 2012). Soil EC in each NaCl treatment was not different between the first pot trial in April (autumn 2016) and the second pot trial in December (summer 2017), ie. CH and L-met seed priming experiments, respectively.

Table 5.3. Soil electrical conductivity (EC) (dS m^{-1}) under different salt stress in glasshouse pot experiment of *L. multiflorum* seed priming during different seasons. Different letters in each column indicate significant differences by Fisher's range test at $p < 0.05$. Difference in EC level between seasons is indicated by * (significant at $p<0.05$) and ns (not significant).

NaCl (mM)	Summer (2017)	Autumn (2016)	Difference between seasons
0	0.16 ± 0.02^{d}	0.17 ± 0.03^{d}	ns
60	1.90 ± 0.14^{c}	1.18 ± 0.06^{c}	*
120	3.11 ± 0.07^{b}	1.57 ± 0.06^{b}	*
180	4.09 ± 0.15^{a}	1.69 ± 0.06^{a}	*

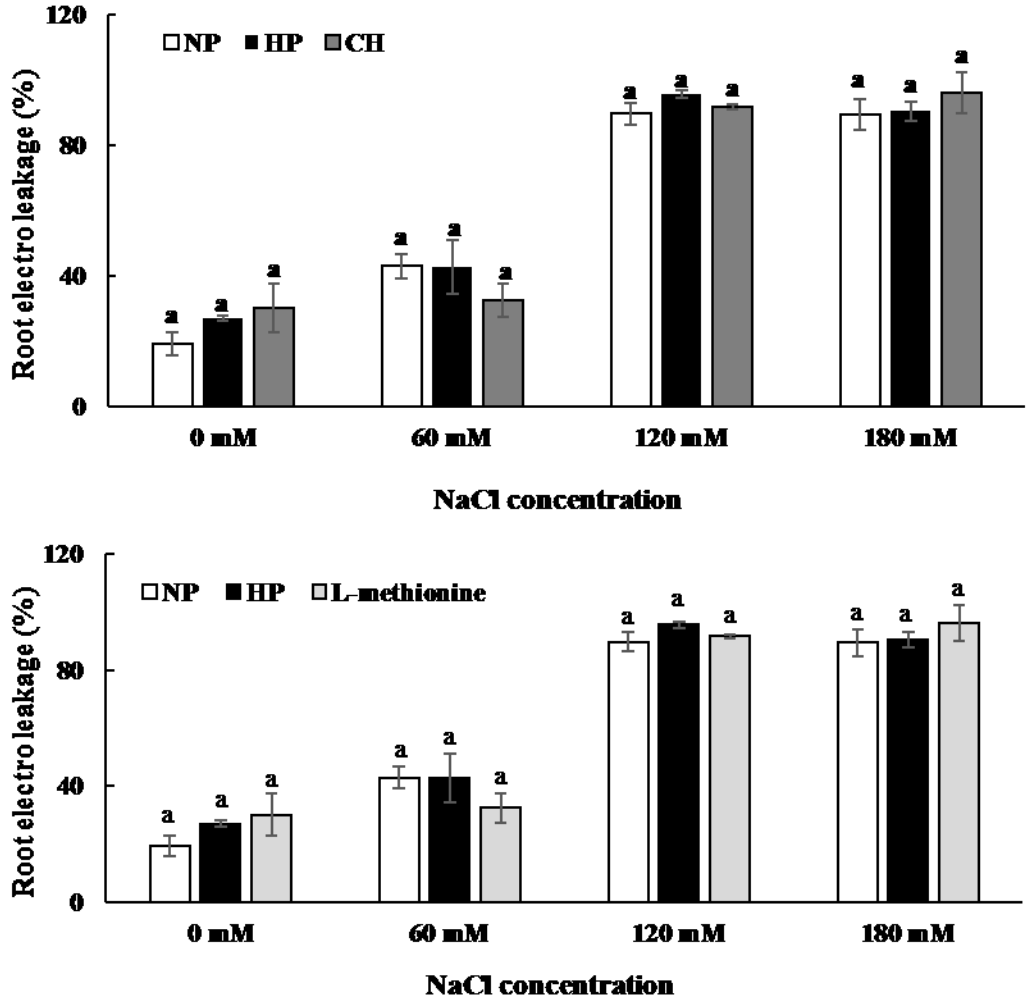


Fig. 5.2. Electrolyte leakage from roots of *L. multiflorum* grown for 6 weeks in different NaCl concentrations (0, 60, 120, and 180 mM) under glasshouse conditions: first pot trial (upper), and second pot trial (bottom). Data indicates means ± standard errors (n= 7). Different letters indicate significant differences within each NaCl concentration (LSD, $p< 0.05$).

5.4.3. Contents of photosynthetic pigments

With varying salt concentrations, total chlorophyll (a+b) and carotenoid contents of *L. multiflorum* leaves showed similar trends in each pot experiment. Increasing NaCl concentration significantly reduced the ryegrass biomass (Table 5.2), but marginally increased or did not change the photosynthetic pigment contents in the leaves (Figs. 5.3 and 5.4). There

was a limitation for sampling leaves from the 180 mM NaCl treatment in the second pot trial as there was not enough plant growth at the second week after germination. According to Ungar (1978), high temperature and evaporation in saline soil lower the water potential and inhibit seed germination. Hence, it might be due to high temperature in that summer so that increased evaporation from soil surface might have impeded the speed of seed germination.

In general, salinity in soil causes degradation of the photosynthetic pigments such as chlorophyll and carotenoid in plant leaves (Sultana et al., 1999). Between Na^+ and Cl^- ions, Cl^- inhibits chlorophyll generation and chlorotic toxicity (Parihar et al., 2015). Indeed, variation in the pigment content was dependent on sensitivity or tolerance of plant species and exposure period to salt stress (Doganlar et al., 2010; Sultana et al., 1999). Nevertheless, the present study showed reverse results; particularly in the first pot trial, leaf samples at the fifth week had considerably greater amounts of total chlorophyll (a+b) and carotenoid than that at the second week in all salt treatments. It may be due to *L. multiflorum* being a relatively salt-tolerant ryegrass species (Marcar, 1987). Thus, the salt-tolerant ryegrass cultivar could display a good capacity to maintain synthesising the photosynthetic pigments in the saline soils, by increasing the number of chloroplasts in the leaves under stress (Doganlar et al., 2010; Stefanov et al., 2016). Moreover, the higher chlorophyll content might be related to enhanced photosynthesis as well as enzyme synthesis participating in carbon reduction and developing chloroplast membrane system (Sanoubar et al., 2016). Similarly, some tolerant crop species such as broad bean (*Vicia faba* L.), tomato (*Lycopersicon esculentum* Mill.), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) showed increases in total chlorophyll and carotenoid content in their leaves in response to salt stress (Abd El-Samad, 1993a, b; Doganlar et al., 2010; Hamada, 1996; Misra et al., 1997).

Both of the two seed priming treatments in the present study influenced photosynthetic responses of *L. multiflorum*. In control pots of unsalted soils, CH and L-met seed priming

applications significantly increased the contents of total chlorophyll and carotenoid in the leaves of Italian ryegrass compared to either NP and HP ($p< 0.05$, respectively) (Figs. 5.3 and 5.4). Indeed, the positive effect of CH priming on the pigment development was likely to be improved more with increasing time of growth. In soils with added NaCl solutions, except in 180 mM NaCl, the CH seed priming treatment appeared to provide more stimulatory effects on pigment production in the leaves than that of L-met seed priming. Compared to NP, total chlorophyll and carotenoid contents were significantly increased by CH seed priming in 60 mM NaCl at the fifth week ($p< 0.05$) and in 120 mM NaCl at the second week ($p< 0.05$), respectively. However, there were no discernible variations in both photosynthetic pigments by L-met seed priming in all salt-amended soils compared to NP and HP, respectively ($p> 0.05$).

Chlorophyll plays a pivotal role in light absorption and energy transduction in plants (Li et al., 2014). However, salinity stress induces ROS production which could cause the demolition of chlorophyll pigments and instability of pigment-protein complex (Jaleel et al., 2008; Li et al., 2014). According to the earlier Chapter 4, CH priming of *L. multiflorum* seeds resulted in less ROS production in the seedling roots exposed to salt stress than that of NP through increases in antioxidant enzyme activities including SOD, POD, and CAT (Fig. 4.2; Fig. 4.5). It can be inferred that, therefore, such effects of the CH seed priming may not only prevent damages of thylakoid membrane in the leaves due to ROS but also enhance the chlorophyll content under salt stress in the present study (Rady et al., 2016). Similarly, Nasibi et al. (2016) reported that cysteine priming of wheat seeds (*T. aestivum* L.) markedly increased total chlorophyll content in those seedlings grown in 300 mM NaCl compared to the control plants.

Moreover, increased carotenoids in the Italian ryegrass leaves by CH seed priming may play significant roles in growth and salt stress tolerance of Italian ryegrass in the pot experiment. Carotenoids can function to collect light energy for photosynthesis as well as scavenge the

excited chlorophyll triplet state from photoinhibition damage and siglet oxygen ($^1O_2$) and lipid peroxidation (Aldesuquy et al., 2014; Gill and Tuteja, 2010; Taïbi et al., 2016). Thus, the increase in carotenoid contents in the leaves of *L. multiflroum* plants after CH seed priming may reduce salinity stress-induced ROS and bring about an improvement in photosynthetic activity (Rady et al., 2016; Verma and Mishra, 2005).

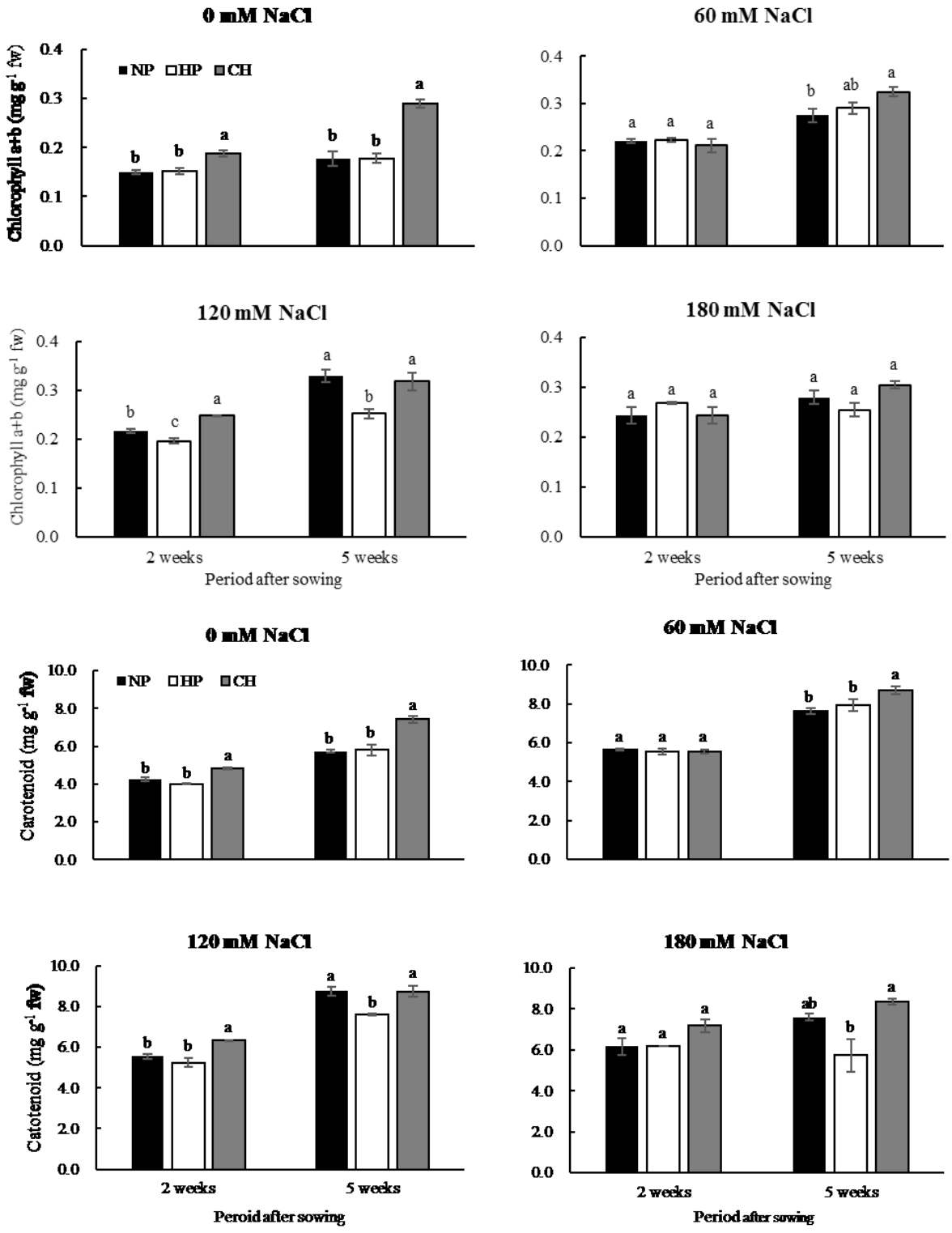


Fig. 5.3. Total chlorophyll (a+b) and carotenoid contents in *L. multiflorum* leaves of the seedlings from non-primed (NP), hydro-primed (HP), and casein hydrolysate (CH) primed seeds grown in different NaCl concentrations (0, 60, 120, and 180 mM NaCl). Data presented are means ± standard errors (n=7) of samples at the second and the fifth weeks. Within each sampling time, different letters indicate significant differences by Fisher's range test at $p < 0.05$.

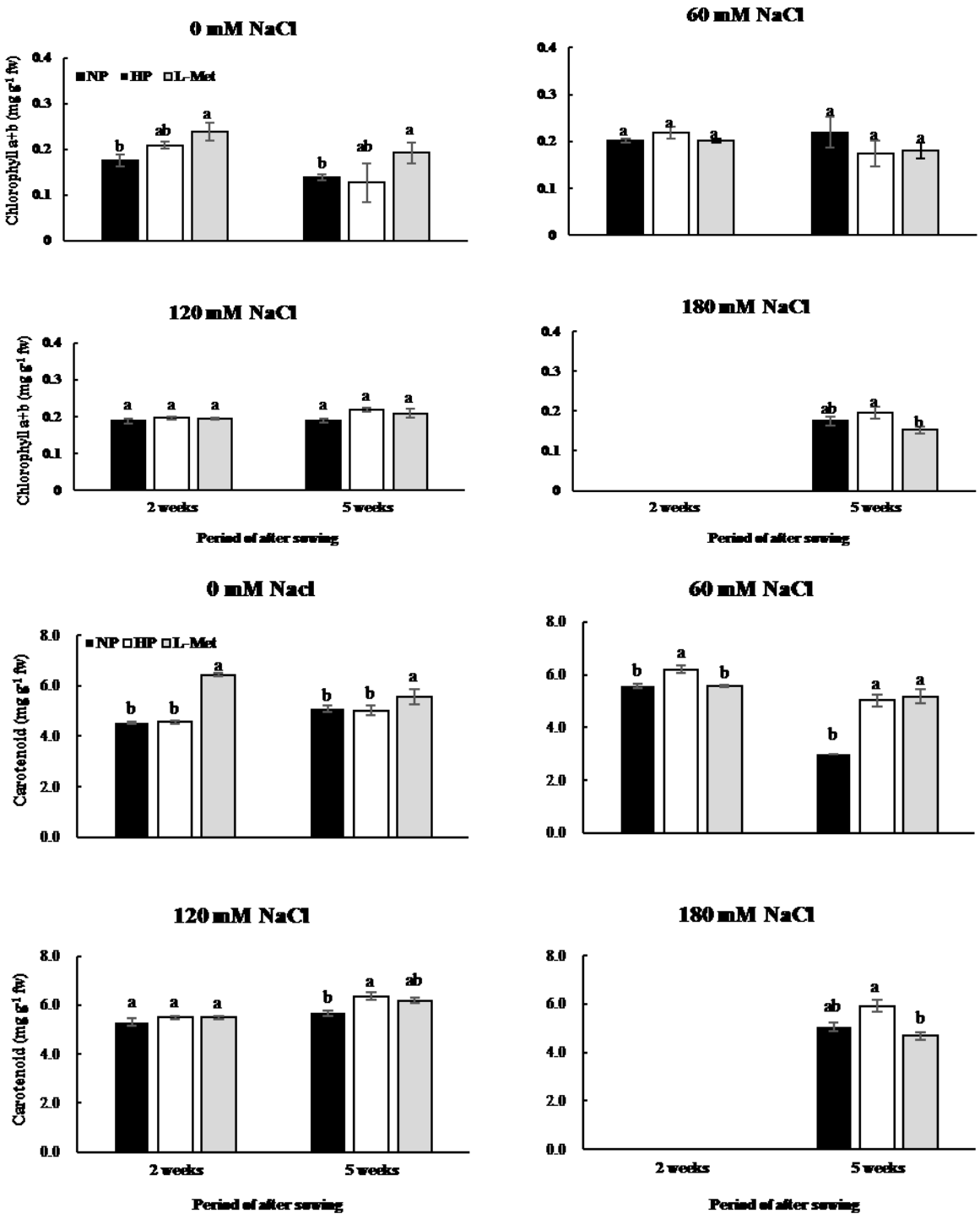


Fig. 5.4. Total chlorophyll (a+b) and carotenoid contents in *L. multiflorum* leaves of the seedlings from non-primed (NP), hydro-primed (HP), and L-methionine (L-met) primed seeds grown in different NaCl concentrations (0, 60, 120, and 180 mM NaCl). Data presented are means ± standard errors (n=7) of samples at the second and the fifth weeks. Within each sampling time, different letters indicate significant differences by Fisher's range test at $p < 0.05$.

5.4.4. Response of antioxidant enzymes

Addition of NaCl and application of seed priming resulted in different effects on antioxidant enzyme activities such as SOD, CAT, and POD in *L. multiflorum* leaves during the cultivation period (Tables 5.4 and 5.5). With increasing salt concentrations, there was a distinct trend in each of the enzyme activities studied in the leaves of plants without seed priming (NP): SOD activity gradually decreased at either the second and the fifth weeks after germination; CAT activity marginally increased at the second week and decreased at the fifth week; POD activity gradually decreased at the second week and increased at the fifth week.

Seed priming of Italian ryegrass seeds with both CH and L-met showed significant effects on increases in these enzyme activities in *L. multiflorum* leaves without added NaCl (0 mM NaCl), particularly at the second week. When the seedlings were grown in soil with added 120 mM NaCl, SOD activity increased significantly in the ryegrass leaves of the seedlings after CH and L-met seed priming at the second week, compared to that of NP ($p< 0.05$). SOD has a function of the first defence mechanism by catalysing conversion reaction of superoxide radicals ($O_2^{\bullet-}$) to H_2O_2 and O_2 in plant cells under salt stress (El-shabrawi et al., 2010; Hu et al., 2012c). In this regard, both CH and L-met seed priming appeared to play a role in removing toxicity of the $O_2^{\bullet-}$, contributing to intensifying salt stress tolerance of *L. multiflorum*, particularly at the beginning of seedling growth in this study. In a similar study, Genisel et al. (2015) found that barely (*Hordeum vulgare* L.) coleoptile of seedlings from seeds primed with cysteine exhibited increased SOD activity when the seedlings were grown in 125 mM NaCl. This may be due to cysteine priming mitigated the oxidative stress injury to membranes and nucleic acids. Hu et al. (2012c) and Verma and Mishra (2005) also demonstrated that the increase in SOD activity in response to salt stress protects cellular membrane from toxic $O_2^{\bullet-}$ attack, contributing to salt stress tolerance. Moreover, Kentucky bluegrass (*Poa pratensis* L.),

and Bermudagrass (*Cynodon dactylon* L.), and Indian mustard (*Brassica juncea* L.) exogenously treated with spermidine, polyamines, and putrescine, respectively, had considerably increased SOD activity in their leaves under salt stress compared to the control plants (Puyang et al., 2015; Shi et al., 2013; Verma and Mishra, 2005).

When the seedlings were grown in soil with added 120 and 180 mM NaCl, significant increases in CAT activity were found in the ryegrass leaves of the seedlings from seeds primed with CH compared to NP ($p< 0.05$) at the second week (Table 5.4). The CAT activities in these NaCl concentrations were about 68 and 28% greater in the CH treatment. At the fifth week, the CAT activity in the leaves of the seedlings in the CH treatment was about twice that in NP when the seedlings were grown in soil with added 180 mM NaCl. However, in the L-met seed priming treatment, CAT activities in the ryegrass leaves of the seedlings were reduced when the seedlings were grown in soils with added 180 mM NaCl over the pot experiment period (Table 5.5). At the second week, CAT activities in the leaves of the seedlings from L-met-primed ryegrass seeds were 15 and 17% lower than that of NP in 60 mM and 120 mM, respectively ($p< 0.05$). Such negative effect of seed priming on CAT activity was reported by Nasibi et al. (2016): wheat (*T. aestivum* L.) seedlings from seeds primed with cysteine had lower CAT activity than those from unprimed seeds when the seedlings were grown in 300 mM NaCl. These results suggest that CH seed priming would encourage better tolerance in plants against severe salinity than seed priming with single amino acids by increasing CAT activity that stimulates catalysis of H_2O_2 to H_2O and O_2 (Hu et al., 2012a).

Among the three antioxidant enzymes activities studied, peroxidase activity in the ryegrass leaves was the most significantly influenced by either CH or L-met seed priming. At the second week of growth of the seedlings in the different salt concentrations, POD activities of the ryegrass leaves in the seedlings in both CH and L-met seed priming treatments were significantly increased compared to that of NP ($p< 0.05$). In particular, when the seedlings were

grown in 180 mM NaCl, POD activity in the CH seed priming treatment were about 100% higher than that in NP and HP (Table 5.4). At the fifth week, unlike SOD and CAT, POD activity in the ryegrass were significantly influenced in both CH and L-met seed priming treatments, except when the seedlings were grown in 180 mM NaCl. In general, H_2O_2 produced in chloroplasts as well as other cell compartments under salinity stress may be inhibited with POD enzyme which detoxifies H_2O_2 in the cytosol (El-Shabrawi et al., 2010). In this regard, therefore, CH and L-met seed priming appeared to play a vital role in encouraging tolerance mechanism to salt stress by increasing POD activity in plasma membrane and cell wall of *L. multiflorum*. As a similar example, Nasibi et al. (2016) reported that POD activity in *T. aestivum* shoot under salt stress was increased following cysteine seed priming.

Table 5.4. Variations in activities of antioxidant enzymes such as SOD, CAT, and POD in *L. multiflorum* leaves of seedlings grown in different NaCl concentrations (0, 60, 120, and 180 mM NaCl). The seeds were non-primed (NP), hydro-primed (HP), or primed with casein hydrolysate (CH) (200 mg L^{-1}). Data presented are means ± standard errors (n=8) of samples at the second and the fifth weeks. Within each NaCl treatment in each sampling time, different letters indicate significant differences by Fisher's range test at $p< 0.05$.

NaCl concentration	Seed-priming treatment	SOD (unit/min/mg protein)		CAT (unit/min/mg protein)		POD (unit/min/mg protein)	
		2 weeks	5 weeks	2 weeks	5 weeks	2 weeks	5 weeks
0 mM	NP	10.05±0.18^{b}	10.85±0.18^{b}	1.92±0.13^{b}	2.60±0.09^{a}	1.32±0.14^{b}	1.34±0.08^{b}
	HP	12.48±0.31^{a}	11.37±0.31^{a}	2.96±0.46^{ab}	2.60±0.30^{a}	1.27±0.07^{b}	1.50±0.14^{ab}
	CH	12.07±0.04^{a}	9.95±0.07^{c}	3.77±0.07^{a}	3.41±0.43^{a}	2.10±0.06^{a}	1.62±0.10^{a}
60 mM	NP	8.39±0.20^{a}	10.68±0.09^{a}	2.49±0.27^{a}	2.32±0.16^{a}	1.30±0.56^{b}	1.89±0.23^{a}
	HP	7.84±0.00^{b}	9.97±0.07^{b}	1.75±0.00^{b}	2.47±0.10^{a}	1.24±0.02^{b}	1.66±0.17^{a}
	CH	8.25±0.05^{b}	9.55±0.10^{c}	1.51±0.09^{b}	2.66±0.10^{a}	1.99±0.01^{a}	1.82±0.19^{a}
120 mM	NP	8.09±0.06^{b}	8.90±0.04^{a}	2.05±0.17^{b}	1.49±0.11^{a}	1.74±0.02^{b}	1.60±0.08^{b}
	HP	7.62±0.10^{c}	8.26±0.04^{a}	2.58±0.00^{b}	1.45±0.04^{a}	1.67±0.11^{b}	1.71±0.16^{b}
	CH	9.52±0.01^{a}	8.08±0.48^{a}	3.44±0.22^{a}	1.03±0.09^{b}	2.12±0.11^{a}	2.11±0.15^{a}
180 mM	NP	8.95±0.65^{a}	9.66±0.33^{a}	2.36±0.10^{a}	0.34±0.00^{c}	1.02±0.04^{b}	1.58±0.04^{a}
	HP	7.93±0.17^{a}	7.87±0.18^{b}	1.60±0.22^{b}	1.62±0.07^{a}	1.06±0.00^{b}	1.64±0.14^{a}
	CH	7.68±0.08^{a}	7.34±0.24^{b}	3.02±0.02^{a}	0.88±0.08^{b}	2.09±0.08^{a}	1.83±0.10^{a}

Table 5.5. Variations in activities of antioxidant enzymes such as SOD, CAT, and POD in *L. multiflorum* leaves of seedlings grown in different NaCl concentrations (0, 60, 120, and 180 mM NaCl). The seeds were non-primed (NP), hydro-primed (HP), or primed with 1 mM L-methionine (L-met). Data presented are means ± standard errors (n=8) of samples at the second and the fifth weeks. Within each NaCl treatment in each sampling time, different letters indicate significant differences by Fisher's range test at $p< 0.05$.

NaCl concentration	Seed-priming treatment	SOD (unit/min/mg protein)		CAT (unit/min/mg protein)		POD (unit/min/mg protein)	
		2 weeks	5 weeks	2 weeks	5 weeks	2 weeks	5 weeks
0 mM	NP	4.67±0.13 ^{c}	8.40±0.18 ^{a}	1.16±0.02 ^{b}	2.90±0.12 ^{a}	1.69±0.04 ^{c}	2.11±0.07 ^{b}
	HP	5.34±0.10 ^{b}	7.76±0.11 ^{b}	1.37±0.02 ^{ab}	1.72±0.05 ^{b}	1.85±0.06 ^{b}	2.59±0.15 ^{a}
	L-met	6.53±0.00 ^{a}	7.16±0.12 ^{c}	1.57±0.03 ^{a}	2.01±0.16 ^{b}	1.90±0.09 ^{a}	2.58±0.04 ^{a}
60 mM	NP	4.93±0.03 ^{a}	8.10±0.05 ^{a}	1.96±0.01 ^{a}	3.22±0.00 ^{a}	1.21±0.04 ^{c}	2.17±0.02 ^{b}
	HP	4.32±0.06 ^{b}	7.88±0.16 ^{a}	1.60±0.05 ^{b}	1.80±0.05 ^{b}	1.45±0.04 ^{b}	1.82±0.09 ^{c}
	L-met	4.52±0.07 ^{b}	7.04±0.08 ^{b}	1.66±0.00 ^{b}	1.30±0.10 ^{c}	1.87±0.11 ^{a}	2.45±0.06 ^{a}
120 mM	NP	4.08±0.06 ^{b}	6.75±0.01 ^{b}	2.56±0.06 ^{a}	1.60±0.00 ^{a}	1.45±0.03 ^{b}	1.86±0.01 ^{b}
	HP	4.04±0.18 ^{b}	6.57±0.03 ^{c}	1.67±0.00 ^{b}	1.72±0.00 ^{a}	1.31±0.03 ^{b}	2.36±0.07 ^{a}
	L-met	4.73±0.06 ^{a}	6.96±0.05 ^{a}	2.12±0.06 ^{b}	1.71±0.06 ^{a}	1.63±0.06 ^{a}	2.34±0.07 ^{a}
180 mM	NP	-	7.60±0.05 ^{a}	-	1.45±0.00 ^{a}	-	2.42±0.01 ^{a}
	HP	-	7.60±0.06 ^{a}	-	0.93±0.04 ^{b}	-	2.55±0.08 ^{a}
	L-met	-	7.36±0.04 ^{b}	-	1.19±0.10 ^{ab}	-	2.31±0.11 ^{a}

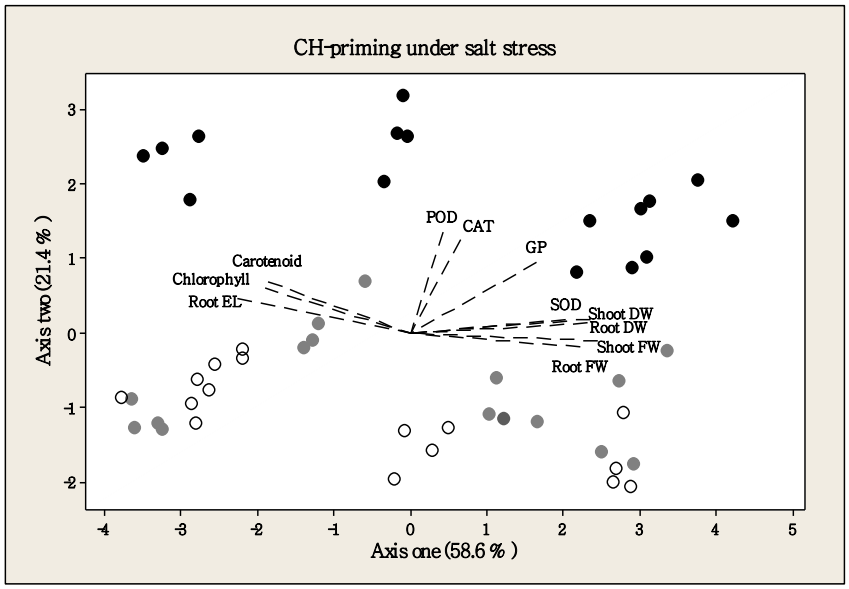
5.4.5. Overall effect of seed-priming

Multivariable analysis was conducted to interpret the overall effects of seed priming applications of CH and L-met on productivity and salt stress tolerance of *L. multiflorum* using a Principle Component Analysis (PCA) (Figs. 5.5 and 5.6). Data for antioxidant enzymes (POD, SOD, and CAT activities) and photosynthetic pigments (total chlorophyll and carotenoid contents) collected at the second week after sowing were used for PCA. This is because it seemed that physiological responses of the ryegrass were more likely to react at this earlier time of exposure against salt stress, as previously shown in this chapter previously. So, the PCA analysis did not include data from 180 mM NaCl treatment (Fig. 5.6) in the second pot trial investigating the effect of L-met seed priming.

There were different trends between CH and L-met seed priming assays (the first and second pot trials). The combined datasets apparently separated the salt treatments of different concentrations (0, 60, 120, and 180 mM NaCl) along Axis 1 in both Figs. 5.5 and 5.6. In the PCA results, generally increasing NaCl concentrations caused an increase in root electrical leakage but a decrease in germination percentage, SOD, and biomass of the ryegrass plants.

On axis 2, the effects of CH seed priming were certainly separated from those of the other two seed priming treatments, NP and HP (Fig. 5.5). Indeed, CH seed priming considerably influenced GP, total chlorophyll and carotenoid contents, and antioxidant enzyme activities (CAT and POD) compared to the other seed priming treatments. The increase in chlorophyll content may indicate that continuous pigment synthesis in the leaves of Italian ryegrass grown in salt-amended soils was related to the response to the CH seed priming. In particular, when the seedlings were grown in 120 mM NaCl, CH-priming showed certain impacts on GP, activities of CAT and POD which could be associated with salt stress tolerance mechanism. However, there was unclear separation in L-met seed priming assay on axis 2 (Fig.

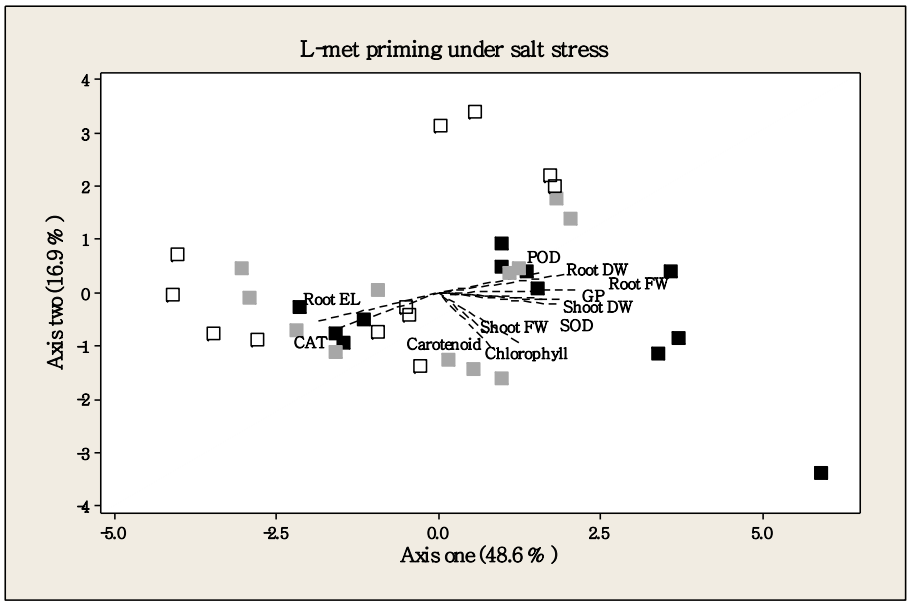
5.6), suggesting that the L-met seed priming did not relatively benefit ryegrass growth and productivity. Therefore, the PCA results may indicate that casein hydrolysate containing 18 amino acid compounds, some vitamins, and nutrients is more beneficial for seed priming than L-met. This might be related to an enhancement in metabolic and biosynthetic pathways associated with salt stress tolerance, particularly at the early post-germinative growth stage of Italian ryegrass.



 # Coefficients of each variable for PC1 and PC2 through the PCA.

Variable	GP	Root EL	Chloro -phyll	Carote -noid	SOD	CAT	POD	Shoot FW	Root FW	Shoot DW	Root DW
PC1	0.257	-0.351	-0.295	-0.289	0.316	0.101	0.067	0.381	0.345	0.365	0.359
PC2	0.404	0.197	0.257	0.293	0.080	0.534	0.580	-0.043	-0.078	0.082	0.058

Fig. 5.5. Principal Component Analysis (PCA) of growth and physiological responses of *L. multiflorum* to salt stress in casein hydrolysate seed priming assay (the first pot trial).



Priming type:

□ : Non-priming (NP)

▒ : Hydro-priming (HP)

■ : L-methionine priming (L-met)

 # Coefficients of each variable for PC1 and PC2 through the PCA.

Variable	GP	Root EL	Chloro -phyll	Carote- noid	SOD	CAT	POD	Shoot FW	Root FW	Shoot DW	Root DW
PC1	0.351	-0.343	0.231	0.153	0.342	-0.288	0.292	0.142	0.393	0.302	0.361
PC2	-0.066	-0.282	-0.493	-0.541	-0.115	-0.354	0.200	-0.402	-0.034	-0.069	0.184

Fig. 5.6. Principal Component Analysis (PCA) of growth and physiological responses of *L. multiflorum* to salt stress in L-methionine seed priming assay (the second pot trial).

5.5. Conclusion

Soil salinity influences morphology and physiology of plants negatively, resulting in reduced crop productivity and quality in agricultural lands. In the present study, salt addition caused reduction in seed germination and early seedling growth of *L. multiflorum*. Especially, a high level of salt (180 mM NaCl) and/or high temperature (in summer) during the greenhouse experiments appeared to retard or inhibit seed germination and seedling growth, with or without seed priming treatments. However, seed priming with casein hydrolysate and L-methionine showed potential benefits for germination, physiological responses, and productivity of Italian ryegrass under salt stress (60 and 120 mM NaCl). The most positive effect of both seed priming agents appeared to be shown when the seedlings were grown in soils amended with 120 mM NaCl. Of the different seed priming treatments, seed priming with CH seemed to induce much better effects in ameliorating severe salt stress-induced damages than seed priming with L-met in this study. In particular, seed priming with CH significantly increased CAT and POD enzyme activities in plants under relatively high levels of salinity (120 or 180 mM NaCl). There might be enhanced photosynthetic responses and yields of Italian ryegrass plants after seed priming with CH. To apply such benefits of the seed priming technique to agricultural industry, a field study should be needed to investigate efficiency and yield variation of Italian ryegrass plants from CH-primed seeds under more variable environmental conditions.

Chapter 6. Effects of drought on biomass production of *L. multiflorum* following casein hydrolysate seed priming: an additional pot experiment

6.1. Introduction

Drought and salinity stresses are commonly associated with regulating water influx and/or efflux and cellular osmotic adjustment for stress adaptation via biosynthesis of osmoprotectant (Golldack et al., 2014; Munns, 2005). Drought reduces losses in global agriculture production as well as causes soil erosion and desertification (Fang and Xiong, 2015). Drought stress generally causes water scarcity to plants, thus can damage morphological, metabolic, and physiological functions of plants (Zheng et al., 2016). During plant growth, seed germination is considerably restricted by drought, which consequently leads to poor seedling establishment (Farooq et al., 2009). In addition, drought stress severely hinders cell elongation and expansion during the early stages of seedling development (Farooq et al., 2009). Also, ROS generated by drought stress can damage plants by resulting in lipid peroxidation, disruption of protein structure, and plant cell death (Zheng et al., 2016).

Soil salinity induces osmotic and ionic stress hindering water uptake roots and growth of plants (Horie et al., 2012; Hu et al., 2012b; Türkan and Demiral, 2009). In particular, highly concentrated Na^+ ions in soil can interrupt osmotic balance and suppress water uptake, causing physiological drought in plants (Türkan and Demiral, 2009). Saline soils accompanying with high water evaporation stimulate more severe osmotic damages to plants. Thus, drought can contribute to the adverse effects of salt stress on plants by lowering water potential in root zone. This may be followed by water deficiency and nutrient imbalance in shoots (Farooq et al., 2009). Hence, understanding of plant response to drought and salinity stresses can aid to stabilise crop yields in under a field conditions.

In Chapter 5, significant impacts of CH seed priming on germination, growth, and tolerance of the Italian ryegrass in response to salt stress were found in pot experiments. Thus, this additional pot trial aimed to identify effect of CH seed priming application to Italian ryegrass on its biomass production under drought stress.

6.2. Methodology

This pot experiment in the glasshouse was carried out similarly as described in Chapter 5 (see section 5.3.1) without salt treatments. Non-priming (NP), hydro-primed (HP), and CH-primed (CHP) seeds were used in this study. There were ten replicates in each seed priming treatment. The pot trial was conducted from May to June in 2016, and it was repeated from June to July in 2016.

The drought stress treatment in this pot trial experiment is outlined in Fig. 6.1. The seeds were sown in pots, and then 30 ml of tap water was supplied every day. After sprouting from the seeds, water supply was stopped for 7 days for the drought stress treatment. After this, water was again supplied for 2 weeks before harvest. At the fourth week after sowing, shoot and root of the ryegrass plants were sampled, and their fresh weights were determined. To determine dry weight, samples of shoots and roots were oven-dried for three days at 65 ˚C.

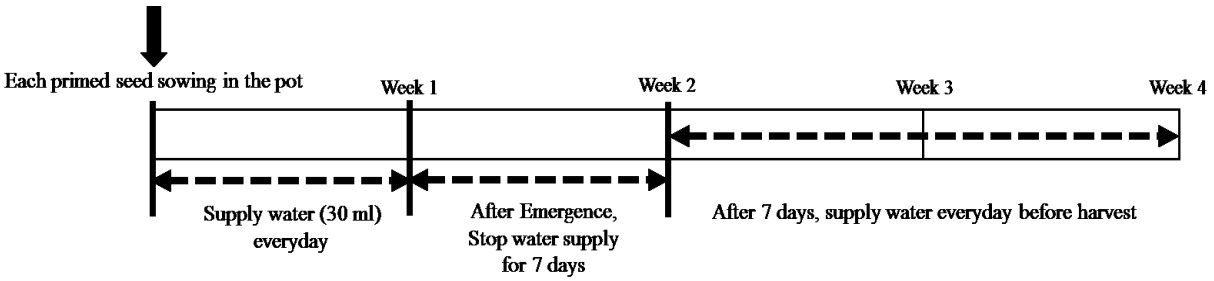


Fig. 6.1. Layout of a greenhouse experiment for 4 weeks: drought stress treatment for a week at the second week after seeds of *L. multiflorum* sprouted.

6.3. Results and Discussion

Seed priming with casein hydrolysate had a positive effect on biomass production of *L. multiflorum* in response to drought stress (Fig. 6.2). Significant increases in fresh and dry weights of Italian ryegrass plants were found in the seed priming treatment with CH compared to NP ($p< 0.05$). FW of the shoots and roots in the CH priming treatment was about 73 and 48% higher than those in NP, respectively. DW of the shoots and roots in the CH priming treatment were also approximately 85 and 54% higher than NP, respectively. Similarly, pre-sowing priming of wheat (*Triticum aestivum*) seeds with proline (20 and 40 mM) increased fresh and dry weights of shoots and roots under drought stress (Kamran et al., 2009). Pinheiro et al. (2018) also found that priming of sorghum (*Sorghum bicolor* L.) with gibberellic acid increased length of shoots and roots in the seedlings under drought stress.

Without either salt and drought stress treatments (see Table 5.2), there was a significant increase only in DW of shoots in the CH seed priming treatment. In this pot trial experiment, however, the increases in both FW and DW of roots in the CH priming treatment were relatively greater than those of shoots exposed to drought stress (Fig. 6.2). Under drought stress, the number and area of leaves are reduced to minimise water loss (Farooq et al., 2009; Farooq et el., 2012). On the other hand, since plants acquire water through roots, the root systems (i.e. size, density, and proliferation) are considerably important to respond to drought stress (Farooq et al., 2009). Upon drought stress, in the present study, the roots of *L. multiflorum* maybe more developed than the shoots to enhance efficiency for uptake of moisture from soil or maintaining the water potential against evaporation from leaves. In this context, seed priming with CH could encourage stronger root system of the Italian ryegrass in response to drought stress.

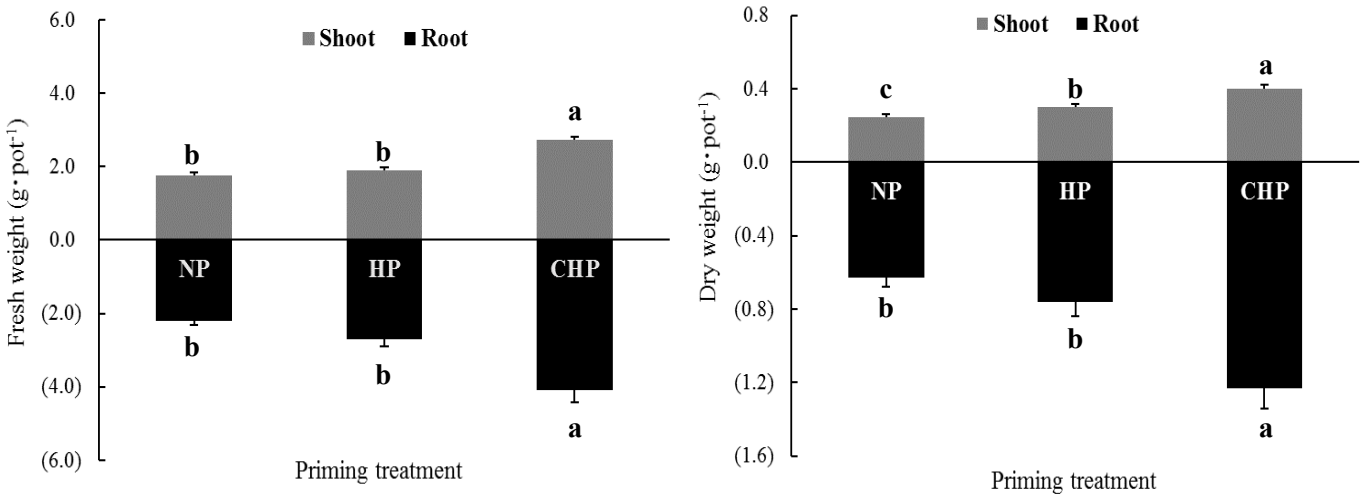


Fig. 6.2. Fresh and dry weights of non-primed (NP), hydro-primed (HP), and casein hydrolysate (CH) primed ryegrass (*L. multiflorum*) after 4 weeks of cultivation. Different colors indicate data for shoots (grey) and roots (black). Different letters mean significant difference between seed priming applications (n=10, $p < 0.05$).

Chapter 7. Synopsis, and Conclusions, and Future directions

7.1. Synopsis

7.1.1. Potential of seed priming using specific amino acids to improve Italian ryegrass seed growth under salt stress

New Zealand's agriculture production is mainly made up of pasture grazing system (Keller et al., 2014). However, growing in a dry season period with reduced rainfalls in New Zealand, particularly in semi-arid region including Canterbury and Otago (Hewitt, 2013), may cause accumulation of some salts such as sodium and calcium released from weathering parent matrials or transferred from subsoils under irrigation system. Based on the laboratory and greenhouse experiments of this PhD research project, it was confirmed that several amino acids could be potential seed priming agents to promote physiological responses and resistance of Italian ryegrass to salinity. Among 20 L-amino acids, lysine, methionine, and tryptophan seemed to have a greater potential for seed priming to enhance germination and seedling establishment under salt-affected conditions. Germination rate and radicle length of Italian ryegrass seedlings primed with these three amino acids were relatively enhanced in NaCl treatments compared to those non-primed, hydro-primed, and the other amino acids-primed seeds. Also, markedly increased POD enzyme activity was found in the roots of the seedlings, which can scavenge excess H_2O_2 products caused by salt stress condition. Among the three L-amino acids, methionine showed relatively more benefits in radicle elongation and POD activity. However, D-amino acid (D-methionine) priming could not alleviate oxidative stress due to salinity.

In Petri-dish experiments, the seedling roots raised from Italian ryegrass seeds primed with L-methionine exhibited considerably increased SOD, CAT, and POD enzyme activities as well as lowered H_2O_2 and MDA contents than the control seedling roots when the seedlings were

grown in salt solutions. In the pot experiment, in addition, root biomass of seedling from the seeds primed with L-methionine were significantly greater in salinised soil applied with 60 mM NaCl solution daily, than that of non-primed and hydro-primed seeds. Also, a considerable increase in SOD and POD activities was found in plants grown in the soil applied with 120 mM NaCl.

Although the 20 amino acids solutions had different pH levels ranging between 3.3 and 9.8, we did not find that the pH difference affects the germination percentage, radicle length, and POD enzyme activity of the Italian ryegrass. In particular, even among priming reagents of some amino acids with similar pH, they showed different effects on all results. Also, use of MES buffer to control pH also did not show any effect of pH on ryegrass seed germination and seedling growth. Thus, it may be concluded that rather than change in pH levels by dissolving amino acids, difference in characteristic and function of each amino acids might result in the difference in seedling growth of *L. multiflorum*.

Amino acids are capable of regulation of seed germination and seeling establishment. During the seed and seedling growth period, amino acids applied for seed priming may play beneficial roles in biosynthesis and catabolism of amino acids, which mediate production of energy, secondary metabolites, and N source, etc. In this study, L-methionine was an effective priming reagent among 20 L-amino acids. It is related to the increase in plant growth substance such as cytokinins, auxins, and brassinosteroids (El-Awadi and Hassan, 2010). Therefore, the results in this study indicated that some L-amino acids like methionine used for seed priming may aid plant metabolism and tolerance mechanism of the Italian ryegrass in response to salt stress by alleviating ROS toxicity, thereby with potential for increasing its yields under salt stress.

7.1.2. Effectiveness of seed priming with casein hydrolysate in improving tolerance of Italian ryegrass seed against salt stress

In this study, seed priming with casein hydrolysate had greater effects than NP control at the early stage of plant development on germination and radicle length, leaf physiology (i.e. photosynthetic pigments), plant biomass production, and mortality of Italian ryegrass grown under salt stress. In particular, enhanced antioxidant metabolism following CH priming treatment of the seeds was associated with improving salt tolerance in this species. In Petri-dish trials, seeds in the CH priming treatment had similarities in germination rate, radicle elongation, and POD activity with that of L-methionine priming treatment during four days after sowing. Indeed, CH priming was more influencial on activating other antioxidant enzymes such as SOD and CAT than the single L-amino acid priming under salt stress. Consequently, the elevated antioxidant enzymes may contribute to reduced accumulation of ROS such as $O_2^{\bullet-}$ and H_2O_2 and lipid peroxidation in the root of 4-day-old Italian ryegrass seedlings. Additionally, CH priming application significantly induced concentration of proline, as a non-enzymatic antioxidant, in the roots.

In saline soils, germination rate and biomass production (fresh and dry weight) during 5 weeks after sprout emergence from the seeds primed with CH were greater than that of non-primed and hydro-primed seeds. An increase in contents of photosynthetic pigments (chlorophyll and carotenoids) was also found in the CH treatment. Two weeks after sowing, in soil watered with 120 mM NaCl, SOD, CAT, and POD activities in leaves of the Italian ryegrass plants from CH-primed seeds were significantly higher than those in leaves of plants from non-primed and hydro-primed seeds. In the 180 mM NaCl treatment, the highest concentrations of CAT and POD were found in the leaves of plants from CH-primed seeds.

Moreover, both fresh and dry weights in root and shoot of plants from CH-primed Italian ryegrass seeds were considerably increased when the plants were grown in soils under drought stress condition, compared to plants from non-primed and hydro-primed Italian ryegrass seeds.

It is well known that casein hydrolysate contains a variety of beneficial nutrients including N, Ca, P, microelements, and vitamins, etc. for plant growth (George et al., 2008). Also, it consists of a mixture of up to 18 amino acids which have already been shown to facilitate root development and antioxidant metabolism (Hildebrandt et al., 2015; Teixeira et al., 2018). In the present study, it is possible that casein hydrolysate priming exhibited superior functions in possibly providing nutrients, enhancing tolerance, and yields of Italian ryegrass under salt stress. In this regard, although casein hydrolysate has never been used in seed priming before, it was hypothesised to be a crucial seed priming agent source rather than each of L-amino acids. CH might regulate several nutrients in *L. multiflorum* seedlings.

7.1.3. Major findings in each research chapter

Objective 1. *Priming Italian ryegrass seeds with specific L-amino acids and casein hydrolysate could induce salt stress tolerance mechanism during germination and early seedling growth in Chapter 3*

The aim of this experimental study was to investigate the beneficial effects, if any, of seed priming with amino acids and casein hydolysate on seed germination and early seedling growth of *L. multiflorum* under salt stress. Among the seed priming techniques, there has been little information of the pre-treatment with twenty L-AAs and CH on Italian ryegrass seeds on plant growth in salt-affected condition so far. The major findings are as follows: (1) seeds primed with several L-AAs (i.e. asparagine, leucine, lysine, methionine, and tryptophan, etc.) and CH showed higher GP; (2) radicle length of the ryegrass seeds primed with several L-AA (i.e.

glutamine, lysine, methionine, tryptophan, and valine, etc.) and CH was longer; (3) POD activity in the seedling roots from seeds primed with some L-AAs (i.e. arginine, lysine, methionine, proline, and tryptophan, etc.) and CH was greater; (4) elevated activities of SOD and CAT enzymes in the seedling roots from seeds primed with L-methionine and CH; (5) less accumulation of ROS in the seedling roots from seeds primed with L-methionine and CH, than those of the non-primed seeds under salt stress. Overall, among the L-AAs mentioned above, methionine, lysine, or tryptophan together with CH seemed to be the best seed priming agents to promote salt tolerance of Italian ryegrass seeds in this study.

Objective 2. *Priming seeds with specific L-amino acids and casein hydrolysate may alleviate osmotic and oxidative stress caused by salinity during early seedling growth in Chapter 4*

The aim of this experimental study was to investigate how seed priming with a specific L-amino acid and casein hydrolysate could regulate oxidative stress during seed germination and early seedling growth of *L. multiflorum* under salt stress. Based on the results of ROS staining in the previous chapter, H_2O_2 and malondialdehyde (for lipid peroxidation) content in the Italian ryegrass roots were determined. Proline content as an osmoprotectant and a non-enzymatic antioxidant was also determined. The major findings are follows: (1) seedling roots from seeds primed with L-methionine or CH had lower H_2O_2 and MDA content than that of NP, but the CH priming effect was considerably greater than that of the L-methionine; (2) CH priming enhanced proline content in the ryegrass roots, while L-methionine priming reduced it; and (3) priming of D-isomer of methionine showed negative effects in regulation of oxidative stress including increasing H_2O_2 and MDA contents and decreasing proline content.

Overall, CH priming would have a better function to enhance salt tolerance of Italian ryegrass seeds than L-methionine by alleviating oxidative stress more effectively.

Objective 3. *The beneficial effects from priming Italian ryegrass seeds with a specific L-amino acid and casein hydrolysate may also be exhibited at plant growth and development stages beyond early post-germinative growth in glasshouse experiments in Chapter 5*

The aim of this experimental study was to investigate how the beneficial effects of seed priming with a specific L-amino acid and casein hydrolysate could affect Italian ryegrass growth and yield in saline soil. Based on the results of earlier studies, L-methionine and CH were used for priming Italian ryegrass seeds and the primed seeds were sowed in pot soils treated with NaCl solution. To determine physiological response and biomass production of the plant in response to salt stress during 6 weeks of the growth period in a greenhouse, germination rate, fresh and dry plant weights, photosynthetic pigments (chlorophyll and carotenoid), and antioxidant enzyme activity (SOD, CAT, and POD) were measured. The major findings are as follows: (1) germination percentage and biomass in the CH priming, but not L-methionine, treatment was higher than that in the NP and HP treatments in soil amended with 120 mM NaCl; (2) chlorophyll and carotenoid contents were considerably higher in leaves of the plants in the CH priming, but not L-methionine, treatment than those in the NP and HP treatments in soil amended with 120 mM NaCl at 2 weeks after sowing; and (3) there were higher levels of antioxidant enzyme activities including SOD, CAT, or POD in the leaves of plants from seeds primed with CH than with L-methionine, NP, and HP at 2 weeks after sowing, when the plants were grown in soil treated with 120 and 180 mM NaCl. In the glasshouse study, seed priming with L-methionine showed no or small effects on Italian ryegrass growth in saline soil, unlike

the results from the Petri-dish experiments. It is thought to be due to more variable environmental conditions such as soil moisture, temperature, light, or humidity in the greenhouse than in the laboratory. These variables might limit the positive effects of priming with L-methionine as shown in the Petri-dish experiments. In addition, high temperature during the pot experiment with seeds primed with L-methionine seemed to lead to high soil evaporation and subsequent drought and increased salt stress in soil, which might be unfavourable for plant germination and growth in the glasshouse. Overall, rather than L-methionine, CH would be a useful supplement for seed priming in terms of enhancement of adaptive capacity and production of Italian ryegrass in salt-affected soil.

7.2. Overall conclusions

Salinisation has been problematic in agricultural land worldwide. The amount of crop and food yield has been continuously reduced due to the increase in soil salinity. Also, oxidative stress due to salinity is often interconnected with other unfavorable factors such as drought and extreme temperature, which may induce similar damages to plants. To mitigate salt stress, seed priming is a useful technique for inducing a physiological state associated with abiotic stress resistance in the seed before germination. Throughout this study, potential of seed priming with casein hydrolysate of Italian ryegrass seed for resisting salt stress was demonstrated. Beneficial effects of seed priming with CH include higher seed germination and early seedling establishment in laboratory and greenhouse experiments. Pre-treatment with CH could be used to ameliorate injurious effects of oxidative stress in Italian ryegrass seedlings. This is the first study to assign a protective role of casein hydrolysate in Italian ryegrass responses to salt stress, although it has been widely used in plant tissue culture and orchid seed germination. In this

study, when the Italian ryegrass seedlings from CH-primed seeds were grown under salt stress, they exhibited higher seed germination percentage, longer radicles, higher levels of chlorophyll and carotenoid, antioxidant enzyme activities (SOD, CAT, and POD), and biomass compared with those from NP and HP. In addition, L-methionine priming appeared to bring about these similar responses in the Italian ryegrass seedlings against salt stress. However, casein hydrolysate priming of *L. multiflorum* seeds seemed to result in a greater increase in salt stress tolerance than L-methionine priming.

In the laboratory experiments, concentrations of H_2O_2 and MDA in the roots of the seedlings from seeds primed with CH and L-methionine were significantly lower than those in NP and HP treatments when the seedlings were grown under salt stress. The lower levels of these oxidative stress markers are closely related to the increase in proline content only in the CH priming treatment when the seedlings were grown under salt stress. The significant accumulation of proine in the CH priming treatment could be associated with salt stress tolerance by counteracting against the toxic effects of salts. On the other hand, the seedlings in D-methionine priming showed a lower proline content, lower activities of antioxidant enzymes (SOD, CAT, and POD), but higher levels of H_2O_2 and MDA when the seedlings were grown under salt stress than those in NP and HP. From these results, pre-sowing treatments with casein hydrolysate and L-methionine had beneficial effects, whereas D-methionine priming showed negative effects when the seedlings were grown under elevated levels of salinity. These results may be used as practical biochemical parameters for identifying the beneficial effect of salt stress tolerance.

In the greenhouse experiments, after seed priming with CH and when the seedlings were grown in 120 mM NaCl, there was a considerable increase in germination percentage, antioxidant enzymes (SOD, CAT, and POD), and biomass compared to the seedlings in the NP and HP treatments. It seems that salt stress induced oxidative injury could be potentially

alleviated in *L. multiflorum* seedlings following priming seeds with CH. A summary of the interpretations of the findings about CH priming of *L. multiflorum* are depicted in Fig. 7.1.

Hence, further study will be needed to understand of the complexity of casein hydrolysate priming of seeds at the biochemical and molecular levels. Furthermore, the application of seed priming plays a role in improving abiotic stress resistance during seed germination as well as early seedling growth. Taken together, further study of the *L. multiflorum* plants grown in moderate saline soil under field conditions after seed priming with CH is needed to confirm improvement of seed germination and stress tolerance resulted from CH priming.

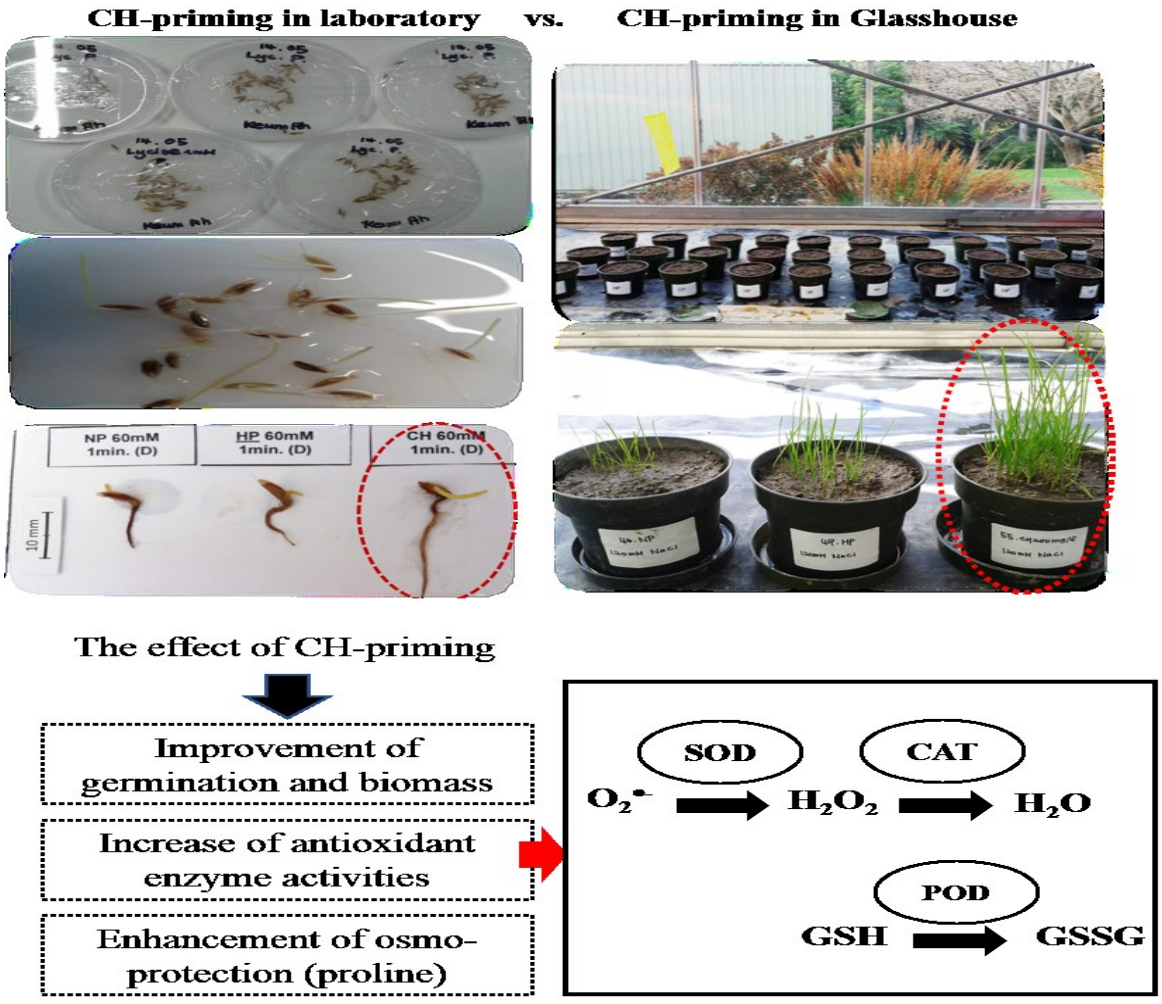


Fig. 7.1. The potential beneficial effect of priming with casein hydrolysate (CH) on Italian ryegrass (*L. multiflorum*) growth under salt stress.

7.3. Future directions

Agriculture in New Zealand largely relies on dairy pasture industry in recent and the future, so that proper management and development of pasture grazing system would be most important for sustainable dairy production (Keller et al., 2014). For improving crop productivity, seed priming technique is widely used, which helps plant species to increase seed production as well as seed quality and breeding. Moreover, it is important for pre-sowing treatment of seeds to enhance stress tolerance of the resultant crops that may face challenges under abiotic stress environment. Further research on casein hydrolysate priming of *L. multiflorum* seeds compared to L-methionine priming is needed in relation to improved resistance to salt stress as well as drought stress. Based on the results obtained in this study, more investigations are proposed below into casein hydrolysate priming in the future (Fig. 7.2):

1. Practical priming with casein hydrolysate of *L. multiflorum* seeds before sowing in the fields for crop growth and production beyond the seedling establishment stage. Of particular interest, seed production would be enhanced in a crop established more uniformly using seeds primed with casein hydrolysate.

2. More investigation of other ryegrass species and other seeds with casein hydrolysate priming: It would be of interest to compare growth and physiological responses of different seeds to CH priming as related to salt stress tolerance mechanism in saline soil.

3. Investigation of changes in additional non-enzymatic antioxidant defence mechanisms such as glutathionine, flavonoids, α-tocopherol as well as other oxidative stress markers including $O_2^{\bullet-}$, and $OH^{\bullet}$ in *L. multiflorum* seeds primed with CH. It would be interesting to relate their changes to resistance to salt stress or/and drought stress during germination and seedling growth in the glasshouse.

4. Molecular work for identifying gene expression related to salt overly sensitive (SOS) pathway signaling (i.e. Soni et al., 2013) and antioxidative defence in unprimed, CH-primed and hydro-primed Italian ryegrass seeds should be initiated.

5. Investigation to determine if priming of *L. multiflorum* seeds with casein hydrolysate would be better than or at least as effective as other well-known seed priming agents such as polyethylene glycol, potassium nitrate, and trace elements. Adding casein hydrolysate with the seeds are unlikely to be of environmental concern when the primed seeds are sown in saline soils.

6. It would be interesting to investigate if other protein hydrolysates have seed priming potential for improving abiotic stress resistance in plants.

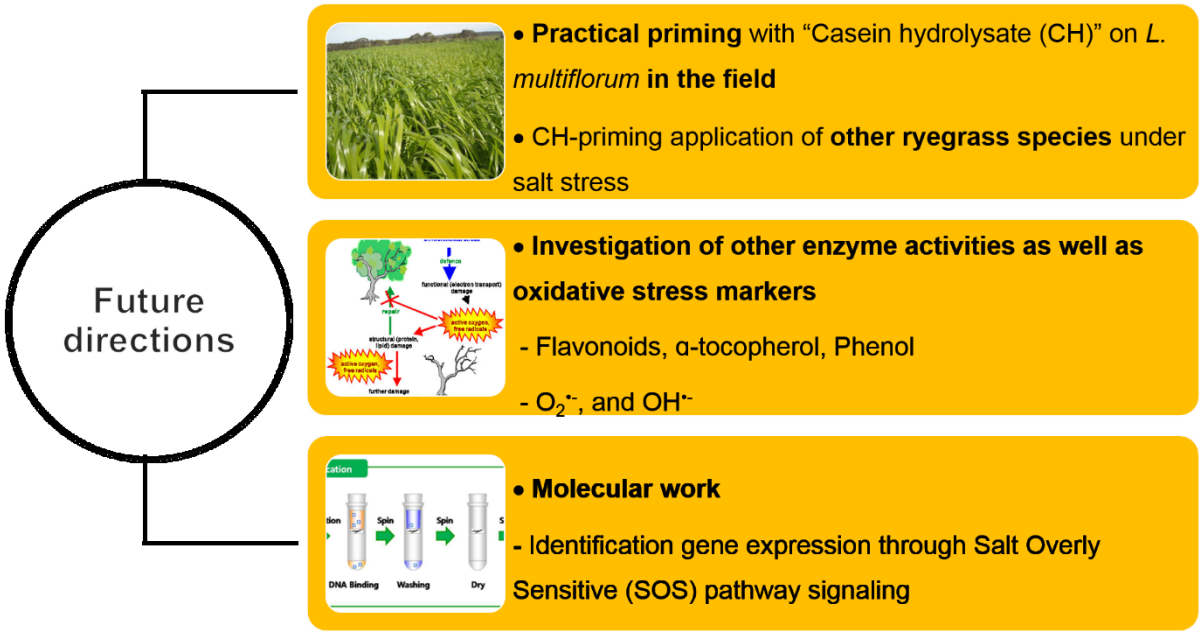


Fig. 7.2. Further research for CH-priming on *L. multiflorum* seeds under salinity stress in the future.

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Appendix 1

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N-Z-Amine A
Product Number **C 0626**
Store at Room Temperature

Product Description

The average MW is approximately 250 Da. The MW distribution is 54% at 100-200 Da, 36% at 200-500 Da, and 9% at 500-1000 Da.

The approximate amino acid content of this product is as follows:

Amino Acid	Amount (mg/g)
Alanine	30
Arginine	31
Asparagine	67
Cysteine	3
Glutamine	186
Glycine	19
Histidine	22
Isoleucine	44
Leucine	75
Lysine	68
Methionine	27
Phenylalanine	40
Proline	88
Serine	51
Threonine	42
Tryptophan	10
Tyrosine	28
Valine	59

The total amino acid content is approximately 890 mg/g.

This product is an enzymatic digest of casein. This product is not vitamin free. The presence of vitamins has been confirmed, but the amount was not determined. These vitamins are present in most casein enzymatic hydrolysates. It is recommended that a yeast extract be included in culture media to insure the presence of sufficient amounts of vitamins for growth of cultures. Alternatively, meat digests such as Product Nos. P7750 or P8388 (peptone type I and type II) can be used in place of N-Z-Amine products as a source of amino acids. The vitamin content will be higher, but it is still advisable to supplement with additional vitamins for fastidious cultures. The type of nutrient used, casein hydrolysate or meat hydrolysate, may influence the type of metabolic byproducts the cultured bacteria will produce.

Precautions and Disclaimer

For Laboratory Use Only. Not for drug, household or other uses.

Preparation Instructions

This product is soluble in water (100 mg/ml) with heating at 95℃ for 5 minutes.

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