USE OF POLYETHYLENE GLYCOL (PEG) 8000 FOR RAPID

SCREENING OF POTATO (*Solanum tuberosum* L)

GENOTYPES FOR WATER STRESS

TOLERANCE

By

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A THESIS

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An Abstract of the Thesis Presented
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The overall goal of this research was to develop new rapid techniques for screening potato genotypes for drought tolerance. A series of approaches namely single-node cutting assay (SNCA), root tip cutting assay (RTCA), microtuberization assay (MA), greenhouse experiment GE), and leaf disc assay (LDA) were employed to answer the questions: (1) can root and shoot growth reduction be used to screen potato for drought-stress tolerance, (2) can tuber production be used to screen potato for drought-stress tolerance, (3) can excised root elongation and leaf disc growth reduction be used to screen potato for drought-stress tolerance? Potato genotypes (Chagllina-INIA, E86.011, Reiche, C89.315, Tacna, Unica, Andover, Superior, Shepody, Kennebec, Katahdin, and Russet Burbank) were exposed to Polyethylene glycol (PEG) solutions (8% PEG8000, except for LDA using 10% PEG8000) and their growth reduction compared to the control

(0% PEG8000) were determined. It was expected that PEG treatments could mimic the effects of water stress and cause significant growth reduction in which genotypes known to be drought tolerant demonstrated less growth reduction than genotypes known to be drought sensitive.

As expected, the results showed that PEG treatments mimicked the effect of water stress in all approaches employed during the study. Kennebec known to be drought tolerant consistently showed less growth reduction than most genotypes tested, while Superior known to be drought sensitive demonstrated the opposite. It was true for root length density reduction (RLDR) and root dry weight reduction (RDWR) in SNCA, and leaf growth reduction (LGR) in LDA, which meant that those dependent variables could be use to select potato genotypes for water stress tolerance. Root growth reduction (RGR) and RDWR in RTCA might be used to select potato genotypes for water stress tolerance in conjunction with LGR of LDA. The GE approach needs lots of improvement to be used for water-stress screening of potato genotypes. To minimize the effect of environmental factors, it was recommended that the screening process be done in the growth chamber regardless of the choice of the approaches.

DEDICATION

I dedicate this dissertation to my mother Sumarni Sastrotenoyo who endlessly prays for my success and expects the best out of me, to my little brother Doso Joko Saptono who thinks that I can do no wrong, to my wife Tunjung Pamekas who is willing to share her dream with me, and to my children Hario Dhanar Anindito, Duhita Pramesthi Hayuningtyas, Dyah Ayu Zahra Sesotya Windya, and Salsabila Khairunnisa, who give me the strength to prevail.

The prophet Muhammad (peace be upon him) said:

Conduct yourself in this world,
As if you are here
To stay forever;
Prepare for eternity
As if you have to die tomorrow.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

INTRODUCTION

Potatoes (*Solanum tuberosum* L.) are grown and eaten in more countries than any other crop (Jackson, 1999). Among the most important crops in the world, potato ranks fourth in annual production behind the cereal species rice (*Oryza sativa*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) (Fernie and Willmitzer, 2001).

Potatoes are classified as very sensitive to water stress (Ekanayake and de Jong, 1992; Vayda, 1994). However, in many countries potatoes are grown in regions where irrigation is not available which causes growth and yield reduction (Heuer and Nadler, 1998). For example, due to recurrent drought, the average yield of potato in Bolivia is reported to be only 5.6 ton per hectare (Friedman and McDonald, 1997). For the same reasons, drought is also reported to be a major environmental constraint in Asia (Maldonado *et al.,* 1998).

Drought stress reduces plant growth (Weisz *et al.,* 1994), marketable yield, tuber number per stem, and average tuber yield (Lynch and Tai, 1989), carbohydrate accumulation and partitioning (Ekanayake and de Jong, 1992), the yielding capacity of potato crops, and subsequent performance of the seed tubers (Karafyllidis, 1996). Moreover, drought stress has been reported to reduce gas exchange, decrease the amount of phosphorylated intermediates, like 3-phosphoglyceric acid (3PGA) (Geigenberger *et al.,* 1997), and inhibit starch synthesis (Geigenberger *et al.,* 1999). Other studies showed

that drought stress increases the incidence of internal tuber defects (Miller and Martin, 1985), the percentage of sugar-end tubers (Kincaid *et al.*, 1993), and total glycoalkaloid content (Papathanasiou *et al.*, 1999).

Growers have two choices to deal with the problem caused by drought stresses; providing irrigation for the crops and/or growing more drought-tolerant crops. In this respect, the development of drought-tolerant lines becomes increasingly important. Some rapid methods for screening drought-tolerance traits in potato have been established (Bansal *et al.*, 1991; Demagante *et al.*, 1995; Levy *et al.*, 1991). Canopy temperature and chlorophyll *a* fluorescence have been reported as potential tools for drought screening of potato germplasm (Jefferies, 1992; Ranalli *et al.*, 1997; Stark *et al.*, 1991). Demagante *et al.* (1995) employed apical cuttings for screening drought tolerance in raised beds. Bansal *et al.* (1991) established a new screening method using the growth reduction of leaf discs floated over different concentrations of polyethylene glycol (PEG) 6000 (now PEG8000, Sigma Aldrich, 2001).

In vitro bioassays have been employed to screen potato genotypes for salinity tolerance (Ochatt *et al.*, 1999; Zhang and Donnelly, 1997), to screen *Prunus* tolerance to osmotic stress (Rajashekar *et al.*, 1995), and to select drought-tolerant rice (Biswas *et al.*, 2002). Even though *in vitro* techniques can potentially be used to screen potato genotypes for drought tolerance, no such research has been reported.

The overall goal of this research was to develop new rapid techniques for screening potato genotypes for drought tolerance. A series of approaches namely single-node cutting assay (SNCA), root tip cutting assay (RTCA), microtuberization assay (MA), greenhouse experiment GE), and leaf disc assay (LDA) were employed to answer

the questions: (1) can root and shoot growth reduction be used to screen potato for drought-stress tolerance, (2) can tuber production be used to screen potato for drought-stress tolerance, (3) can excised root elongation and leaf disc growth reduction be used to screen potato for drought-stress tolerance?

The reports were organized in the following chapters: 1. Introduction, 2. Literature Review, 3. Use of polyethylene glycol (PEG) 8000 for rapid screening of potato (*Solanum tuberosum* L.) genotypes for water stress. I. Root and shoot growth, 4. Use of polyethylene glycol (PEG) 8000 for rapid screening of potato (*Solanum tuberosum* L.) genotypes for water stress. II. Tuberization, 5. Use of polyethylene glycol (PEG) 8000 for rapid screening of potato (*Solanum tuberosum* L.) genotypes for water stress. III. Excised root tip and leaf disc growth reduction, and 6. Synthesis.

Chapter 2

LITERATURE REVIEW

2.1. Potato Family

Taxonomically, potatoes belong to family *Solanaceae*, genus *Solanum*, subgenus Potatoe (formerly Pachystermonum), section Petota (formerly Tuberarium), and subsection Potatos (formerly Hyperbasarthum) (Hanneman, 1989). The subsection has been further divided into 18 series that includes cultivated *tuberosa* having ploidy levels of 2x, 3x, 4x, and 5x and wild *tuberosa* having ploidy levels of 2x, 3x, 4x, and 6x.

Solanum has about 230 species, including *tuberosum, demissum,* and *stoloniferum.* They are wild and cultivated, diverse in morphology and physiology, and found in all altitudes from the high mountains to the coastal areas (Huaman and Ross, 1985). Among them, *Solanum tuberosum* is the most common cultivated species. It has two subspecies (ssp.), *Solanum tuberosum* ssp. *andigena* and *Solanum tuberosum* ssp. *tuberosum,* each of which has different climatic adaptation. While the former is adapted to short photoperiods, the later is adapted to long photoperiods. It is believed that *Solanum tuberosum* ssp. *tuberosum* originated from tubers of *Solanum tuberosum* ssp. *andigena* collected from Peru and Coloumbia and then introduced to Europe (Peloquin *et al.,* 1989). In addition to *andigena* and *tuberosum* groups, there is a new group called Neo-*tuberosum,* which refers to *andigena* potatoes selected through several cycles of recurrent selection for tuberization under long-day conditions.

The optimum daylength for potato growth and development depends upon the temperature and cultivar (Monrique *et al.,* 1990). Typically, *andigena* cultivars require

12 to 14 hours for tuber initiation provided that temperatures are relatively cool (Ewing, 1981) while *tuberosum* cultivars tuberize under much longer daylength even though their growth habits also respond to daylength and temperature (Monrique *et al.*, 1990). Under short days and cool temperatures, *tuberosum* cultivars may perform poorly because tuber induction is excessively strong (Monrique *et al.*, 1989), leading to early maturity (Ewing, 1981). Under this condition, *tuberosum* cultivars produce a small leaf area that senesces early, leading to low tuber yield (Burton, 1966; Monrique *et al.*, 1990). Cultivars that perform in this manner are known as 'long day" because they produce higher tuber yield under long days (Monrique *et al.*, *1990)*. Other *tuberosum* cultivars yield as well under short days as under long days, and they are known as "day neutral" (Ewing, 1981).

According to the length of time required for maturity, potato cultivars are classified as late (160 to 180 days), medium (130 to 150 days), early (90 to 120 days), and precocious (60 to 80 days) cultivars (Manrique *et al.*, 1990).

2.2. Potato Growth and Tuberization

A potato plant is a cluster of true main stems, each of which may develop into a complicated shoot structure. A true main stem may develop diageotropic shoots (stolons, botanically called rhizomes), below-ground branches from below-ground buds, and above-ground branches from aerial buds (Struik and Ewing, 1995). Based on the morphology of shoot and tuber development, Kleinkopf (1983) has divided the growth of potato into four distinct stages. They are early vegetative growth, tuberization, tuber bulking, and maturity. The early period (Stage I) includes early plant development from planting to initiation of tubers. This stage varies from 30 to 60 days, depending on potato

cultivar and environmental conditions. Tuberization (Stage II) is the period during which the stolon tips swell to form visible tubers. It generally takes 2 to 4 weeks. Tuber bulking (Stage III) includes the stage of linear tuber dry matter accumulation to near maturity, and this stage takes about 60 days. At this stage, flowers appear on the main and secondary stems. Leaf area index (LAI) reaches its maximum 3.5-6.0 during stage III. The maturation stage (Stage IV), represents the final 10-24 days growth, and is characterized by senescence of the shoot, along with the decline in leaf, shoot, and root dry weight. Stressful environmental conditions can change the time required to complete each stage of development. One of the environmental factors that significantly affect the growth and yield of potato is drought stress, which is discussed in another section.

The life cycle of potato tubers, induction, initiation, enlargement, dormancy and sprouting, has been studied by many workers (Ewing and Struik, 1992; Fernie and Willmitzer, 2001; Galis *et al.*, 1995; Jackson, 1999; Kolomiets *et al.*, 200). Tuberization consists of two processes known as tuber induction and tuber initiation. O' Brien *et al.* (1998) defined tuber induction as a physiological change of the plant which results in the characteristic swelling of stolons while tuber initiation is defined as the visible appearance of tubers. Furthermore, appearance of a tuber is defined as a swelling of the stolon tip that is at least twice the diameter of the subtending stolon. Potato stolons, botanically rhizomes, are diagravitropic stems, arising as branches from underground nodes (Ewing and Struik, 1992). Tuber initiation is believed to lead to an abrupt preferential diversion of assimilate to the tubers which causes either reduction in the growth rate or cessation in growth of foliage and roots (Ewing and Struik, 1992; O'Brien

6

et al., 1998). Thus, early tuber initiation often results in small plants with limited leaf area and low final tuber yields.

Potato tubers are derived from lateral underground buds developing at the base of the main stem, which finally develop into stolons when kept underground (Fernie and Willmitzer, 2001). Under favorable conditions, which are called inducing conditions, the subapical part of the stolons swell after the stolons stop elongating and the cells located in the pith and the cortex of the apical region of the stolons first enlarge and then divide longitudinally (Fernie and Willmitzer, 2001. Longitudinal division stops and is replaced by randomly-oriented division and cell enlargement when the swollen portion has attained a diameter of approximately 2 to 4 mm (Xu *et al.,* 1998). The work of Muller-Rober *et al.* (1992) shows that starch formation is not required for tuber initiation and enlargement. They found that reducing ADP-Glc pyrophosphorylase activity by antisense repression significantly reduced the starch level. However, the plant still displayed normal tuber formation. Unlike starch, protein biosynthesis plays an important role in tuber formation. The protein composition of the tuber dramatically changes during stolon-tuber transition resulting in the formation of much-simplified protein complement consisting of only a few highly abundant proteins such as patatin (Fernie and Willmitzer, 2001). Physiologically mature potato tubers contain approximately 80% water, between 15% and 25% of starch, and nearly 2% of protein (Fernie and Willmitzer, 2001).

Detailed reviews on the significant effects of environmental factors on potato tuberization have been provided by Ewing and Struik (1992), Jackson (1999), and O'Brien *et al.* (1998). Even though there are many factors affecting potato tuberization, it has been shown that light, temperatures, and nitrogen levels have the greatest effect

(Jackson, 1999). It has been established that potato is well adapted to mean temperatures of 17-20 °C, with the optimum temperature for tuber formation in the range of 10-17 °C (O'Brien *et al.*, 1998). High temperatures have inhibitory effects on tuberization regardless of the photoperiods, even though the effects are much more pronounced under long photoperiods (Ewing and Struik, 1992; Jackson, 1999). Tuber formation is inhibited when the mean daily temperature is >30 °C, or mean night soil temperature is >24 °C (O'Brien *et al.*, 1998), or the mean daily temperature is <6 °C. In general, low temperatures have been reported to hasten tuberization (O'Brien *et al.*, 1998). High temperatures have been reported to alter the partitioning of assimilates by reducing the amount that goes to the tubers and increasing the amounts allocated to other parts of the plant (Jackson, 1999). Menzel (1981) found that bud removal from the shoot could ameliorate the effects of high soil temperatures on tuberization. It was also reported that under high temperatures buds produced very high levels of gibberellin-like substances, which are known to inhibit tuberization (Menzel, 1983).

Plant growth regulators have been known to play a key role in tuberization. Grafting of a flowering-induced plant of tobacco onto a potato stock leads to formation of tubers (Gregory, 1956). This experiment demonstrated that the stimulus for tuber induction is received in the leaves of the scion plant and is graft-transmissible, and the tuber-inducing stimulus and the flowering stimulus must be related or similar (Gregory, 1956; Fernie and Willmitzer, 2001). Using transgenic techiniques, Jackson *et al.* (1998) reported that phytochrome B was involved in the production of a graft-transmissible inhibitor of tuber formation. More recent work on transgenic plants showed that phytochrome A was involved in resetting the circadian clock and delaying tuber

formation under noninducing condition (Yanofsky *et al.*, 2000). Fernie and Willmitzer (2001) conclude that concerted action of both phytochromes is involved in the repression of tuber formation. Unlike cytokinin and jasmonic acid derivatives, the evidence that ABA has role in tuber induction is less convincing (Jackson, 1999; Fernie and Willmitzer, 2001).

2.3. Drought and Its Effects on Potato Growth and Yield

Plants experience drought when transpiration is excessive and/or when water supply is limited (Frensch, 1997). Drought reduces the rate and duration of growth, the size of leaves produced (Hang and Miller, 1986) and the number of leaves produced (Jefferies, 1993). Leaf growth is one of the first processes affected by water stress (Hsiao, 1973), and leaf extension is more sensitive to tissue water stress than stomatal resistance and CO_2 assimilation (Harris, 1992). Because leaf expansion depends mostly on cell expansion, the principles that underlie the two processes are similar. Taiz and Zeiger (1997) defined cell expansion as $GR = m \ (\psi_p - Y)$ where GR is growth rate, Y is the yield threshold (the pressure below which the cell wall resists plastic deformation), ψ_p is turgor potential, and m is wall extensibility. This equation indicates how significant the relationship between turgor pressure and cell growth is. It also emphasizes that cell expansion and leaf growth are turgor-driven processes that are extremely sensitive to water deficit. A study by Gandar and Tanner (1976) supports this statement. The rate of leaf extension decreased linearly as leaf water potential (ψ_l) decreased to -2 bars (-0.2 MPa) and it was completely stopped at -5 bars (-0.5 MPa). Lower ψ_l was reported by

Jefferies (1979) in which the average daily leaf extension was completely stopped when the midday ψ_l reached -1.0 MPa.

In addition to turgor pressure, cell expansion is affected by cell wall loosening triggered by *expansin*. This protein is responsible for cell wall acidification that leads to cell wall loosening under normal conditions (Taiz and Zeiger, 1997). However, drought stress causes alterations in the chemical composition and physical properties of the cell wall (Ingram and Bartels, 1996), which is believed to involve the genes encoding *S*-adenosylmethionine synthetase, SAM1 and SAM3, the enzymes whose expression are correlated to cell wall lignification (Espartero *et al.*, 1994). These facts suggest that water deficit induces the synthesis of SAM1 and SAM3 that promote lignification of cell walls which in turn stops cell elongation. Inhibition of cell and leaf expansion results in decreased leaf area of crops. As a result, light interception is reduced (Gardner *et al.*, 1991) along with photosynthetic capacity of the crops (Weisz *et al.*, 1994; Olesinski *et al.*, 1989). Shekhar and Iritani (1979) showed that potato plants exposed to moisture stress fixed less labeled $^{14}CO_2$ than the controls. However, the researchers did not provide supporting data to answer whether the decrease in $^{14}CO_2$ was due to stomatal closure or decreased light interception.

Unfavorable environmental factors caused by drought or heat stress bring about physiological disorders of potato tubers, which markedly affect the physical appearance of the tubers. Physiological disorders could be in the form of tuber cracking, tuber malformation, surface abrasions, hollow heart, brown center, internal brown spot, vascular discoloration or bruise (Hiller *et al.*, 1985; Eldredge *et al.*, 1996). Physiological disorders could also appear in the form of greening, secondary growth, enlarged lenticels,

or translucent ends. Some of these tuber defects are caused by high temperature or/and water deficits (Hiller *et al.*, 1985; Levy, 1985). The effects of high temperature stress on tuber defects are often difficult to separate from the effects of water stress during the growing season (Hiller *et al.*, 1985). A short period of high temperature has been reported to cause tuber cracking, promote secondary growth, and cause tuber knobiness (Levy, 1985). Coupled with dry soil conditions, high soil temperatures induce internal brown spot or heat necrosis and increase vascular discoloration (Hiller *et al.*, 1985).

In addition to promoting tuber defects, drought and heat stresses also influence total dry matter, sugar content, sugar and starch distribution, mealiness, texture, and flesh color (Iritani, 1977) all of which are very important for tuber processing quality. High temperature and water deficit increased reducing sugar content in the stem ends of cv. Russet Burbank (Eldredge *et al.*, 1996). According to Hiller *et al.* (1985) and Eldredge *et al.* (1996) sugar-end tubers are caused by either failure in converting sugars translocated to the tuber's stem end into starch, or degradation of starch in the tuber stem end to sugars that resulted in sugar accumulation. The time when plants are exposed to stress also affects the quality of the tubers (Shock *et al.*, 1993).

Like other *Solanaceae* family members, potato tubers contain glycoalkaloids in the form of α-solanine and α-chaconine. Glycoalkaloids have been reported to cause death when consumed in high concentrations (McMillan and Thompson, 1979; Papathanasiou *et al.*, 1999). Papathanasiou *et al.* (1999) found that drought stress significantly increased total glycoalkaloid concentration in British Queen potato tubers. A more recent study showed that drought stress increased glycoalkaloid concentration (α-solanine and α-chaconine) of potato tubers, with an average increase of 43 and 50% for

drought-tolerant and control cultivars, respectively (Bejarano *et al.*, 2000). The glycoalkaloid concentration ranged from 52.4 to 100 mg kg^{-1} F.W. in drought-tolerant cultivars and 55.6 to 122.3 mg kg^{-1} in the controls. These levels are still lower than the recommended food safety standard, 2000 mg kg^{-1} (Friedman and McDonald, 1997).

Low temperature and high salt content are other factors that may reduce water availability to plant roots (Taiz and Zeiger, 1998). Even though water deficit reduces plant water potentials, it affects root and leaf growth differently (Frensch, 1997). Many studies have shown that root growth is more resistant to water stress than shoot growth (Hsiao and Jing, 1987; Kramer and Boyer, 1995; Frensch, 1997).

Frensch and Hsiao (1994) have demonstrated that lowering water potentials by adding mannitol to the watering solutions reduced shoot growth more than root growth. Root elongation stopped almost immediately upon addition of the mannitol, but resumed after a few minutes at a new rate smaller than prior to the treatment. On the other hand, leaf elongation remained strongly inhibited, at least within the first hour of exposure to water stress. Further, when transpiration rate was increased artificially, leaf elongation was significantly reduced while root elongation was unaffected (Frensch and Hsiao, 1994). Hsiao and Jing (1987) believed that the contrasting responses of roots and shoots to water stresses are related to the different mechanical and hydraulic properties of those organs.

2.4. The Role of ABA on Maintaining Root Growth at Low Ψ_W

In the past, it was thought that ABA generally inhibits plant growth, and this inhibition increases during water stress as ABA levels in the plant increase. However, it

now appears that roots and shoots differ in their response to ABA, and the size of response may depend on the degree of water stress. Recent work also indicates that ABA may even stimulate growth via inhibition of ethylene production. The growth is stimulated via relief from ethylene inhibition.

Saab *et al.* (1990) noted that speculation on the involvement of ABA in growth responses to water stress has relied on the results of ABA application to well-watered plants. While such applications have generally inhibited shoot growth, they have been reported to promote, inhibit, or have little effect on root growth (Bensen *et al.*, 1988; Creelman *et al.*, 1990; Jones *et al.*, 1987; Mulkey *et al.*, 1983). The results of earlier work on measuring the effects of exogenously supplied ABA on root growth have been conflicting. For example, at concentration of 10^{-6} to 10^{-5} M in culture solution ABA slowed the growth of excised or intact roots of *Zea mays* (Pilet and Chanson, 1981), *Lens culinaris* (Pilet, 1970; Gaither *et al.*, 1975), and *Allium cepa* (de la Torre *et al.*, 1975). In contrast, the same ABA concentration enhanced the growth of excised roots of *Glycine max* (Yamaguchi and Street, 1977), *Pisum sativum* (Gaither *et al.*, 1975), and *Phaseolus coccineus* (Abou-Mandour and Hartung, 1980).

Pilet and Barlow (1987) suggested that the contrasting effects of ABA on root growth are likely due to the differences inherent to the material and to the techniques used. For example, each study used different methods of applying ABA to roots (ABA-containing agar block, droplets, solution supplied to the whole root, or ABA-loaded beads applied to localized zones of the root) and this may result in different growth reactions.

Saab *et al.* (1990) employed different approaches to study the effect of ABA on root growth at low water potential (ψ_w) by manipulating the concentration of endogenous ABA as an alternative to external application of hormones. An inhibitor of carotenoid biosynthesis (hence ABA biosynthesis inhibitor), fluridone (FLU), and a mutant deficient in carotenoid biosynthesis (*vp5*) were used to reduce the endogenous ABA content in the growing zone of the primary root at low ψ_w. Maize seedlings (30 to 60 h old) were transferred into vermiculite that had been preadjusted to water potentials of approximately -0.03 MPa (highψ_w) and -1.6 MPa (low ψ_w), and put in the dark at near saturation humidity. Saab *et al.* (1990) reported that for the first 30 h after transplanting, treatment with fluridone was associated with reduced primary root growth (length) at low ψ_w. Compared with untreated roots at high ψ_w, the rate of root elongation in FLU-treated seedlings was reduced by 83% at low ψ_w. On the other hand, at the same ψ_w without fluridone application, the rate of root growth was only reduced by 55%. Results of the experiments using the mutant *vp5* were very similar to that of experiments using fluridone (FLU). Saab *et al.* (1990) pointed out that the reduction of root growth rate of FLU-treated seedlings and mutant *vp5* seedlings at low ψ_w was likely due to the reduction in ABA synthesis. This argument was based on the fact that ABA concentrations were significantly lower in the growing zone of FLU-treated seedling and mutant *vp5* seedlings at low ψ_w.

Saab *et al* (1990) demonstrated that ABA accumulation is required to maintain root elongation at a low ψ_w. A study done by Sharp et al. (1994) confirmed the results reported by Saab *et al.* (1990). Fluridone-treated maize seedlings growing at low water potential (-1.6 MPa) demonstrated a significant reduction in root growth. When ABA was

applied to the fluridone-treated maize seedlings, the seedlings showed root growth recovery. Likewise, when treated with ABA (0.7 mM), seedlings of mutant *vp5* demonstrated comparable root growth to untreated wild-type seedlings.

In agreement with the above, Creelman *et al.* (1990) found that root response to water deficit is not uniform. By 24 h after treatment, when a portion of the root had turned brown and the hypocotyl decreased in diameter, the root tip (terminal 10-15 mm) was indistinguishable from a well-watered root tip (Creelman *et al.*, 1990). They also reported that the concentration of endogenous ABA increased differentially, depending on the seedling sections, when 2-d soybean seedlings were transported from well-watered vermiculite to low-water potential. At 24 h after transfer to low-water-potential vermiculite, there was a 5- to 10-fold difference in ABA concentration compared with well-watered seedlings, and the root-tip contained 5-fold higher ABA than the mature root. For example, the concentration was 4.5 μg/g root dry weight in mature roots and 27.9 μg/g in the root tip. When supplied with exogenous ABA, increasing concentration of ABA progressively inhibited hypocotyl growth. In contrast, root elongation was unaffected by any ABA concentration applied.

Sharp and LeNoble (2002) pointed out that root elongation of well-watered seedlings was inhibited when the content of ABA in the root tip was increased by applying exogenous ABA. However, other studies indicated that root elongation was further inhibited when the ABA content of water-stressed roots was decreased genetically by using the *vp5* mutant (mutant that can not produce ABA) or chemically by applying fluridone, an ABA inhibitor. But, when exogenous ABA was applied to the water-

stressed root, root elongation was restored, indicating that ABA promotes root elongation at low water potentials (Sharp and LeNoble, 2002).

Furthermore, Sharp and LeNoble (2002) indicated that shoot growth was more inhibited than root under drought stress because the ABA levels were optimal for root elongation, but were insufficient for maximal elongation of the shoot. Evidence to support the claim came from an experiment demonstrating that shoot elongation was greater in ABA-deficient maize seedlings than in the control in early germination stages (Saab *et al.*, 1990). Shortly after that, the growth was reversed to a point that ABA deficiency causes shoot growth inhibition, as in the root. The application of exogenous ABA to the seedling promoted substantial shoot growth (Sharp *et al.*, 1994; Sharp, 2001).

2.5. Mode of Action of ABA during Drought Stress

There are least four different mechanisms by which ABA acts during drought stress. These are discussed below.

2.5.1. ABA and Osmotic Adjustment

In addition to ABA accumulation, plant responses to drought stress also include proline accumulation required for osmotic adjustment (Voetberg and Sharp, 1991). The concurrent accumulation of proline and ABA in response to drought stress has led to speculation that ABA may trigger proline increases (Stewart, 1980; Dallmier and Stewart, 1992). Robertson *et al.* (1990) reported that when excised apical roots (3.0 mm segments) from well-watered sunflower plants were desiccated or treated with ABA solution (10^{-2} mmol m^{-3}), root ψ_w dropped sharply from -0.08 MPa to -1.46 MPa, but the

root apices were able to adjust osmotically. At this point the turgor pressure did not decrease, instead it increased from 0.15 MPa to 0.32 Mpa. Robertson *et al.* (1994) also found that the osmotic adjustment process in sunflower root apices apparently does not come from the body of the plant. These data suggest that ABA may be involved in regulating osmotic adjustment of roots at low ψ_w.

Proline is one of the well-documented osmolytes that accumulates in the cells when plants are exposed water deficit or salt stress. For proline to accumulate either synthesis from glutamic acid must be enhanced or the rate of oxidation must be decreased, or both (Dallmier and Stewart, 1992). Proline dehydrogenase (PDH) is the first enzyme in the proline oxidation pathway, whose activity has been shown to decline in response to water stress (Rayapati and Stewart, 1991).

Studies to elucidate the effect of ABA on proline accumulation have been done by measuring the effect of exogenous ABA (0, 11, 33, and 100 μmol ABA) on the decline of extractable PDH in well-watered maize seedlings (Dallmier and Stewart, 1992). The results showed that there was no effect of ABA on PDH activity at 33 and 100 μmol ABA, but there was a 38% decline at 11 μmol ABA. However, PDH activity was reduced up to 69% under drought treatment (Dallmier and Stewart, 1992). Dallmier and Stewart (1992) concluded that ABA might not be involved in proline accumulation at low ψ_w. However, a recent study shows otherwise (Ober and Sharp, 1994).

It is likely that the differences in the results are caused by the methods employed. While Dallmier and Stewart (1992) applied exogenous ABA to study its effect on proline accumulation, Ober and Sharp (1990) manipulated endogenous ABA concentration by using fluridone (FLU) and the *vp5* mutant to study its effect on proline accumulation of

maize roots at ψ_w. of -1.6 MPa. The results showed that a high level of ABA is required

to maintain high rates of net proline deposition in the apical root. However, the metabolic

basis for the increase in proline at low ψ_w is not yet understood. One may speculate that

it could be through the increased net proline synthesis, decreased proline oxidation, or

increased proline import.

2.5.2. ABA and Cell Cycle

In response to drought stress and ABA, sunflower roots demonstrated a three-

phase growth response. First an initial phase of promoted growth, then a phase of

complete inhibition between 6 h and 72 h, and then finally a third phase of partial

recovery in the rate of root elongation   (Robertson *et al.*, 1990a). Root elongation during

the period of resumed growth at low ψ_w was considerably greater in ABA-treated plants

than in controlled plants, but still well below values in well-watered plants, confirming

the role of ABA in root elongation when plants are exposed to water deficit.

Further evidence indicated that the three phases of root growth corresponded with

changes at the meristem (Robertson *et al.,* 1990a). The initial phase of promoted root

elongation was contemporary with a sharp transitory decline in osmotic potential (ψ_π)

and an initial increase in ψ_p in the root apex. In the second phase, between 6 h and 72 h,

root elongation stopped almost completely as the merismatic region decreased in length.

Cells in the proximal region of the apex, which at the same position had been densely

cytoplasmic at the beginning of treatment and had remained so in control plants,

vacuolated and elongated. Root elongation that resumed after 72 h was contemporary

with a partial recovery in the length of the meristematic region. A similar three-phase growth response to ABA has been reported by Mulkey *et al.* (1983) for *Zea mays*.

Drought stress decreased the size of the apical meristem as cells in the proximal region of the meristem became vacuolated and elongated (Robertson *et al.*, 1990a). Root-to-shoot biomass ratio (*R:S*) increased initially but declined after 72 h. The inhibition of root elongation and anatomical changes in the root apices were not determined by loss of turgor or lack of photosynthate, but rather appeared to be an active response by the meristem to a drop in external water potential (ψ). Robertson et al. (1990a) also reported that ABA triggered a three-phase growth response, increased R:S, and triggered the same initial changes in water potential (ψ_w), osmotic potential (ψ_π), *and* increased turgor pressure (ψ_p) in excised 3.0 mm apical segments, and induced the same pattern of anatomical changes in the root apices as drought stress.

A study of the effect of drought and ABA on mitotic activity of sunflower roots has been conducted by Robertson *et al.* (1990b). The results showed that both drought stress and ABA-treatment (at a concentration of 10^{-2} mol m^{-3}) inhibited DNA synthesis and mitosis within the first 6 h of treatment. The depression of mitotic activity was first seen in the proximal regions of the meristem (1000-5000 μm from the cap junction), followed by a general depression of mitotic activity throughout the meristem. There was a partial recovery of mitotic activity in the distal regions of the meristem. The beginning of this partial recovery of mitotic activity was concurrent with the activation of the quiescent center. These findings support the hypothesis that ABA mediates drought-induced changes in the primary development. Cells in the proximal regions of the root

meristem elongated and went out of cycle as the size of meristem decreased (Robertson *et al.*, 1990b).

In control plants, mitotic activity extended to the interface of cytoplasmic region and the zone of elongation. In drought-stressed plants, the mitotic index fell within 6 h of treatment, especially in the regions greater than 1000 μm from the cap junction. The decline in mitotic index was gradual in the proximal regions (less than 1000 μm). At 24 h after treatment, there was general depression of mitosis throughout the meristem. After 96 h of treatment, a partial mitotic recovery (40-60% of control values) was detected in the regions within 500 μm from the cap junction. Afterward, mitotic indices remained at 40-60% of the control value in the cytoplasmic regions of meristem. This study demonstrated that the effect of ABA application on root growth mimicked the effect of drought (Robertson *et al.*, 1990b).

In summary, Robertson *et al.* (1990b) found that drought and ABA inhibited mitotic activity by arresting the cell cycle in G_1, and consequently halting DNA synthesis. Further, the authors also reported that the recovery of mitotic activity in the cells of distal regions of the meristem was concomitant with the activation of the quiescent center (QC) (Robertson *et al.*, 1990b). It is likely that regeneration of root mitotic activity after damage is one of the functions of QC, as demonstrated in an earlier study by Clowes (1970) in which halting cycling in the meristems induced the activity of QC. In addition, a recent study by Muller *et al.* (1994) confirmed that ABA application retarded the completion of the cell cycle and acted upon the exit from either G_1 or G_2 phase. Decapitating of primary roots preferentially shortened the G1 phase of the cycle in the QC. When supplied to decapitated root, ABA reversed the effect.

2.5.3. ABA and Cell Wall Loosening

Nonami and Boyer (1989) have revealed that transfer of soybean seedlings to low-water potential vermiculite induces a transient decrease in cell turgor in the inner cortical cells of the elongating region of the hypocotyl and causes disruption of water potential gradients. The change in water status is correlated with the inhibition of shoot and root growth (Nonami, 1986). With time, the water potential of growing zones declines to a similar extent and turgor pressure is re-established in these regions. However, root growth recovers while shoot growth remains inhibited (Nonami, 1986). These phenomenon suggest that changes in water status alone cannot explain the pattern of growth observed in plants exposed to water deficit. It is likely that biochemical differences exist between hypocotyl and root of the seedlings, which contributed to the differential response to water deficit. Creelman *et al.* (1990) reported that seedlings transferred to low-water potential vermiculite accumulate 5- to 10-fold higher levels of ABA than are found in well-watered seedlings, with the highest ABA content found in the root tip. This evidence supports earlier studies concluding that ABA may modulate differential inhibition in shoot *versus* root growth (Caldwell, 1976; Davies *et al.*, 1986). Because ABA plays an important role in modulating growth, Creelman *et al.* (1990) suggested that both water deficit and ABA treatment alter wall properties. However, the metabolic basis for increases in cell wall-loosening of roots at low ψ_w is unknown (Wu *et al.*, 1994).

Wu *et al.* (1994) have proposed that low ψ_w may decrease xyloglucan endotransglycosylase (XET) activity in the maize primary root elongation zone. XET is

believed to cause cell wall-loosening by cleaving xyloglucan molecules that tether adjacent cellulose microfibrils (Fry $et\ al.$, 1992; Nishitani and Tominaga, 1992). The net effect would be to relieve tension in the wall and promote expansion by allowing microfibril separation (Wu $et\ al.$, 1994).

Early studies showed that XET activity correlated with spatial distribution of elongation rate at high ψ_w in $Pisum\ sativum$ stems (Fry $et\ al.$, 1992) and maize primary roots (Pritchard $et\ al.$, 1993). Wu $et\ al.$ (1994) have demonstrated that ABA modulates XET activity of maize seedlings at low ψ_w. In their experiment, Wu $et\ al.$ (1994) showed that the activity of XET per unit fresh weight in the apical 10 mm (encompassing the elongation zone) was constant at high ψ_w, but decreased by more that 2-fold at a ψ_w – 1.6M Pa. Treatment with FLU to decrease ABA accumulation greatly delayed the decrease in activity of XET activity of maize seedlings at low ψ_w; but these effects were overcome when internal ABA levels were restored by exogenous ABA application (Wu $et\ al.$, 1994).

2.5.4. ABA and Ethylene

The work of Spollen $et\ al.$ (2000) demonstrated that the role of ABA accumulation in the maintenance of maize primary root elongation at low ψ_w is to restrict ethylene production. In the absence of ABA, ethylene inhibits elongation. They found that FLU-treated and $vp5$ roots grown at low ψ_w exhibited additional symptoms of excess ethylene. Moreover, a study with ethylene-deficient mutants showed that excess ethylene production was the cause for shoot growth reduction in wild-type tomato plants, and

treatment with ABA reduced the ethylene production and partly restored the shoot growth of the wild-type tomatoes (Sharp and LeNoble, 2002).

2.6. Breeding for Drought Tolerance

The potato plant is more sensitive to drought than most other crops (Horton, 1987). Yet, only a small part of the water taken up by the plant is used directly in photosynthesis. The primary role of water uptake is to replace water lost when stomata open to allow CO_2 into the leaf. The secondary roles of water uptake are to cool the plants by transpiration and to provide a medium for transporting organic compounds and minerals within plants (Horton, 1987). The supply of water to shoots depends not only on the soil moisture status, but also on the ability of roots to exploit available soil moisture (Gregory and Simmons, 1992; Weisz *et al.*, 1994). In well-watered crops, hydraulic conductance between the bulk soil and the base of the stem was proportional to the root length in the soil (Jefferies, 1995).

One explanation of the acute sensitivity of potato to drought is its relatively shallow root system, and the inability of potato roots to penetrate deeper soil layers to get more water (Miller and Martin, 1987; Vayda, 1994). In fact, the root system of potato is fibrous and all roots are adventitious (Kleinkopf, 1983), most of which are confined to the plow layer (Kleinkopf, 1983) or the upper 30-cm soil layer (Opena and Porter, 1999). The effective rooting depth of potato plants is considered to be 40 to 50 cm (Horton, 1987). Drought-adapted plants are characterized by deep and vigorous root systems. These rooting systems are associated with extensive rooting depth, high root length density, and low resistance to water flow within the root (Monevaux and

Belhassen, 1996). Drought stress increases both root:shoot ratio and root length:root weight ratio (Jefferies, 1993a), which enable the plants to maximize the soil volume exploited and allow water uptake to be sustained (Jefferies, 1995). However, if evapotranspiration exceeds the rate at which the roots can grow and exploit the potential rooting volume, the roots may fail to extract all the available water in the soil profile.

Many studies indicate that root growth is controlled by soil water status. Lesczynski and Tanner (1976) reported that root length and mass of irrigated potato grown on loamy sand continued to increase in size until early senescence. With minimum irrigation and only when soil moisture deficit approached, Asfary *et al.* (1983) found that root growth occurred early in the life of crops grown on sandy loam soil and root length hardly changed between 14 days after emergence and harvest. Vos and Greenwood (1986) reported that in a dry season without irrigation (unirrigated) potato root length and root mass increased rapidly during early growth and were maximal at 45 days after emergence. Afterward, root length and root mass decreased in size (30%), and then remained constant until 102 days after emergence. In contrast, root growth continued in a wet season through 80 days after emergence.

Jefferies (1993b) has classified plant strategies for drought tolerance into three groups by: 1) improving available water supplied either by increasing the rooting depth or by improving the water use efficiency; 2) improving leaf growth by increasing the potential extension rate of individual leaves (F); and 3) improving photosynthetic performance by improving the relation between the coefficient for conversion of intercepted radiation into dry matter (C) and the soil moisture status or by improving the

slope of the relation between F and C. This model suggested the importance of earliness and sustained leaf growth under stress.

Potato breeders are expected to produce improved cultivars that give high yields of high quality tubers (Hermsen and Swieynski, 1997). New cultivars should have pest and disease resistance during growth and storage, resistance to stress and mechanical damage, and specific tuber properties required for various processing industries. The development of drought-tolerant potato cultivars is one of the major objectives of breeding programs in hot tropical environments, where moisture is insufficient during growing seasons and average daily temperature may reach 40 °C. Since water stress is often accompanied by heat stress, which complicates field studies and cultivar assessment (Vayda, 1994), selecting for both traits is highly recommended.

In many cases, the ability of plants to withstand severe moisture stress is negatively correlated with productivity. Therefore, obtaining drought-tolerant potatoes should not be done by restricting transpiration. Instead, the goal should be to maintain transpiration at maximum rate without undergoing water stress (Burton, 1981). For example, breeding for improved drought tolerance should be directed to improve the phenology of potato water supply that includes increasing rooting to increase water uptake capacity, early vigor which is very important in areas where water loss though evaporation is high, and improved osmotic adjustment (Tuner et al., 1996). Osmotic adjustment enables plants to carry out photosynthesis is at low leaf water potential, delay senescence, and improve tuber yield under water-limited conditions (Turner, 1997; Turner et al., 1996).

Typically a potato breeding program will take 10 to 15 growth cycles from crossing of parental lines to the final release of a new cultivar (Caligari, 1992). The program includes choosing parental lines for crossing, evaluating progenies, identifying superior genotypes, and evaluating promising cultivars in the most appropriate areas. Parental lines should have good general combining ability (GCA) for the traits in question (Bradshaw and Mackay, 1994). It is well known that within *Solanum tuberosum* spp *tuberosum* sources of tolerance or resistance to the main biotic and abiotic potato yield constraints are either scarce or completely absent (Peloquin *et al.*, 1989). Tarn and Tai (1983) have demonstrated that the progenies of *andigena* x *tuberosum* are superior to those of *tuberosum* x *tuberosum* in total yield, but have smaller tuber size, later maturity, and more persistent stolons. Also, it is common experience that when *andigena* clones are used as males to pollinate *tuberosum* clones, most of the resulting hybrids are male sterile while reciprocal crosses do not show this response (Vilaro *et al.*, 1989*)*.

Problems may arise when breeders cross two or more parental lines in which the hybridization does not produce flowers, because of incomplete matching between two parental lines caused either by prezygotic barriers or by postzygotic barriers. To overcome the former problem Hermensen (1994) suggests breeders use a mixture of pollen from many male genotypes and select matching genotypes within parent species. Another option is to use somatic hybridization (Pehu, 1996). Somatic hybridization is a biotechnological tool for combining genomes of distantly related plant species (Jacobsen *et al.*, 1994). This method has been intensively used in several crops to introduce desired traits like cytoplasmic male sterility or herbicide resistance through introduction of mitochondrial and chloroplast DNA. Resistance to biotic factors and tolerance to abiotic

factors, which can be inherited chromosomally, can also be introduced using this technology. Bradshaw and Mackey (1994) have described seedling or tuber progeny tests that provide the means to rapidly and efficiently identify progeny with the greatest likelihood of containing desirable clones. But, large-scale, clonally replicated trials are necessary to select the best clones from the better progenies.

Breeding strategies in potato include introgression from wild species, breeding at the diploid level, and breeding at tetraploid levels (Caligari, 1992). In breeding programs, wild species have been used as donors of some specific traits that are not available in the standard *tuberosum* varieties. For example, *andigena* has been used as a source of resistance to potato blights, scab, root knot and golden nematodes, wart, and viruses as well as processing quality and tolerance to stresses (Plaisted, 1987). In addition, unadapted species have also been used as a source of added genetic variability for all traits. Evaluation of drought sensitivity of potato is conventionally done in the field by assessing the variation in yield due to stress. A linear moisture gradient created by installing line-source sprinkler irrigation is often used for evaluating drought sensitivity of potato in the field. This method depends not only on seasonal weather changes, but also involves considerable space, time, labor, equipment and planting material resources. Therefore, some workers have developed alternative techniques for screening the resistance of potato to drought.

Demagante *et al.* (1995) recommended that a screening method be fast, simple, and repeatable, as well as nondestructive (Bansal *et al.*, 1991). The assessment of the method involves consideration of cost, simplicity, and repeatability in the response to the stress over time. Beekman and Bouma (1986) employed a screening method on the basis

27

of drought recovery using eyepieces grown in pots in the glasshouse. A rapid screening technique for drought resistance in potato (*Solanum tuberosum* L.) has been developed by Bansal *et al.* (1991). Using the growth reduction of leaf discs floated over polyethylene glycol 6000 solution of ψ -0.4 M.Pa compared to that of floated discs over distilled water. This simple rapid and nondestructive technique has proven to be reliable and gives results which are in general agreement with what is known about the drought resistance of genotypes. For example, Desiree, a drought-resistant genotype showed only 18% growth reduction, while Kufri Kunda, a drought-susceptible genotype showed 73% growth reduction. Demagante *et al.* (1995) developed another method for screening drought tolerance in potato by using apical cuttings in raised beds. Demagante *et al.* (1995) used the degree of reduction in plant growth rate of ten genotypes to study whether apical cutting is a reliable technique for screening drought tolerance. Among ten genotypes tested, Berolina I showed the most growth reduction under drought stress and P-7 showed the least. These results are in agreement with the fact that Berolina is a drought-sensitive genotype and P-7, bred from *S tuberosum* x *S. andigena* hybridization, is a drought-tolerant genotype (Demagante *et al.*, 1995). Demagante *et al.* (1995) also reported that there was significant positive association between the field and the bed experiment for total plant, tuber, and root dry matter over all sampling dates across stressed and unstressed treatments. This indicated that apical cutting is reliable enough to use for screening potato to drought stress.

The improvement of potato (*Solanum tubersoum* L) has been largely confined to conventional breeding approaches (Shahin and Simpson, 1986). In this regard, genetic engineering potentially offers an excellent tool for introducing traits, such as disease

resistance, insect resistance, drought and heat tolerance, or high protein value into the already-adapted cultivars (Shahin and Simpson, 1986). It is a promising method for improving drought tolerance in potatoes, as the introduction of foreign genes into a number of plant species has become possible (Stiekema *et al.*, 1988).

Chapter 3

USE OF POLYETHYLENE GLYCOL (PEG) 8000 FOR RAPID SCREENING OF POTATO (*Solanum tuberosum* L) GENOTYPES FOR WATER STRESS TOLERANCE

I. ROOT AND SHOOT GROWTH

Abstract

Attempts to develop drought-tolerant potato (*Solanum tuberosum* L.) cultivars have shown slow progress, partly because conventional breeding programs are painstaking and time consuming. It is of importance to develop rapid screening techniques to shorten the time spent in breeding. The overall goal of this study was to evaluate whether growth reduction of potato genotypes, expressed in root length density reduction (RLDR), root dry weight reduction (RDWR), shoot dry weight reduction (SDWR), leaf area reduction (LAR), and root-to-shoot ratio reduction (RSR) could be used to select potato genotypes for drought tolerance. Twelve potato genotypes grown either *in vitro* or in the greenhouse were exposed to 0% and 8% PEG8000, their growth reductions at 8% PEG8000 were calculated, and then were ranked from the least and the most reduced in growth.

The results showed that Kennebec, known to be drought tolerant in previous studies, showed the least growth reduction in the *in vitro* study as seen in the RLDR (12.4% and 20.9%), RDWR (20.4% and 38.1%), SDWR (11.7% and 47.8%, and RSR (-120.1% and −18.2%). Furthermore, the linear correlations of RLDR ($r^2 = 0.89*$) and RDWR ($r^2 = 0.78*$) results over runs of the experiment were significant, suggesting that the results were consistent. RLDR and RDWR show promise for selecting potato

genotypes grown *in vitro* for drought tolerance. The linear relationship between RLDR and RDWR was also significant ($r^2 = 0.86^*$), suggesting that either one was good for screening.

The results of greenhouse experiments were not conclusive, even though Reichie consistently was at the top rank among genotypes tested and showed the lowest growth reduction in some dependent variables (SDWR in 2002, LAR and RDWR in 2003). As a consequence, the LAR, SDWR, RDWR, and RSR might not be used to screen potato genotypes grown in the greenhouse for drought tolerance.

There was no significant correlation between SDWR ($r^2 = 0.02^{ns}$, RDWR ($r^2 = 0.16^{ns}$), and RSR ($r^2 = 0.06^{ns}$) results over runs of experiments. Kennebec outperformed Reichie in the *in vitro* study, while Reichie outperformed Kennebec in the greenhouse study.

Key words: PEG, water stress, potato, root-to-shoot ratios

3.1. Introduction

Potato (*Solanum tuberosum* L.) is well known to be very sensitive to drought stress (Ekanayake and de Jong, 1992; Vayda, 1994). Part of the reason is due to its poor soil water extraction (Weisz *et al.*, 1994) as a result of the shallow and ineffective rooting system (Fulton, 1970). Most of the roots are confined at the upper 30 cm soil layer (Kleinkopf, 1983; Opena and Porter, 1999). On the other hand, drought-adapted plants are characterized by deep and vigorous root systems. These rooting systems are

associated with extensive rooting depth, high root length density, and low resistance to water flow within the root (Monneveux and Belhassen, 1996).

Plants experience drought by excessive transpiration and/or by a limitation of water supply (Frensch, 1997). Although drought stress reduces plant water potentials (ψ_s), it affects root and leaf growth differently (Frensch, 1997). Many studies have shown that root growth is more resistant to water deficit than shoot growth (Frensch, 1997; Hsiao and Jing, 1987; Hsiao and Xu, 2000; Kramer and Boyer, 1995; Sharp, 2002). Furthermore, drought stress increases both root-to-shoot ratio and root-length-to-root weight ratio (Jefferies, 1993).

It has been documented that drought stress reduces plant growth (Weisz *et al.*, 1994), marketable yield, tuber number per stem, and average tuber yield (Lynch and Tai, 1989), carbohydrate accumulation and partitioning (Ekanayake and de Jong, 1992), the yielding capacity of potato crops, and subsequent performance of the seed tubers (Karafyllidis, 1996). Moreover, drought stress has been reported to reduce gas exchange, decrease the concentration of phosphorylated intermediates, like 3-phosphoglycerate acid (3PGA) (Geigenberger *et al.*, 1997), and inhibit starch synthesis (Geigenberger *et al.*, 1999). Other studies have shown that drought stress increases the incidence of internal tuber defects (Miller and Martin, 1985), increases the percentage of sugar-end tubers (Kincaid *et al.*, 1993), and increases total glycoalkaloid content (Papathanasiou *et al.*, 1999).

So significant is the effect of drought stress on potato growth and yield that the need for genotypes adapted to drought has become urgent (Maldonado *et al.*, 1998; Rajashekar *et al.*, 1995). In fact, there have been major efforts to develop drought-

tolerant cultivars. Through intensive breeding programs, researchers have successfully released some potato cultivars that are drought tolerant. However, conventional breeding techniques are considered to be painstaking and time consuming. It may take 10 to 15 growth cycles from crossing of parental lines to the final release of new cultivars (Caligari, 1992). Therefore, it is of importance to develop rapid screening techniques to shorten the time spent in breeding.

Some rapid methods for screening drought-tolerance traits in potato have been established (Bansal *et al.*, 1991; Demagante *et al.*, 1995; Levy *et al.*, 1991). Canopy temperature and chlorophyll *a* fluorescence have been reported as potential tools for drought screening of potato germplasm (Jefferies, 1992; Ranalli *et al.*, 1997; Stark *et al.*, 1991). Demagante *et al* (1995) employed apical cuttings for screening drought tolerance in raised beds. Bansal *et al.* (1991) established a new screening method by using the growth reduction of leaf discs floated over different concentrations of polyethylene glycol (PEG) 6000 (now PEG8000, Sigma Aldrich, 2001).

In vitro bioassays have been employed to screen potato genotypes for salinity tolerance (Ochatt *et al.*, 1999; Zhang and Donnelly, 1997), to screen *Prunus* tolerance to osmotic stress (Rajashekar *et al.*, 1995), and to select drought-tolerant rice (Biswas *et al.*, 2002). Even though *in vitro* techniques can potentially be used to screen potato genotypes for drought tolerance, no such research has been reported

The purposes of this experiment were to study whether genotypes, PEG8000 concentration, and their interaction affected the growth of potato crops, and to evaluate whether root and shoot growth of potato genotypes grown at low water potentials (ψ_w) can be used as tools for screening potato for drought tolerance. It was expected that

33

drought-tolerant genotypes would demonstrate less growth reduction than the drought-sensitive ones when they were exposed to high concentrations of PEG8000.

3.2. Materials and Methods

Plant materials used in this experiment were obtained from the International Potato Center, CIP, (Chagllina-INIA, E86.011, Reiche, C89.315, Tacna, and Unica) and from Dr. Feridoon Mehdizadegan, the Maine Seed Potato Board (Andover, Superior, Shepody, Kennebec, Katahdin, and Russet Burbank). Based on personal communication with a CIP researcher, the CIP selections were presumed to be drought tolerant. While no information is available for the maturity of CIP genotypes, Andover and Superior are known as early-mid season, Shepody and Kennebec middle season, and Katahdin and Russet Burbank late-season cultivars.

3.2.1. Single Node Cutting Assay

The potato genotypes were exposed to different artificially imposed water potentials by adding 0 or 8% polyethylene glycol (PEG) 8000 to the culture media. Two single-node cuttings, 1-cm long with one leaf and one axillary bud, taken from the medial part of 3-week-old micropropagated plantlets, were cultured in 25mm x 125mm Pyrex glass test tubes, containing 10 ml of potato micropropagation culture media at designated PEG8000 concentrations. The plant materials were previously grown in test tubes containing 10 ml solid media (Zhang and Donnelly, 1997) and subcultured every 8 weeks since they arrived at the University of Maine from either CIP in February 2000 or the Maine Seed Potato Board in fall 1999.

The culture media were prepared by following Zhang and Donnelly (1997) in which a modified MS (Murashige and Skoog, 1962) basal salt solution was supplemented with inositol (100 mg l^{-1}), pyridoxine·HCl (0.5 mg l^{-1}), thiamine·HCl (1.0 mg l^{-1}), niacin (0.5 mg l^{-1}), Ca-pantothenate (2.0 mg l^{-1}), glycine (2.0 mg l^{-1}), 3% sucrose and 0.6% agar. The medium was adjusted to pH 5.7 prior to autoclaving at 121 $^{\circ}$C for 20 minutes.

The cultures were incubated at 25 $^{\circ}$C with 16/8 day/night at 40 μmol m^{-2} s^{-1} photon flux density of cool-white fluorescent light. After six weeks of incubation, the plantlets were harvested and shoot dry weight (SDW), root length density (RLD), and root dry weight (RDW) measured. In addition, root-to-shoot ratio on a dry-weight basis (RS), and the growth reduction compared to the control (0% PEG8000) was calculated. The root length was measured to the nearest mm individually with a ruler. Roots and shoots were dried at 70 $^{\circ}$C for 7 days.

The experiment was conducted twice and arranged in a randomized complete block design (2 factors) with five replications per treatment. The first experiment was carried out from May to July 2001 and the second set from September to November 2002. Data analyses were done using Proc. GLM (SAS Institute, Cary, NC) for analysis of variance, followed by mean separation with Duncan's Multiple Range Test, in addition to linear correlation analysis done with Microsoft Excel (with *r critical* of *0.58; α = 0.05; n = 12*).

3.2.2. Greenhouse Study

Two replicate experiments were carried out in the University of Maine, Roger Clapp greenhouse from late September to December 2002 and from January to April

2003. Nearly sprouting microtubers were grown in 10.2 cm (diameter) plastic pots, one tuber per pot. The tubers were selected for uniformity of size within each replication. The media used for filling the pots was a mix of peat moss, perlite, vermiculite, dolomite, and calcitic limestone (Promix ®). At planting, the media were flushed with 1/8 strength Hoagland's solution containing 0 and 8% of PEG8000. The PEG solutions were prepared according to Michael and Kufmann (1973), except that the PEG8000 was diluted in 1/8 strength Hoagland's solution instead of water.

Plastic pots were wrapped in aluminum foil to minimize water loss via evaporation. The media were watered every three days to maintain soil moisture. Each pot was watered until the solution dripped from the media. To prevent PEG8000 accumulation, the media were flushed with tap water every other week, immediately followed by application of fresh PEG8000 solution.

The day length was set at 14 hours by supplemental halogen lighting. The temperature of the greenhouse was set at 26 °C during the day and 20 °C at night. No pests or diseases were found during the period of the experiments. Twelve weeks after planting, the plants were harvested for root length density (RLD), root dry weight (RDW), leaf area (LA), and shoot dry weight (SDW). The growth reduction compared to the control (0% PEG8000) and root-to-shoot ratios (RS) were calculated. The leaf areas were measured with an Agvision root and leaf analysis system (Decagon Devices Inc.). Roots and shoots were dried at 70 °C for 7 days. Data were analyzed with PROC GLM SAS (SAS Institute, Cary, NC) followed by Duncan's mean separation, as well as linear correlation analysis with Microsoft Excel (with *r critical* of *0.58;* α*= 0.05; n = 12*).

3.3. Results and Discussion

3.3.1. Single Node Cutting Assay

3.3.1.1. Effect of Genotypes.

Root length density (RLD), root dry weight (RDW), shoot dry weight (SDW), and root-to-shoot ratio (RS) were all significantly affected by potato genotypes (Table 3.1) in both years. The RLD values ranged from 1.99 to 4.94 in 2001 and from 0.59 to 2.66 in 2002 (Table 3.1). Five genotypes (E86.011, Andover, Chaglina-INIA, Reichie, and Unica) consistently belonged to the group with high RLD values over the years. There was no consistency regarding which genotype showed the lowest RLD values over years. For example, in 2001 the lowest RLD was found in Shepody, while in 2002 it was found in Kennebec. So far, there has been no published information on the effect of water stress on RLD of potato crops grown *in vitro* to which the author might compare the results of current study. From their field studies, Vos and Greenwold (1986) reported RLD values of 1 and 2 cm cm^{-3} in the uppermost soil layer, and Lesczynski and Tanner (1976) found the typical RLD values of 2 to 6 cm cm^{-3} in the uppermost soil layer. In a more recent field study, Opena and Porter (1999) reported that the RLD values of Superior in the 0-15 soil layer of control treatments were 1.23 and 1.96 cm cm^{-3} in 1993 and 1994. In this experiment, the RLD values of Superior were 3.89 and 1.95 cm cm^{-3} for 2001 and 2002, respectively (Table 3.1). However, one should keep in mind that the growing environment of a test tube with culture media is very different from that of a soil.

37

Table 3.1. The effect of potato genotypes (G), polyethylene glycol (PEG) 8000, and the interaction of G x PEG on root length density (RLD, cm cm^{-3}), RLD reduction (RLDR, %), root dry weight (RDW, mg), RDW reduction (RDWR, %), shoot dry weight (SDW, mg), SDW reduction (SDWR, %), root-to-shoot ratio (RS), and RS reduction (RSR, %) of potatoes grown *in vitro*.

Treatments	2001								2002							
	RLD (cm cm^{-3})	RLDR (%)	RDW (mg)	RDWR (%)	SDW (mg)	SDWR (%)	RS	RSR (%)	RLD (cm cm^{-3})	RLDR (%)	RDW (mg)	RDWR (%)	SDW (mg)	SDWR (%)	RS	RSR (%)
Genotypes (G)																
E86.011	4.94 a	-	10.05 b	-	36.15 bc	-	0.28 ab	-	2.38 a	-	1.27 def	-	6.14 d	-	0.21 c	-
Andover	4.92 a	-	16.82 ab	-	51.24 ab	-	0.33 ab	-	2.66 a	-	3.57 a	-	11.38 b	-	0.31 bc	-
Chaglina-INIA	4.90 a	-	10.27 b	-	37.85 bc	-	0.27 ab	-	2.36 a	-	2.13 bc	-	3.98 ef	-	0.53 ab	-
Russet Burbank	4.82 a	-	4.32 b	-	7.36 c	-	0.58 ab	-	0.75cd	-	0.96 f	-	2.91 fg	-	0.32 bc	-
Reichie	4.73 a	-	29.74 a	-	76.65 a	-	0.39 ab	-	2.51 a	-	2.74 b	-	21.21 a	-	0.13 d	-
Unica	4.23 a	-	27.23 a	-	31.48 bc	-	0.86 a	-	2.49 a	-	1.89 cde	-	5.34 de	-	0.35 bc	-
Superior	3.89 ab	-	13.91 ab	-	72.50 a	-	0.19 b	-	1.95 ab	-	1.57 cdef	-	4.09 ef	-	0.38 b	-
Tacna	3.78 ab	-	7.14 b	-	71.48 a	-	0.09 c	-	1.45 bcd	-	1.68 cdef	-	2.39 fg	-	0.70 a	-
Kennebec	3.69 ab	-	1.34 b	-	11.20 c	-	0.12 b	-	0.59 d	-	0.98 f	-	1.69 g	-	0.58 ab	-
C89.315	3.30 abc	-	6.88 b	-	35.33 bc	-	0.19 b	-	2.52 a	-	1.95 cd	-	3.28 fg	-	0.59 ab	-
Katahdin	2.34 bc	-	1.39 b	-	15.05 c	-	0.09 c	-	0.76 cd	-	1.11 f	-	8.41 c	-	0.13 d	-
Shepody	1.99 c	-	1.66 b	-	13.51 c	-	0.12 b	-	1.59 bc	-	1.21 ef	-	4.00 ef	-	0.30 bc	-
Polyethylene Glycol (PEG)																
0%	7.07	0.0	17.56	0.0	61.87	0.0	0.28	0.0	3.23	0.0	3.03	0.0	9.96	0.0	0.31	0.0
8%	0.86	87.8	4.40	74.9	14.76	76.1	0.29	-3.6	0.42	86.9	0.47	84.5	2.52	74.6	0.19	38.7
Analysis of variances, Pr >F																
Source of variation																
G	**	-	**	-	**	-	**	-	**	-	**	-	**	-	**	-
PEG	**	*	**	**	**	**	NS	NS	**	**	**	**	**	**	**	**
G x PEG	**	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**

Means within the same column followed by the same letter are not significantly different at the 0.05 (*) or 0.01 (**) levels (Duncan's Multiple Range Test, DMRT). NS is not significantly different at the 0.05 level; RLDR, RDWR, SDWR, and RSR apply to effect of 8% PEG8000 compared to the control (0%).

The highest values of root dry weight (RDW) were found in Reichie, Unica, and Andover, and the RLD of these varieties were also among the highest (Table 3.1). Furthermore, Katahdin whose RLD was among the lowest in both years, showed the lowest RDW value, followed by Shepody. These results were consistent with a previous study of Opena and Porter (1999), in which increasing RLD due to soil amendment also increased RDW. However, this kind of consistency was not found in the other genotypes used in this experiment. For example, E86.011 whose RLD values were consistently among the highest for both years, took the sixth and eighth highest place in RDW for year 2001 and year 2002, respectively, suggesting that the roots were long and slender.

Shoot dry weight (SDW) was significantly affected by potato genotypes for both years. It ranged from 7.36 to 76.65 mg in 2001 and from 1.69 to 21.21 mg in 2002, with the highest values was found in Reichie, Superior, Tacna, and Andover in 2001 and Reichie in 2002 (Table 3.1). Considering the main function of the root in *in vitro* culture, which absorbs water, nutrients, and sugars (Kyte, 1999), it is likely that the performance of rooting systems directly contribute to the growth of the plants, as seen in Reichie, Andover, Katahdin, and Russet Burbank.

The root-to-shoot ratio (RS) values of this experiment ranged from 0.09 to 0.86 in 2001 and from 0.13 to 0.70 in year 2002, which were much higher than those reported by Opena and Porter (1999) in their field experiment (0.04 and 0.08 for two consecutive years). The difference in the growing environment might contribute to these differences. In our experiment, potato plants were grown in very rich and soft growth media (Zhang and Donnelly, 1994). In the field experiment, potato root growth may be inhibited by poor soil texture, lack of nutrients, and the presence of physical impedance (Opena and

39

Porter, 1999; Vos Greenwold, 1986). Furthermore, under favorable field conditions, potato crops would allocate more dry weight to the shoot than to the root (and hence low RS values) to maximize the light harvesting capacity of the crops, required for high tuber yield. This was unlikely the case for potato plants grown *in vitro* that did not need to produce their own sugars (Kyte, 1987).

In general, the genotypes performed better in 2001 than in 2002 as shown by the RLD, RDW, and SDW. The reason was not clear. It might have been due to the differences in the physiological age and/or the number of subcultures of plant materials used to start the experiment. Plant materials used in 2002 were at least a year older and had three more subcultures than those used in 2001. A previous study showed that an increase in the number of subcultures of potato plantlets and/or the physiological age of mother tubers significantly reduced plantlet growth (Villafranca *et al.*, 1998).

3.3.1.2. Effect of PEG8000.

As expected, PEG treatments significantly reduced plant growth (Table 3.1) for both years, except for RS in 2001. When applied to growth media, PEG8000 acted to hold water mimicking the effect of water stress (Bansal *et al.*, 1991; Michel and Kaufmann, 1973), which might result in the reduction in nutrient and water absorption by the roots. The results of this experiment confirmed previous studies using PEG for introducing water stress (Steuter *et al.*, 1981; Bansal *et al.*, 1991).

It was expected that water stress would increase the RS values of the crops. However, the results of this experiment indicated that water stress (8% PEG8000) did not significantly increase RS value, even though there was a slight increase in RS for 2001

40

(Table 3.1). In 2002, water stress (8% PEG8000) significantly reduced RS. Perhaps it was due to the increase in the number of subcultures of the plants used to start the experiment as mentioned above (Villafranca *et al*, 1998).

3.3.1.3. Interaction between Genotype and PEG8000.

The effects of PEG8000 on root length density (RLD), root dry weight (RDW), shoot length (SL), shoot dry weight (SDW), and root-to-shoot ratio (RS), were dependent on the potato genotypes (Table 3.1). The ultimate goal of this experiment was to evaluate whether root and shoot growth of potato crops grown *in vitro* could be used as tools for screening potato genotypes for drought-stress tolerance. Therefore, the authors focused the discussion on the growth reduction of the genotypes at 8% PEG8000, instead of the actual growth at that level of PEG. The growth of potato crops at 8% PEG8000 was compared to the control (0% PEG8000) treatments, then the genotypes were ranked from the least (1) to the most (12) reduced to determine their relative degree of drought tolerance (Bansal *et al.*, 1991). It was expected that the more tolerant genotypes would show less growth reduction than the more sensitive ones, as the degree of reduction in growth was considered as an index of drought stress (Demagante *et al.*, 1995).

As seen in Table 3.2, water stress (8% PEG8000) caused a significant reduction in root length density (RLDR), in which Kennebec was the lowest in both years. The RLDR values for Kennebec in this experiment were comparable to that reported by Bansal *et al.* (1991) in their leaf disc experiment, in which Kennebec showed growth reduction of 27% when exposed to −0.4 MPa. In their experiment, Bansal *et al* (1991) also found that the

Table 3.2. Root growth reduction of potato genotypes grown *in vitro* as affected by water stress (8% PEG8000), measured in root length density reduction (RLR, %) and root dry weight reduction (RDWR, %), and the rank of the genotypes from the least (1) to the most (12) reduced.

| Genotypes | 2001 | | | | 2002 | | | |
| | RLDR | | RDWR | | RLDR | | RDWR | |
	(%)	Rank	(%)	Rank	(%)	Rank	(%)	Rank
Kennebec	13.4 a	1	20.4 a	1	20.9 a	1	38.1 a	1
C89.315	79.5 b	2	82.4 b	2	85.9 bc	5	82.7 bcd	5
E86.011	86.5 b	3	91.1 b	6	80.9 bc	4	67.3 bc	3
Reichie	87.1 b	4	89.2 b	5	88.0 bc	7	82.9 bcd	6
Tacna	87.3 b	5	85.5 b	3	86.5 bc	6	88.3 cd	7
Katahdin	90.5 b	6	92.3 b	7	98.6 c	12	98.9 d	12
Andover	92.7 b	7	95.7 b	11	89.6 bc	8	93.6 d	10
Shepody	93.0 b	8	94.5 b	9	78.8 bc	2	88.5 cd	8
Unica	93.1 b	9	86.3 b	4	97.1 c	11	62.6 b	2
Russet Burbank	94.1 b	10	93.6 b	8	79.3 bc	3	98.1 d	11
Superior	94.2 b	11	98.7 b	12	94.9 bc	9	81.2 bc	4
Chaglina-Inia	95.2 b	12	96.7 b	10	95.2 bc	10	90.5 cd	9

Note: mean followed by the different letters were significantly different at 0.05 of Duncan's Multiple Range Test.

hybrid HC294, bred for heat and drought tolerance (Khanna, 1966) and the wild species *S. pheruja*, selected as a parent in breeding cultivars adapted to lowland tropics (Mendoza and Estrada, 1979), showed growth reductions of 31% and 10% respectively, while Kufri Sindhuri, known to be drought sensitive, showed a growth reduction of 55%. This indicated that the method employed by Bansal *et al.* (1991) was valid for screening potato genotypes for drought tolerance, in which Kennebec was grouped with drought-tolerant varieties. In fact, Kennebec has been listed as a drought-tolerant cultivar (Barclay and Scott, 1997). Furthermore, the linear regression analysis showed that there was a significant relationship between RLDR 2001 and RLDR 2002 ($r^2 = 0.88*$), suggesting that the results were repeatable, and hence RLDR may be used to select potato genotypes grown *in vitro* for water-stress-tolerance.

Water stress (8% PEG 8000) also caused severe reduction in root dry weight (RDWR) for some genotypes, but a much smaller reduction for Kennebec (Table 3.2). The RDWR values of Kennebec were 20.4 % (2001) and 38.1% (2002), respectively, which were comparable to the results of Bansal *et al.* (1991). According to the criteria of Demagante *et al* (1995), Kennebec would be the most and the only water-stress-tolerant cultivar tested in this experiment. The linear relationship between RDWR 2001 and RDWR 2002 was significant ($r^2 = 0.79*$), indicating that the results were repeatable. As a consequence, the RDWR of potato genotype grown *in vitro* may be used as a trait to select potato genotypes for drought tolerance. The linear relationship between the average values of RLDR and RDWR was also significant ($r^2 = 0.86*$), suggesting that either one can be used to select potato genotypes grown *in vitro* for water stress. From a practical standpoint, however, the author recommends RDWR over RLDR, especially when

43

dealing with a large number of samples, unless a quick method to measure root length is available, since measuring root length is more time consuming than weighting the roots.

Kennebec also showed the lowest shoot dry weight reduction (SDWR) in 2001 (11.7%), but was not statistically different from Tacna, Shepody, Reichie, and Andover (Table 3.3). The last four genotypes were also statistically equal to the rest of the genotypes. In 2002, the lowest SDWR was found in Superior, even though it was not statistically different from Unica, Kennebec, Russet Burbank, and Shepody (Table 3.3). There was no consistency in the ranking of the genotypes. For example, Superior showing the lowest SDWR value in 2002 and was in the 6th rank in 2001, while Andover had the highest SDWR in 2002 and was the 2nd lowest in 2001. This inconsistency was further confirmed by the result of the linear regression analysis of SDWR, which was not significant over experiments ($r^2 = 0.0003^{ns}$). It was not clear what caused the inconsistency in the ranking of SDWR. Perhaps, it was attributed to the differences in the physiological age between plants used in the experiments, as mentioned in previous section (Villafranca, 1998). Another factor that might also contribute to the inconsistency was the seasonal change, which might affect the room temperature used to incubate the plant materials. The experiment was carried out from May to July in 2001 and from September to November in 2002; and the plant materials were incubated in a room (not in the growth chamber) whose temperature was controlled by an air conditioner. Regardless of the inconsistency, the average SDWR of Kennebec was comparable to the growth reduction of leaf disc reported by Bansal *et al.* (1981), confirming that Kennebec was tolerant to water stress. Furthermore, the linear correlation between RDWR and SDWR

Table 3.3. Shoot dry weight reduction (SDWR, %) and root-to-shoot ratio reduction (RSR, %) of potato genotypes grown *in vitro* under water stress (8% PEG8000) compared to the control treatments (0% PEG 8000), and the ranking of the genotypes from least (1) to most (12) reduced.

Genotypes	2001 SDWR		2001 RSR		2002 SDWR		2002 RSR	
	(%)	Rank	(%)	Rank	(%)	Rank	%	
Kennebec	11.7 a	1	-120.1 a	1	47.8 ab	3	-18.2 a	1
Andover	35.7 ab	2	84.2 b	11	87.9 c	12	37.7 ac	6
Tacna	40.7 ab	3	-4.8 ab	3	68.1 bc	7	60.4 bc	9
Shepody	55.3 ab	4	79.9 b	10	56.8 abc	5	76.1 c	10
Reichie	69.8 ab	5	62.8 b	8	84.1 bc	11	-14.1 a	2
Katahdin	77.9 b	8	50.6 b	7	76.5 bc	9	94.3 c	11
Unica	77.4 b	7	70.6 b	9	46.1 ab	2	24.2 abc	4
Superior	73.9 b	6	95.6 c	12	20.9 a	1	49.8 abc	8
E86.011	80.5 b	9	50.1 b	6	67.5 bc	6	-10.7 ab	3
Russet Burbank	88.3 b	10	28.1 b	5	48.9 ab	4	97.2 c	12
C89.315	91.3 b	11	-49.6 ab	2	70.2 bc	8	34.1 abc	5
Chaglina_INIA	95.1 b	12	2.9 ab	4	77.9 bc	10	39.0 abc	7

Note: mean followed by the different letters were significantly different at 5% of Duncan Multiple Range Test.

45

was significant ($r^2 = 0.50*$), with a low to moderate relationship suggesting that both RDWR and SDWR should be used simultaneously should some one to use them for *in vitro* screening.

When exposed to water stress, Kennebec demonstrated a significant increase in root-to-shoot ratio (RS), shown by the negative values of RS reduction (RSR), -120% in 2001 and -18% in 2002 (Table 3.3.). Along with Kennebec, C89.315, and Tacna in 2001 and Reichie and E86.001 in 2002, also demonstrated an increase in RS when exposed to water stress, in one of the two years. On the other hand, the rest of the genotypes demonstrated a significant reduction in root-to-shoot ratio (RSR), ranging from 2.9% to 95.6% in 2001 and from 24.2% to 97.2% in 2002. The reduction in root-to-shoot (RS) was contradictory to the general knowledge, in that plants tend to increase their RS when exposed to water stress (Struik and Bray, 1970; Jefferies, 1993). In addition to the age factor as previously mentioned, this discrepancy might also be attributed to the differences in the environmental conditions where the plants were grown. One should keep in mind that the tendency of plants to increase the RS by allocating more assimilates to the roots when exposed to water stress is the normal response of plants grown in the field (Struik and Bray, 1970). This enhances their ability to explore deeper soil layers to extract more water (Gardner *et al.*, 1991). In this experiment, plants were grown *in vitro* (very humid) and supplied with sugar, vitamins and minerals (Zhang and Donnelly, 1994). Therefore, the demand for water by the shoots would have been much lower than for field-grown plants and consequently root growth was not proportionately stimulated as it is in field studies.

The ranking of RSR was not consistent over the years and the linear correlation between RSR 2001 and RSR 2002 was not significant ($r^2 = 0.15^{ns}$), suggesting that RSR might not be consistent enough to screen potato genotypes grown *in vitro* for drought tolerance. However, Kennebec known to be drought tolerant in a previous study (Bansal *et al.*, 1981) consistently demonstrated the characteristics of a drought-tolerant variety over the experiments in this study, while Superior known to be responsive to irrigation (Porter *et al.*, 1999) showed the characteristic of a drought-sensitive variety with RSR of 72.7%. This suggested that RSR could be used to evaluate potato genotypes grown *in vitro* for drought tolerance, probably in accordance with other techniques to verify the results. The fact that the CIP genotypes demonstrated the tendency to be in the top of the groups, as seen in C89.315, Tacna, and Chaglina-INIA in 2001 and Reichie, E86.011, and Unica in 2002 (Table 3.3), supported the claim.

3.3.2. Greenhouse Study

3.3.2.1. Effect of Genotypes.

Except for root-to-shoot ratio (RS) in 2003, our results showed that genotypes significantly differed in growth, as measured in root dry weight (RDW), leaf area (LA), shoot dry weight (SDW), and root-to-shoot ratio (RS) (Table 3.4). The RDW values were very similar for both years, ranging from 0.08 to 0.23 g in 2002 with Chaglina-INIA the highest and from 0.08 to 0.29 g in 2003 with Reichie the highest (Table 3.4). On the other hand, Katahdin, along with Andover, Shepody, and Superior consistently showed the lowest RDW values (0.08-0.10 g) for both years, even though they were not

Table 3.4. The effect of potato genotypes (G), 8% polyethylene glycol (PEG) 8000, and the interaction of G x PEG on the root-to-shoot ratio (RS), RS reduction (RSR, %), root dry weight (RDW, g), RDW reduction (RDWR, %), shoot dry weight (SDW, g), SDW reduction (SDWR, %), leaf area (LA, mm^2), and LA reduction (LAR, %) per plant of potatoes grown in the greenhouse.

Treatments	2002								2003							
	RS	RSR (%)	RDW (mg)	RDWR (%)	SDW (mg)	SDWR (%)	LA (mm^2)	LAR (%)	RS	RSR (%)	RDW (mg)	RDWR (%)	SDW (mg)	SDWR (%)	LA (mm^2)	LAR (%)
Genotypes (G)																
Chaglina-INIA	0.43 a	-	0.23 a	-	0.54 bcd	-	136.43 bc	-	0.32 a	-	0.16 b	-	0.50 b	-	77.71 bcdef	-
E86.011	0.35 ab	-	0.16 bcd	-	0.46 cde	-	106.21 cd	-	0.38 a	-	0.16 b	-	0.42 bcd	-	82.18 bcde	-
Andover	0.32 b	-	0.10 de	-	0.31 de	-	78.72 d	-	0.29 a	-	0.09 bc	-	0.31 de	-	58.27 def	-
Unica	0.31 b	-	0.22 ab	-	0.72 ab	-	94.82 cd	-	0.25 a	-	0.13 bc	-	0.53 b	-	99.46 b	-
Superior	0.31 b	-	0.11 de	-	0.36 cde	-	73.17 d	-	0.25 a	-	0.09 bc	-	0.36 cde	-	70.45 def	-
Russet Burbank	0.31 b	-	0.15 cde	-	0.48 bcde	-	83.69 d	-	0.26 a	-	0.12 bc	-	0.45 bcd	-	85.10 bcd	-
Katahdin	0.28 b	-	0.08 e	-	0.29 e	-	74.17 d	-	0.33 a	-	0.08 c	-	0.24 e	-	50.05 f	-
C89.315	0.26 b	-	0.14 cde	-	0.54 bcd	-	158.07 bc	-	0.23 a	-	0.11 bc	-	0.47 bc	-	88.83 bc	-
Tacna	0.23 b	-	0.13 de	-	0.56 bc	-	157.23 bc	-	0.33 a	-	0.15 bc	-	0.45 bcd	-	78.78 bcde	-
Kennebec	0.23 b	-	0.11 de	-	0.47 bcde	-	95.54 cd	-	0.38 a	-	0.12 bc	-	0.32 de	-	54.42 ef	-
Reichie	0.22 b	-	0.19 abc	-	0.86 a	-	202.89 a	-	0.40 a	-	0.29 a	-	0.72 a	-	151.32 a	-
Shepody	0.19 b	-	0.09 e	-	0.47 bcde	-	109.49 cd	-	0.30 a	-	0.10 bc	-	0.33 de	-	58.62 def	-
Polyethylene glycol (PEG) 8000																
0%	0.31	0.0	0.21	0.0	0.76	0.0	143.25	0.0	0.32	0.0	0.21	0.0	0.67	0.0	122.46	0.0
8%	0.32	-3.2	0.07	66.7	0.25	67.1	85.15	40.5	0.37	-15.6	0.06	71.4	0.19	71.6	36.76	69.9
Analysis of variance, Pr>F																
Source of variation																
G	**	-	**	-	**	-	**	-	NS	-	**	-	**	-	**	-
PEG	NS	NS	**	*	**	**	**	**	NS	*	**	**	**	**	**	**
G x PEG	NS	NS	**	NS	NS	**	**	**	NS	NS	**	NS	**	**	**	**

Means within the same column followed by the same letter are not significantly different at the 0.05 (*) or 0.01 (**) levels (Duncan's Multiple Range Test). NS is not significantly different at the 0.05 level. RLDR, RDWR, SDWR, and LAR apply to effect of 8% PEG8000 compared to the control (0%).

statistically different from most other genotypes (Table 3.4). The fact that Katahdin showed similar RDW values to Andover and Superior was interesting, since the former is known as a late-maturing variety while the latter are early-maturing varieties (Barclay and Scott, 1997). Genotype maturity might contribute to the low RDW values in Andover and Superior, as suggested by Opena and Porter (1999) from their field studies for Superior. On the other hand, it might not be the case for Katahdin, expected to have higher RDW values as a late-maturing crop. Furthermore, the author could not relate Chaglina-INIA root growth to genotype maturity since no information on its maturity was available.

The significant linear correlation between RDW and LA ($r^2 = 0.68*$) might explain why Andover, Katahdin, and Superior showed low RDW while Chagllina-INIA and Reichie demonstrated the opposite. As seen in Table 3.4, the highest LA was found in Reichie (202.89 mm^2 in 2002 and 151.31 mm^2 in 2003) and the lowest LA values were found in Andover, Katahdin, and Superior even though some genotypes were not statistically different. These experiments were carried out in the greenhouse; therefore, the plants had to be photosynthetically active to support themselves. Leaves play important role in photosynthesis because they affect light interception and gas exchange of the crops (Gardner et al., 1991; Taiz and Zeiger, 2001). Plants with high LA harvest more light and absorb more CO_2 than those with low LA (Gardner et al., 1991), and hence produce more assimilates and show more growth, including root proliferation, provided that other growth factors are available. In return, a large rooting system will promote plant growth due to an increase in plant ability to absorb nutrients and water

(Steudle, 2000). This might explain why genotypes with high LA values produced more RDW while those with low LA values produce less RDW.

The observed SDW values corresponded to the RDW and LA values, in which genotypes with high LA and RDW showed high SDW while those with low LA and SDW demonstrated low SDW (Table 3.4). These results came out as expected, considering the role of leaves and roots in plant growth (Gardner *et al.*, 1995; Steudle, 2000; Taiz and Zeiger, 2001), and corresponded with the results of regression analysis between RDW and SDW ($r^2 = 0.83*$).

As seen in Table 3.4, the analysis of variance (ANOVA) showed that genotypes significantly affected root-to-shoot ratio (RS) only in year 2002, Chaligna-INIA was the highest, followed closely by E86.011 which was not statistically different from the rest of the genotypes (Table 3.4). Even though genotypes did not significantly affect RS in 2003, the observed RS values in both years were very much in the same range, from 0.19 to 0.43 in 2002 and from 0.23 to 0.40 in 2003. These RS values were about ten times higher than those reported by (Opena and Porter, 1999) for Superior, and comparable to those reported by Jefferies (1993). Perhaps, it was due to the differences in the growth media, the microenvironment where the crops were grown, and the seeds used for the experiment. In the current study, potatoes grown in 10-cm plastic pots from microtuber seeds, produced small plants with a single stem and that was usually without branches, because of this the ratios of root-to-shoot were relatively high. On the other hand, other studies were most likely carried out in the field using normal tubers, which produced very vigorous plants with many stems and branches, and a reduced root-to-shoot ratio.

3.3.2.2. Effect of PEG8000.

As expected, water stress (8% PEG8000) significantly affected the growth of the potato crop (Table 3.4). Water stress reduced root dry weight (RDW) up to 73%, shoot dry weight (SDW) nearly 77 and 80%, and leaf area (LA) about 37 to 60%. The root-to-shoot ratio (RS) was not affected by drought treatment in either year (Table 3.5), confirming the finding of Jefferies (1993). Furthermore, in field experiments, Opena and Porter (1999) reported that irrigation did not significantly affect RS.

3.3.2.3. Interaction between Genotype and PEG8000.

The interaction between genotypes and water stress (8% PEG8000) was significant for RDW, SDWR, LA, and LAR in both years and for SDW in 2003 (Table 3.4). When exposed to water stress, Reichie demonstrated the highest LA over the years, although it was statistically equal to Chagllina-INIA in 2002 (Table 3.5). However, Reichie showed substantial leaf growth reduction (LAR) in both years even though it had the lowest LAR in 2003 (Table 3.5). In 2002, the lowest LAR was found in Shepody (1.8%), Unica (2.8%), Chagllina-INIA (11.1%) and Katahdin (13.8%), whose LAR in 2003 was 65.5, 76.1, 54.9%, and 84.6%, respectively (Table 3.5).

The evidence to determine whether Shepody, Unica, and Katahdin were drought tolerant or drought sensitive was also inconclusive. According to the criteria of Demangate *et al.* (1995), in which a genotype showing the least growth reduction is considered to be the most drought-tolerant genotype, Shepody, Unica, and Katahdin should be considered as the most drought-tolerant genotypes in 2002. However, based on their LAR values in 2003, these genotypes could not be grouped as drought-tolerant

51

genotypes. On the other hand, Reichie consistently showed substantial LAR over the years and the LAR values were statistically equal to those found in Kennebec (48.1% and 70.1% for 2002 and 2003, respectively). Although shown to be drought tolerant in a previous study (Bansal *et al.*, 1991) and listed as a drought-tolerant cultivar (Barclay and Scott, 1997), Kennebec did not appear to be a drought-tolerant genotype in this study. This raised a question whether LAR was a valid trait to screen potato genotypes grown in the greenhouse for drought tolerance. Even though, the correlation between LA 2002 and LA 2003 was significant ($r^2 = 0.57*$), the linear relationship between LAR 2002 and LAR 2003 was not significant ($r^2 = 0.0005^{ns}$), suggesting that LAR might not be reliable for screening. We were not sure what might cause this inconsistency. Perhaps, there were other factors than PEG solutions, such as the seasonal changes, that might affect the greenhouse microclimate and contribute to this inconsistency.

In general, the observed LA values in 2002 were higher than those in 2003, and the LAR values in 2002 were lower than those in 2003 (Table 3.5). The differences were even more significant in the case of Shepody, Unica, and Katahdin. It was not clear what caused the differences. Perhaps, it was attributed to the seasonal changes (from fall to winter), which might affect the amount of solar radiation available for the crops and the temperature of the greenhouse. The experiments were carried out from September to December, 2002, and from January to April, 2003. The 2002 plants were believed to receive more solar radiation than the 2003 crops, especially during their early stage of growth, which might affect the production of assimilate and its investment to the shoots. The seasonal changes might also affect the temperature of the greenhouse, due to the heating system during the winter. Unlike the 2002 crops exposed to the heated

Table 3.5. Shoot growth reduction (%) of potato genotypes as affected by water stress (8% PEG8000), measured in leaf area (LA), leaf area reduction (LAR), shoot dry weight (SDW), and shoot dry weight reduction (SDWR), and the rank of the genotypes from the least (1) to most (12) reduced in growth. Data were obtained from the greenhouse experiment.

Genotypes	2002			2003			2002			2003		
	LA (mm^2)	LAR (%) (%)	Rank	LA (mm^2)	LAR (%)	Rank	SDW (g)	SDWR (%)	Rank	SDW (g)	SDWR (%)	Rank
Reichie	143.71a	41.4 bcd	6	109.06 a	44.9 a	1	0.60 a	44.7 a	1	0.37 a	65.3 ab	3
Chaglina-INIA	125.89ab	11.1 ab	3	40.18 bc	54.9 ab	2	0.26 bc	64.9 abc	5	0.22 bc	70.1 abc	6
Shepody	103.06bcd	1.8 a	1	28.18 bc	65.5 abc	4	0.25 bc	63.1 abc	4	0.15 bcd	68.1 abc	4
Tacna	92.09 bcd	50.7 cd	11	44.42 b	57.5 abcd	3	0.35 b	53.0 ab	2	0.26 b	54.8 a	1
Unica	91.79 bcd	2.8 a	2	38.24 bc	76.0 bcd	9	0.23 bc	78.5 c	11	0.20 bc	72.1 bc	7
C89.315	89.31bcde	55.6 d	12	27.33 bc	83.5 cd	11	0.24 bc	70.3 bc	8	0.18 bcd	78.2 bc	11
E86.011	73.50 cde	48.0 bcd	10	40.36 bc	66.5 abcd	5	0.23 bc	65.4 bc	6	0.19 bc	69.8 abc	5
Katahdin	67.90 cde	13.8 abc	4	12.03 c	84.6 d	12	0.12 c	73.1 bc	9	0.06 d	79.6 bc	12
Kennebec	64.44 cde	48.0 bcd	9	23.61 bc	70.1 abcd	6	0.23 bc	68.6 bc	7	0.16 bcd	64.7 ab	2
Russet Burbank	60.73 de	40.2 bcd	5	25.76 bc	81.3 cd	10	0.27 bc	58.2 abc	3	0.15 bcd	74.2 bc	9
Andover	57.41 de	41.4 bcd	7	24.17 bc	74.7 bcd	7	0.11 c	79.9 c	12	0.13 cd	75.8 bc	10
Superior	51.94 e	44.3 bcd	8	28.18 bc	75.8 bcd	8	0.13 c	77.4 c	10	0.15 bcd	72.3 bc	8

Means within the same column followed by the same letter are not significantly different, at 5% of Duncan's Multiple Range Test. Growth reduction was calculated by the formula $Y = \{(p-q)/p\} \times 100\%$, where Y is growth reduction, p as growth at 0% PEG800, and q was growth at 8% PEG8000.

greenhouse only in the end of growing season, the 2003 crops were exposed to the heated greenhouse for the whole growing season. This might amplify the effect of water stress on the plant growth, especially for Shepody, Unica, and Katahdin. For future study, we recommend the use of growth chambers to avoid the effect of uncontrolled environmental factors.

The observed SDW values under water stress (8% PEG8000) indicated that Reichie also consistently demonstrated the highest SDW for both years (0.60 g in 2002 and 0.37 g in 2003), while Katahdin, Andover, and Superior were consistently at the bottom of the groups (Table 3.5). This consistency was also reflected in the significant linear relationship between SDW 2002 and SDW 2003 ($r^2 = 0.84*$). It appeared that the high SDW value in Reichie was closely related to its high LA values ($r^2 = 0.85*$). The results corresponded with the common knowledge that to a certain degree, greater LA leads to greater growth (Gardner *et al.*, 1991; Salisbury and Ross, 1992).

The effect of interaction between PEG and genotypes on shoot dry weight reduction (SDWR) was significant for both years (Table 3.4). The lowest SDWR at 8% PEG8000 was Reichie (in 2002) and Tacna (in 2003), while the highest was Andover (in 2002) and Katahdin (in 2003) (Table 3.5). There was a dramatic change in the ranking over the years, as reflected in the significant but weak linear correlation analysis ($r^2 = 0.38*$). Regardless of the change in the ranking, Kennebec showed consistent SDWR values over the years (68.6% in 2002 and 64.7% in 2003). However, these values did not indicate that Kennebec was a drought-tolerant genotype, which contradicted the report of Bansal *et al.* (1981). In addition, even though the data suggested that Reichie and Tacna were the most water-stress tolerant among the genotypes tested, their growth reductions

were relatively high, up to 50% in average (Table 3.5), indicating that these genotypes were not water-stress tolerant. As a consequence, SDWR might not be used to screen potato genotype grown in the greenhouse for drought tolerance.

The interaction between PEG and genotypes was significant on RDW in both years (Table 3.4). When exposed to water stress (8% PEG8000), Reichie showed the highest RDW in both years, while Andover consistently showed the lowest RDW even though it was not statistically different from some of the genotypes (Table 3.6). The RDW values were closely related to their LA values, as seen in their significant linear correlation between the average LA and SDW values over years $(r^2 = 0.86*)$.

There was no interaction between PEG and genotypes on root dry weight reduction (RDWR) in either year (Table 3.4). However, Reichie and Kennebec were consistently in the top of the rank when exposed to water stress (8% PEG8000) (Table 3.6). RDWR was not consistent over experiments, as shown by the low r^2 for the linear correlation analysis $(r^2 = 0.33^{ns})$. This raised a question concerning whether RDWR was a valid trait to screen potato genotypes grown in the greenhouse for drought tolerance.

Both root-to-shoot ratio (RS) and root-to-shoot ratio reduction (RSR) were not affected by the interaction between PEG and genotype (Table 3.4). However, the results showed that water stress (8% PEG8000) increased the RS values of most genotypes tested, indicated by the negative values of RSR (Table 3.6). Moreover, the linear relationship between RSR over experiments was not significant $(r^2 = 0.15^{ns})$, suggesting that the order of the ranking over experiments was not consistent and that RSR might not be useful to select potato genotypes grown in the greenhouse for drought tolerance.

Table 3.6. Plant growth reduction measured in root dry weight (RDW, g), and root dry weight reduction (RDWR, %), root-to-shoot ratio (RS), and root-to-shoot ratio reduction (RSR, %) of potato crops grown in greenhouse pots. Growth reduction was ranked from the least (1) to the most (12).

Genotypes	2002			2003			2002			2003		
	RDW (g)	RDWR (%)	Rank	RDW (g)	RDWR (%)	Rank	RS	RSR (%)	Rank	RS	RSR (%)	Rank
Reichie	0.15 a	39.9 a	1	0.16 a	58.6 abc	2	0.28 ab	-6.4 a	9	0.43 a	-20.6 ab	7
Chaglina-INIA	0.12 ab	65.1 ab	8	0.07 bcd	65.6 abc	3	0.49 a	-417.0 a	8	0.40 a	-74.4 ab	2
Shepody	0.05 cd	62.0 ab	7	0.05 bcde	67.6 abc	5	0.20 b	+5.7 a	10	0.32 a	-7.3 ab	8
Tacna	0.09 bc	40.4 a	2	0.07 bc	65.9 abc	4	0.26 a	-39.6 a	6	0.29 a	+14.1 b	12
Unica	0.08 bc	74.5ab	11	0.07 bcd	72.1 bc	7	0.37 ab	-51.8 a	3	0.41 a	-162.7a	1
C89.315	0.07 bcd	58.7 ab	4	0.05 bcde	72.8 abc	8	0.29 ab	-56.7 a	1	0.34 a	-28.4 ab	6
E86.011	0.08 bc	61.2 ab	5	0.09 b	69.8 abc	6	0.37 ab	-46.5 a	4	0.45 a	-47.8 ab	5
Katahdin	0.05 cd	61.5 ab	6	0.03 de	78.7 abc	10	0.40 ab	-45.8 a	5	0.48 a	-59.9 ab	3
Kennebec	0.07 cd	53.5 ab	3	0.08 bc	49.0 a	1	0.30 ab	-54.3 a	2	0.48 a	-53.3 ab	4
Russet Burbank	0.06 cd	71.1 ab	9	0.02 e	83.7 c	12	0.26 ab	+26.1 a	12	0.26 a	+1.8 b	10
Andover	0.02 d	85.4 b	12	0.02 e	81.1 bc	11	0.20 b	+5.7 a	11	0.29 a	-1.2 b	9
Superior	0.05 cd	72.8 ab	10	0.04 cde	75.7 abc	9	0.35 ab	-24.1 a	7	0.25 a	+6.6 b	11

Means within the same column followed by the same letter are not significantly different, at 5% of Duncan's Multiple Range Test. Growth reduction was calculated by the formula $Y = \{(p-q)/p\} \times 100\%$, where Y is growth reduction, p as growth at 0% PEG800, and q was growth at 8% PEG8000.

3.3.3. Relationship between Single Node Cutting Assay and Greenhouse Study

There were no close significant linear relationships between RDWR in the greenhouse study and RDWR of the single node cutting assay ($r^2 = 0.16^{ns}$). Similarly, the results of the greenhouse and single node cutting assay were not significant for SDWR ($r^2 = 0.02^{ns}$) or RSR ($r^2 = 0.06^{ns}$). Even though it did not always demonstrate the lowest growth reduction, Reichie was consistently in the top ranking among the genotypes tested in 2003 (Table 3.5 and Table 3.6) and outperformed of Kennebec in the greenhouse study. In the *in vitro* study, Kennebec consistently demonstrated the lowest growth reduction for any dependent variables measured, except for SDWR in 2002 (Table 3.2 and Table 3.3). Based on the *in vitro* the growth reductions, Kennebec (13.4 to 47%) were considered to be a drought-tolerant variety, while Reichie (62.8 to 89.2%) was a drought-sensitive genotype (Table 3.5 and Table 3.6). However, based on the greenhouse study, Reichie would be classed as drought tolerant.

The above evidence suggested that Kennebec and Reichie perform differently in different studies. There were at least two factors that might contribute to these differences. First of all, it might be due to the differences in the plant materials used to start the experiments. In the greenhouse study, the plants were grown from microtuber seeds produced by *in vitro* culture, while in the *in vitro* study the plants were grown from single-node cuttings. The author suspected that the size of the microtubers used in the study might affect the results. Even though the microtubers were relatively uniform within the genotypes, it was not always the case for different genotypes, especially for Reichie. Reichie produced very few microtubers, because of which there were not many

to select. Secondly, it might be due to the differences in the environments to which the plants were exposed. Plants grown in the greenhouse were exposed to changes in relative humidity (RH), temperature, and solar radiation, which might amplify the effect of water stress, while plants grown in the test tubes were exposed to relatively constant RH, temperature, and artificial radiation. Finally, it might be attributed to the differences in the media where the plants were grown, as described in the methods. It was not clear which factor was more dominant than the others. It is recommended to conduct the study in the growth chamber to minimize the effect of environmental factors.

3.4. Summary

The results showed that genotypes significantly differed in growth *in vitro*. Likewise, PEG8000 also significantly affected the growth of potato genotypes, except for RS and RSR in 2001. The effects of PEG8000 on the plant growth were dependent on the genotypes. When exposed to water stress (8% PEG8000), Kennebec consistently demonstrated the lowest reduction in growth over the years, as measured in RLDR, RDWR, SDWR, and RSR, because of which it was considered to be the most drought-tolerant genotype. Furthermore, the evidence indicated that RLDR and RDWR could be used to select potato genotypes grown *in vitro* for drought tolerance, while SDWR and RSR might or might not be used to select potato genotype grown *in vitro* for drought tolerance because of their inconsistency.

The greenhouse experiments showed that genotypes significantly differed in growth, except for RS in 2003. PEG8000 also significantly affected the growth of potato crops, except for RS and RSR in 2002 and RS in 2003. Interactions between PEG8000

and genotypes were significant for RDW, SDWR, LA, and LAR in both years and SDW in 2003. Based on the growth reduction at 8% PEG8000 and the inconsistency of the results (reflected in the r^2 values of the linear correlation analysis), it was hard to conclude whether LAR, RDWR, SDWR, and RSR could be used to select potato genotypes grown in the greenhouse for drought tolerance. Furthermore, the linear correlations between the RDWR, SDWR, and RSR results of *in vitro* study versus the greenhouse study were not significant, so the results were not consistent across experimental systems. Kennebec performed better than Reichie in the *in vitro* study, while Reichie showed best growth under water stress in the greenhouse study.

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Chapter 4

USE OF POLYETHYLENE GLYCOL (PEG) 8000 FOR RAPID SCREENING OF POTATO (*Solanum tuberosum* L) GENOTYPES FOR WATER STRESS TOLERANCE. II. TUBERIZATION

Abstract

Drought stress reduces plant growth, marketable yield, tuber number per stem, average tuber yield, and subsequent performance of the seed tubers. Because of these effects, the objective of several breeding programs is to obtain drought-tolerant cultivars. The objectives of these studies were to evaluate whether *in vitro* and greenhouse tuber production could be used as tools to select potato genotypes for drought tolerance. PEG8000 was used to impose water stress on twelve potato genotypes. Genotypes, PEG8000, and their interaction significantly affected potato tuberization in both the *in vitro* and greenhouse studies. Results from the *in vitro* study indicated that Kennebec and Katahdin fit into the category of drought-tolerant cultivars. The linear correlations over experiments were significant for tuber number ($r^2 = 0.68*$) and tuber number reduction ($r^2 = 0.59*$). The results also suggested that the *in vitro* technique is a promising method to select potato genotypes for drought tolerance. On the other hand, improvements are needed if the greenhouse method is to be used to select potato genotypes for drought tolerance.

Key words: *PEG, water stress, potato, microtubers*

4.1. Introduction

Potato (*Solanum tuberosum* L.) is well known to be very sensitive to drought stress (Ekanayake and de Jong, 1992; Vayda, 1994), due to its poor soil water extraction (Weisz *et al.*, 1994), which is a result of the shallow and ineffective root system (Fulton, 1970). While drought-adapted plants are characterized by deep and vigorous root systems, most of the potato root system is confined in the upper 30-cm soil layer (Kleinkopf, 1983; Opena and Porter, 1999).

Drought stress reduces plant growth (Harris, 1978; Weisz *et al.*, 1994), marketable yield, tuber number per stem, average tuber yield (Lynch and Tai, 1989), carbohydrate accumulation and partitioning (Ekanayake and de Jong, 1992), and subsequent performance of the seed tubers (Karafyllidis, 1996). Drought stress has also been reported to reduce gas exchange, decrease the concentration of phosphorylated intermediates (Geigenberger *et al.*, 1997), and inhibit starch synthesis (Geigenberger *et al.*, 1999). Other studies show that drought stress increases the incidence of internal tuber defects (Miller and Martin, 1985), the percentage of sugar-end tubers (Kincaid *et al.*, 1993), and total glycoalkaloid content (Papathanasiou *et al.*, 1999).

The effect of drought stress on potato tuberization depends on the physiological stage at which crops are exposed to water stress (Harris, 1978). Drought stress during the stolonization stage is crucial. It reduces the number of tubers and total yield (Harris, 1978), due to a decrease in the number of stolons formed (Haverkort *et al.*, 1990). In long-term field studies, early drought stress significantly reduced tuber number, up to 50% (Ewing and Struik, 1992). Likewise, pot experiments demonstrated that drought stress during tuber initiation significantly reduced tuber number per stem while similar

64

stress after tuber initiation did not affect tuber number per stem (Ewing and Struik, 1992; MacKerron and Jefferies, 1986). Demagante *et al.* (1995) working on apical cuttings grown in raised beds reported that drought sensitivity based on % reduction in tuber and total dry matter at maturity was similar to the results found in the field at maturity. So far, there is no such information published from *in vitro* studies.

There have been major efforts to develop drought-tolerant cultivars via conventional breeding programs, which are considered to be painstaking and time consuming (Caligari, 1992). *In vitro* techniques, on the other hand, offer an effective alternative to conventional plant breeding programs (Fernanda *et al.*, 1997). *In vitro* bioassays have been employed to screen potato genotypes for salinity tolerance (Ochatt *et al.*, 1999; Zhang and Donnelly, 1997), to screen tolerance of *Prunus* to osmotic stress (Rajashekar *et al.*, 1995), and to select drought-tolerant rice (Biswas *et al.*, 2002). Even though *in vitro* techniques can potentially be used to screen potato genotypes for drought tolerance, no such research has been reported. Therefore, it is of interest to explore whether *in vitro* techniques could be incorporated into breeding programs.

Zhang and Donnelly (1998) have used *in vitro* techniques to select potatoes for salt tolerance at the microtuber level. Gopal and Minocha (1997) found that selection at the microtuber level is highly effective for some potato traits, like stem habit, plant height, tuber color, and general impression. Selection at the microtuber level overcomes the existing problem of low efficiency of selection in early generations (Gopal and Minocha, 1997). Furthermore, Gopal and Minocha (1997) have also outlined how breeders may use selection at the microtuber level in breeding programs. In these studies, water stress was induced by applying polyethylene glycol (PEG) 8000.

65

The purpose of this experiment was to study whether genotypes, PEG8000, and their interaction affected the tuberization of potato crops grown *in vitro* and in the greenhouse. The ultimate goal of the study was to evaluate whether selection at the microtuber level could be used to select potato genotypes for drought tolerance.

4.2. Materials and Methods

The plant materials used in these experiments were six potato genotypes, obtained from the International Potato Center (CIP) (Chagllina-INIA, E86.011, Reiche, C89.315, Tacna, and Unica) and six others from Dr. Feridoon Mehdizadegan, the Maine Seed Potato Board (Andover, Superior, Shepody, Kennebec, Katahdin, and Russet Burbank). Based on personal communication with a CIP researcher, the CIP selections were presumed to be drought tolerant. While no information is available for the maturity of CIP genotypes, Andover and Superior are known as early-mid season, Shepody and Kennebec as middle season, and Katahdin and Russet Burbank as late-maturing cultivars. While Kennebec has been reported to be relatively drought tolerant by Bansal *et al.* (1991), Russet Burbank has been reported to be a water-stress-sensitive cultivar (Shock *et al.*, 1993).

Both the *in vitro* and greenhouse study were carried out in randomized complete block designs (RCBD) arranged in factorial with two factors (potato genotypes and PEG8000 concentrations) and five replications. Each experiment was conducted twice.

4.2.1. *In Vitro* Study

The *in vitro* study was done at the University of Maine tissue culture lab from January to August 2001 and from January to October 2002. The experiment was conducted by modifying the method used by Zhang and Donnelly (1997) and Leclerc *et al* (1994), consisting of two major steps, which were micropropagation and microtuberization. The potato genotypes were micropropagated aseptically for four weeks, under 25 °C with 16/8 day/night at 40 μmol m^{-2} s^{-1} photon flux density of cool white fluorescent light. The plant materials were previously grown in test tubes containing 10 ml solid media (Zhang and Donnelly, 1997) and subcultured every 8 weeks since they arrived at the University of Maine from either CIP in February 2000 or the Maine Seed Potato Board in fall 1999.

The culture media were prepared by following Zhang and Donnelly (1997) in which a modified MS (Murashige and Skoog, 1962) basal salt solution was supplemented with inositol (100 mg l^{-1}), pyridoxine·HCl (0.5 mg l^{-1}), thiamine·HCl (1.0 mg l^{-1}), niacin (0.5 mg l^{-1}), Ca-pantothenate (2.0 mg l^{-1}), glycine (2.0 mg l^{-1}), 3% sucrose and 0.6% agar. The medium was adjusted to pH 5.7 prior to autoclaving at 121 °C for 20 minutes.

Microtuberization was conducted in a 2-step procedure. In step 1, 3 plantlets, with root and apex severed, trimmed to 5 nodes each, were layered in 50 ml of liquid potato micropropagation medium containing 6-benzylaminopurine (0.5 mg l^{-1}), GA (0.4 mg l^{-1}), and with sucrose reduced to 2%, in each GA-7 tissue culture container. Plantlets were incubated for 4 weeks at 25 ± 2 °C and 16/8 h D/N cycle (16 hours of day and 8 hours of night).

In step 2, the plantlets were transplanted to 50 ml solid media in GA-7 tissue culture containers, without growth regulators, with sucrose increased to 8%, and with 0 or 8% PEG8000. Plantlets were incubated at 15 ± 1 °C with 8/16 h D/N cycle for another 8 weeks.

Data collected included total tuber number (TN), total tuber dry weight (TDW), average tuber dry weight (ADW), and their growth reduction compared to the control treatments (0% PEG8000). Dry weight was obtained by drying plant materials at 70 °C for 7 days. Data were subjected to analysis of variance followed by mean separation with Duncan's Multiple Range Test using PROC GLM (SAS Institute, Cary, NC), in addition to linear correlation analysis with Microsoft Excel (*r critical* of *0.58; α= 0.05; n = 12*).

4.2.2. Greenhouse Study

Two experiments were carried out in the University of Maine, Roger Clapp greenhouse from late September to December 2002 and from January to April 2003. Nearly sprouting microtubers were grown in 10-cm plastic pots, containing a mix of peat moss, vermiculite, perlite, calcitic limestone, and dolomite (Promix®). One microtuber was planted per pot. The microtubers were selected for uniformity in each block.

The media were flushed at planting with 1/8 strength Hoagland's solution containing 0 or 8% PEG8000. The PEG8000 solutions were prepared according to Michael and Kufmann (1973), except that PEG8000 was diluted in 1/8 strength Hoagland's solution. In 0% PEG treatments, the plants were watered with 1/8 strength Hoagland's solution. Plastic pots were wrapped in aluminum foil to minimize water loss via evaporation. The media were watered with Hoagland's solution with or without PEG

8000 every three days to maintain soil moisture. Each pot was watered until the solution dripped from the media. To prevent PEG8000 accumulation in the PEG-treated pots, the media was flushed with tap water every other week, immediately followed by application of fresh PEG8000 solution. Additional artificial light (Halogen lamp at 100 μmol m^{-1} s^{-1} PPFD) was installed and set at 14 hours. The temperature of the greenhouse was set to 26 °C during the day and 20 °C during the night. No pests and diseases were found during the period of the experiments. Twelve weeks after planting, the plants were harvested and tuber number (TN), total tuber dry weight (TDW), average tuber dry weight (ADW), and their relative reduction compared to the control treatment (0% PEG8000) determined. Dry weight was obtained by drying plant materials at 70 °C for 7 days. Data were subjected to analysis of variance followed by mean separation with Duncan's Multiple Range Test using PROC GLM (SAS Institute, Cary, NC), in addition to linear correlation analysis with Microsoft Excel (*r critical = 0.58; α= 0.05; n = 12*).

4.3. Results and Discussion

4.3.1. Effect of Genotypes.

In vitro study. Our results showed that tuber number (TN), total tuber dry weight (TDW), and average tuber weight (ADW) were significantly affected by potato genotypes, in both years (Table 4.1). The highest TN was found in Kennebec, Andover, and Russet Burbank for 2001. Kennebec and Russet Burbank demonstrated consistent results in the following year (Table 4.1). Reichie, on the other hand, showed poor tuberization, producing only 2.4 tubers in 2001 and 3.6 tubers in 2002. Furthermore,

69

some genotypes produced more tubers in 2001 than in 2002, while the others produced fewer tubers in 2001 than in 2002 (Table 4.1). It was not clear what caused this inconsistency. It might be due to the differences in the number of subcultures of the plant sources between the years. In this study, the 2002 plants were subcultured at least four more times than the 2001 plants. Villafranca *et al.* (1998) reported that the capacity of Kennebec explants to produce microtubers decreased with increased subculturing. The results indicated that genotypes producing more TN also generally demonstrated higher TDW than those producing fewer TN (Table 4.1). In this regard, the results of this experiment confirmed previous studies done by Deblonde and Ledent (2001). Exceptions were seen in Tacna, Shepody, and Unica, whose TNs were much fewer than those of Kennebec, and yet their TDWs were comparable to that of Kennebec. Considering the ADW values of those genotypes, it was likely that the fewer TN were compensated for by higher average dry weight (ADW). The end result was the same values of TDWs, as reported previously by Deblonde and Ledent (2001), who found that a reduction in tuber number (TN) was compensated for by increased average tuber dry weight (ADW).

Greenhouse study. Greenhouse experiments demonstrated that TN, TDW, ADW were significantly affected by potato genotypes for both years (Table 4.2). Tuber number ranged from 2.8 and 2.5 for Andover in 2002 and 2003 down to 1.2 and 1.1 for Chagllina-INIA. In both years, the relationship between TN and TDW in the greenhouse experiment was similar to that of the *in vitro* study. As seen in Table 4.1, some of the genotypes having fewer tubers, such as Tacna and Superior, compensated with higher ADW.

4.3.2. Effects of PEG8000.

The *in vitro* experiment demonstrated that PEG8000 significantly reduced TN, TDW, and ADW for both years (Table 4.1). The observed tuber number reduction (TNR), tuber dry weight reduction (TDWR), and average tuber dry weight reached more than 40% (Table 4.1). The fact that PEG8000 mimicked the effects of water stress and significantly reduced TN, hence, inducing a large positive TNR was consistent with previous studies, in which drought significantly reduced tuber numbers (Lynch and Tai, 1989; Karafyllidis *et al.*, 1996).

The greenhouse experiment showed similar results to the *in vitro* experiment, in which PEG8000 significantly reduced TN, TDW, ADW, and caused a significant relative reduction compared to controls in both years (Table 4.2). PEG8000 was applied early in the growing season, which might explain why potato genotypes produced fewer tubers, due to decreased stolon formation (Haverkort *et al.*, 1990).

4.3.3. The Interaction between PEG8000 and Genotype.

In vitro study. Greater interaction between PEG8000 and genotype was observed in 2001 than in 2002 (Table 4.1). The reason for this was not clear, but for some reason TDW and ADW values were less reduced by PEG8000 in the 2002 experiment, with the result that differential effects of PEG8000 on genotypes were not found in these variables. It might be attributed to the differences in the tissue used to initiate the cultures (as mentioned above). The number of subcultures did not reduce the capacity for producing tubers as reported by (Villafranca *et al*, 1998). Instead, it reduced the dry weight of the tubers produced (Table 4.1).

Table 4.1. The effect of potato genotypes (G), polyethylene glycol (PEG) 8000 concentrations, and their interactions on tuber number per container (TN), TN reduction per container (TNR, %), total tuber dry weight per container (TDW, mg), TDW reduction per container (TDWR, %), average tuber dry weight (ADW, mg), and ADW reduction (ADWR, %) for the *in vitro* experiments.

Treatments	2001						2002					
	TN	TNR (%)	TDW (mg)	TDWR (%)	ADW (mg)	ADWR (%)	TN	TNR (%)	TDW (mg)	TDWR (%)	ADW (mg)	ADWR (%)
Genotypes (G)												
Kennebec	23.25 a	-	0.308 ab	-	0.014 cdef	-	12.63 ab	-	0.198 bc	-	0.016 bcd	-
Andover	21.31 a	-	0.373 ab	-	0.018 cde	-	10.75 bc	-	0.177 c	-	0.014 cd	-
Russet Burbank	19.19 ab	-	0.286 bc	-	0.015 cdef	-	14.25 a	-	0.317 a	-	0.019 bcd	-
Katahdin	15.54 b	-	0.186 cd	-	0.012 cdef	-	11.88 ab	-	0.202 bc	-	0.016 bcd	-
Tacna	11.50 c	-	0.501 a	-	0.047 a	-	8.13 cd	-	0.274 b	-	0.028 a	-
Shepody	10.88 c	-	0.471 a	-	0.029 b	-	13.25 ab	-	0.280 ab	-	0.020 bcd	-
Chagllina-INIA	9.23 cd	-	0.177 d	-	0.011 def	-	12.50 ab	-	0.166 cd	-	0.012 d	-
C89.315	8.38 cd	-	0.087 d	-	0.009 ef	-	12.38 ab	-	0.153 cd	-	0.013 d	-
Unica	8.02 cd	-	0.471 a	-	0.020 cd	-	7.00 bc	-	0.107 cd	-	0.014 cd.	-
Superior	6.44 de	-	0.055 d	-	0.008 f	-	5.38 ef	-	0.127 cd	-	0.023 ab	-
E86.011	6.34 de	-	0.105 d	-	0.015 cdef	-	10.37 bc	-	0.140 cd	-	0.013 d	-
Reichie	2.37 e	-	0.052 d	-	0.021 c	-	3.58 f	-	0.081 d	-	0.023 abc	-
Polyethylene Glycol (PEG)												
0%	15.24	0.0	0.448	0.0	0.031	0.0	15.03	0.0	0.289 a	0.0	0.022	0.0
8%	9.09	40.4	0.119	73.4	0.011	65.1	7.26	51.7	0.114 b	60.6	0.013	40.9
					Analysis of variances, Pr >F							
Source of variation												
G	**	**	**	**	**	**	**	**	**	**	**	NS
PEG	*	*	*	*	*	*	*	**	**	**	*	*
G x PEG	**	**	**	**	**	*	**	**	NS	NS	NS	NS

Means within the same column followed by the same letter are not significantly different at the 0.05 level (*) or 0.001 (**) for Duncan's Multiple Range Test (DMRT). NS is not significantly different at the 0.05 level. TNR, TDWR, and ADWR values apply to effect of 8% compared to the control (0%).

72

Table 4.2. The effect of potato genotypes (G), polyethylene glycol (PEG) 8000 concentrations, and G x PEG interactions on tuber number per plant (TN), tuber number reduction per plant (TNR, %), total tuber dry weight per plant (TDW, g), total tuber dry weight reduction per plant (TDWR, %), average tuber dry weight (ADW, g), and average tuber dry weight reduction (ADWR, %). The experiment was conducted in the greenhouse using plants grown from microtubers.

Treatments	2002						2003					
	TN	TNR (%)	TDW (mg)	TDWR (%)	ADW (mg)	ADWR (%)	TN	TNR (%)	TDW (mg)	TDWR (%)	ADW (mg)	ADWR (%)
Genotypes (G)												
Andover	2.80 a	-	4.134 abc	-	1.596 c	-	2.51 a	-	3.943 abcd	-	1.521 ef	-
E86.011	2.70 a	-	3.592 abc	-	1.410 c	-	2.07 ab	-	4.241 abcd	-	2.003 cde	-
Shepody	2.40 ab	-	4.730 abc	-	2.026 abc	-	1.82 bcd	-	4.882 ab	-	2.382 bcde	-
Russet Burbank	2.00 bc	-	4.344 ab	-	2.194 abc	-	1.88 bcd	-	5.334 a	-	2.661 bc	-
Unica	2.00 bc	-	4.376 ab	-	2.200 abc	-	1.81 bcd	-	4.542 abc	-	2.363 bcde	-
Kennebec	1.95 bcd	-	3.280 abc	-	1.434 c	-	2.18 ab	-	3.721 bcde	-	1.722 def	-
C89.315	1.90 bcd	-	3.280 abc	-	1.778 bc	-	1.99 bc	-	3.483 cde	-	1.844 cdef	-
Katahdin	1.90 bcd	-	3.346 abc	-	1.818 bc	-	1.19 ef	-	2.361 e	-	1.042 f	-
Tacna	1.80 cd	-	5.110 a	-	2.588 ab	-	1.57 cde	-	5.244 a	-	3.242 ab	-
Superior	1.70 cde	-	4.242 a	-	2.626 a	-	1.74 bcd	-	4.913 ab	-	2.723 bc	-
Reichie	1.30 de	-	2.986 bc	-	2.002 abc	-	1.44 def	-	5.342 a	-	3.584 a	-
Chaglina-INIA	1.20 e	-	2.836 c	-	2.166 abc	-	1.13 f	-	2.922 de	-	2.543 bcd	-
Polyethylene Glycol (PEG)												
0%	2.40	0.0	6.06	0.0	2.76	0.0	2.54	0.0	4.61	0.0	1.99	0.0
8%	1.65	31.2	2.52	58.4	1.62	41.3	1.39	45.3	1.18	74.4	0.98	50.8
					Analysis of variances, Pr >F							
Source of variation												
G	**	**	**	**	**	**	**	**	**	**	**	*
PEG	*	*	*	*	*	*	*	*	*	*	**	*
G x PEG	NS	NS	*	NS	NS	*	**	NS	NS	NS	NS	NS

Means within the same column followed by the same letter are not significantly different at the 0.05 level (*) or 0.001 (**) for Duncan's Multiple Range Test (DMRT). NS is not significantly different at the 0.05 level. TNR, TDWR, and ADWR values apply to the effect of 8% compared to the control (0%).

73

The genotype x PEG interaction was found in TN and TNR in both years. As seen in Table 4.3, several varieties were in the top group for TN in both years, but only Katahdin was in the top group for TN and TNR in both years. With regard to TNR, Superior consistently showed less growth reduction than other commercial genotypes in both years, while Russet Burbank and Shepody consistently demonstrated the opposite. Among the CIP genotypes, Reichie consistently demonstrated the lowest TNR for both years (Table 4.3), while C89.315 and E86.011 consistently showed higher TNR (and TN) than commercial genotypes. According to the criteria of Demagante *et al.* (1995) for drought tolerance, in which a genotype showing the least growth reduction is considered as the most drought-tolerant genotype, Reichie, Superior, and Katahdin should be considered as drought-tolerant genotypes. While Katahdin (Barclay and Scott, 1997) and Reichie (CIP personal communication) have been listed as tolerant genotypes, Superior is known to be responsive to additional irrigation (Opena and Porter, 1997). A linear correlation was found between TN in 2001 and 2002 ($r^2 = 0.68*$), as well as between TNR in both years ($r^2 = 0.59*$), indicating that the effects of PEG on TN and TNR were relatively consistent and that TNR may be used to select potato genotypes grown *in vitro* for water stress.

With regard to the 2001 genotypes x PEG interactions that were not observed in 2002, Katahdin, Kennebec, and Tacna demonstrated the highest TDW, and were also in the groups with the least TDWR (Table 4.3). In contrast, E86.011 and C89.315 were in the groups with the lowest TDW and highest TDWR. The high value for TDW for Tacna under PEG stress may have been largely due to its high ADW value in 2001. Tacna was also in the highest group for ADWR in 2001.

Table 4.3. The effects of water stress (8% PEG8000) on genotype tuberization of plants grown *in vitro*, expressed in tuber number per container (TN), tuber number reduction per container (TNR, %), tuber dry weight per container (TDW, mg), tuber dry weight reduction per container (TDWR, %), average dry weight (ADW, mg), and average dry weight reduction (ADWR, %). TN, TDW, and ADW are values for 8% PEG8000.

Genotypes	TN		TNR		TDW		TDWR		ADW		ADWR	
	2001	2002	2001 (%)	2002 (%)	2001 (mg)	2002 (mg)	2001 (%)	2002 (%)	2001 (mg)	2002 (mg)	2001 (%)	2002 (%)
Katahdin	14.7 a	10.5 ab	25.2 abc	32.6 a	0.19 abc	0.13 a	35.1 a	63.2 b	0.013 b	0.011 ab	13.8 ab	47.3 b
Kennebec	14.2 ab	11.0 a	48.3 cd	45.7 ab	0.22 ab	0.15 a	40.6 ab	65.3 b	0.015 b	0.013 ab	-13.9 a	36.2 b
Andover	12.8 ab	8.0 abcd	35.5 bcd	43.9 ab	0.14 abc	0.17 a	59.9 abc	-38.2 a	0.012 b	0.019 ab	46.9 bc	-209.6 a
Russet Burbank	11.8 abc	10.5 ab	38.7 bcd	50.5 bc	0.11 bcd	0.19 a	65.6 bc	60.2 b	0.009 b	0.014 ab	38.2 abc	11.5 ab
Tacna	10.6 abcd	5.5 bcd	4.6 ab	51.3 bc	0.31 a	0.16 a	59.9 abc	67.6 b	0.034 a	0.023 ab	37.7 abc	37.3 b
Chaglina-INIA	8.0 bcde	9.0 abc	47.2 cd	57.6 ab	0.06 cd	0.10 a	77.0 c	64.8 b	0.007 b	0.012 ab	55.1 bc	38.0 ab
Shepody	6.2 cde	7.5 abcd	70.3 d	61.6 bc	0.08 cd	0.13 a	83.8 c	70.9 b	0.011 b	0.014 ab	57.6 bc	31.6 b
Superior	5.8 cde	6.0 abcd	15.0 abc	12.5 a	0.03 d	0.05 a	61.2 abc	76.1 b	0.005 b	0.007 b	51.9 bc	63.3 b
Unica	5.5 cde	7.0 abcd	36.1 bcd	56.3 bc	0.09 bcd	0.07 a	74.4 c	76.6 b	0.008 b	0.009 ab	45.3 bc	46.2 b
E86.011	4.3 de	4.5 cd	52.0 cd	66.7 bc	0.01 d	0.07 a	84.7 c	62.7 b	0.009 b	0.014 ab	66.9 bc	-17.9 ab
C89.315	4.1 de	3.0 d	56.2 cd	84.5 c	0.04 d	0.11 a	82.9 c	56.7 b	0.004 b	0.032 a	49.0 bc	-168.6 ab
Reichie	3.0 e	3.3 d	-10.4 a	10.6 a	0.03 d	0.08 a	69.0 bc	26.7 ab	0.008 b	0.027 ab	72.1 c	-0.2 ab

Means within the same column followed by the same letter are not significantly different at the 0.05 level (Duncan's Multiple Range Test, DMRT). Growth reduction was calculated with the formula, $Y = \{(p-q)/p\} \times 100\%$, where Y = growth reduction, p = growth in 0% PEG8000, and q = growth in 8% PEG8000. A negative value in TNR, TDWR, or ADWR indicated that the genotypes gained TN, TDW, or ADW in response to 8% PEG8000.

Table 4.4. The effects of water stress (8% PEG8000) on genotype tuberization of plants derived from microtubers and grown in the greenhouse, expressed in tuber number per plant (TN), tuber number reduction per plant (TNR, %), tuber dry weight per plant (TDW, g), tuber dry weight reduction per plant (TDWR, %), average dry weight (ADW, g), and average dry weight reduction (ADWR, %). TN, TDW, and ADW are values for 8% PEG8000.

Genotypes	TN		TNR		TDW		TDWR		ADW		ADWR	
	2002	2003	2002 (%)	2003 (%)	2002 (g)	2003 (g)	2002 (%)	2003 (%)	2002 (g)	2003 (g)	2002 (%)	2003 (%)
Kennebec	2.5 a	1.40 bcd	-6.7 a	46.7 a	1.56 b	0.88 cde	60.1 b	56.3 ab	0.79 bcde	0.67 ab	41.9 abcd	28.2 a
C89.315	2.0 ab	1.40 bcd	0.0 ab	20.0 a	1.41 b	0.62 de	56.1 b	73.9 b	0.73 bcde	0.39 b	54.9 abcd	67.0 a
Andover	2.50 a	2.60 a	23.2 abc	23.0 a	1.49 b	1.62 ab	62.9 b	43.9 ab	0.61 cde	0.61 ab	53.9 abcd	17.7 a
Superior	1.50 bcd	1.40 bcd	24.7 abc	30.0 a	1.62 b	1.55 ab	67.3 b	48.8 ab	1.26 bcd	1.36 a	37.9 abc	11.8 a
Reichie	1.26 bcd	0.80 d	30.3 abc	50.0 a	2.59 a	0.53 e	32.3 a	33.3 ab	2.27 a	0.37 b	2.2 a	29.1 a
Shepody	1.76 abc	2.20 ab	33.8 abc	8.4 a	1.33 b	1.77 a	75.7 bcd	48.5 ab	0.72 bcde	0.97 ab	65.2 bcd	3.6 a
Unica	1.46 bcd	1.60 abcd	35.4 abc	20.0 a	1.31 b	1.50 ab	72.7 bc	52.8 ab	0.89 bcde	0.86 ab	57.2 abcd	45.3 a
Tacna	1.00 bcd	1.80 abcd	48.9 abc	13.3 a	1.84 ab	1.65 ab	63.9 b	42.1 ab	1.57 ab	1.05 ab	39.4 abc	27.1 a
Russet Burbank	1.26 bcd	2.00 abc	53.0 abc	0.00 a	1.57 b	1.22 abc	68.8 bc	63.0 b	1.32 bc	0.67 ab	17.0 ab	55.6 a
E86.011	1.50 bcd	2.20 ab	56.7 bc	20.0 a	1.32 b	1.51 ab	67.7 bc	-4.9 ab	1.04 bcd	0.93 ab	11.3 ab	43.9 a
Chagllina-INIA	0.76 bc	1.00 cd	59.1 bc	19.8 a	0.42 c	1.07 bcde	91.4 d	-94.5 a	0.43 de	1.07 ab	88.1 cd	-95.1 a
Katahdin	0.60 c	1.40 bcdc	79.5 c	13.3 a	0.15 c	1.19 abcd	96.8 d	25.9 ab	0.08 e	0.95 ab	93.6 c	2.8 a

Means within the same column followed by the same letter are not significantly different at the 0.05 level (Duncan's Multiple Range Test, DMRT). Growth reduction was calculated with the formula, $Y = \{(p-q)/p\} \times 100\%$, where Y = growth reduction, p = growth in 0% PEG8000, and q = growth in 8% PEG800. A negative value in TNR, TDWR, or ADWR indicated that the genotypes gained in TN, TDW, or ADW in response to 8% PEG8000.

A linear correlation was found between TN in 2001 and 2002 ($r^2 = 0.68^*$), as well as between TNR in both years ($r^2 = 0.59^*$), indicating that the effects of PEG on TN and TNR were relatively consistent and that TNR may be used to select potato genotype grown *in vitro* for water stress.

Greenhouse experiment. Very few tubers were formed at 8% PEG8000 in the greenhouse experiments (Table 4.4). Perhaps, it was due to the poor tuberization of the plants because they grew from microtubers. In a preliminary study (not reported), we found that microtuber seeds grown in different sizes (from 7.5 to 15 cm) of plastic pots produced no more than 3 tubers per microtuber seed, regardless of the pot size. Furthermore, as mentioned above, this may have been because the PEG stress was applied starting at the time of planting, which resulted in less stolon formation and, hence, lower tuber numbers (Ewing and Struik, 1992; MacKerron and Jefferies, 1986). Growth limitation by low winter light levels in the greenhouse might also have been a factor. Perhaps for these reasons, there were no genotype x PEG interactions that were consistent between 2002 and 2003. This was supported by the correlation analysis, which showed that there was no significant linear correlation between 2002 and 2003 for any dependent variables measured. The r^2 values ranged from 0.03 (TNR and TDW) to 0.35 (ADWR).

However, interactions were found for TDW and ADWR in 2002 and for TN in 2003. In agreement with the *in vitro* results, it is of note that Tacna was in the most stress-resistant group for all three of these interactions (Table 4.4). Chagllina-INIA was in the least stress-resistant groups in each of these characters, but this was not the case for the *in vitro* experiment.

Comparison of the in vitro, greenhouse and previous results. Improvements are clearly needed in the greenhouse method if it is to be used in screening for drought tolerance. These might include using normal tubers instead of microtubers, conducting the experiment during the summer time or conducting experiment in the growth chamber, and/or using larger containers for growing the potato to avoid growth restriction. In contrast, the *in vitro* method shows more promise, especially if high levels of stress are imposed. Otherwise, variation among replicates makes it difficult to detect genotype effects. Another critical variable may be allowing for a sufficiently long growth period, as suggested by Villafranca *et al.,* (1998). Otherwise the differences between genotypes may not become large enough to be detected.

Among the genotypes from CIP, our experiments suggest that Tacna may be the most water-stress resistant, whereas C89.315 and E86.011 may be less resistant. Among the Maine varieties tested, both Kennebec and Katahdin are thought to be drought tolerant, and this is supported by the results from the *in vitro* study. Both achieved relatively high TDW in 8% PEG8000 and were in the group with the lowest ADWR. Kennebec has been reported as a drought-tolerant cultivar by Bansal *et al.* (1991), while Katahdin has been listed as a drought-tolerant cultivar by the New Brunswick Department of Agriculture and Rural Development (Barclay and Scott, 1997). No significant linear correlation was found between the *in vitro* and the greenhouse experiments for TN, TNR, TDW, TDWR, ADW, and ADWR.

4.4. Summary

The results indicated that genotype significantly affected the tuberization of potatoes grown both *in vitro* and in the greenhouse. Potato tuberization in both studies was significantly reduced by 8% PEG8000 treatments. In the *in vitro* study, the interaction between genotype and PEG significantly affected potato tuberization, except for TDW, TWDR, ADW and ADWR in 2002. In the greenhouse study, the interaction was only significant for TDW and ADW in 2001, and TN in 2002.

The results of the *in vitro* study suggested that Kennebec and Katahdin were drought tolerant and that the technique was a promising method to select potato genotypes for drought tolerance. In contrast, improvements are needed before using the greenhouse technique for screening.

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Chapter 5

USE OF POLYETHYLENE GLYCOL (PEG) 8000 FOR RAPID SCREENING OF POTATO (*Solanum tuberosum* L) GENOTYPES FOR WATER STRESS TOLERANCE. III. ROOT TIP CUTTING AND LEAF DISC GROWTH REDUCTION

Abstract

Using conventional breeding methods to select a drought-tolerant variety is painstaking and time consuming. Therefore, a fast and effective method to select drought-tolerant genotypes is needed. The objective of this study was to evaluate whether the growth reduction of excised-root tips and leaf discs of potato genotypes exposed to PEG8000 could be used to select potato genotypes for drought tolerance. The growth reduction of excised root tips of twelve potato genotypes exposed to 8% PEG8000 solutions (w/v) and leaf discs of twelve potato genotypes exposed to 10% PEG8000 solutions (w/v) were determined by comparing them to the growth reduction of those at the control treatments (0% PEG). The results showed that PEG treatments were able to mimic the effects of drought stress and demonstrated that Kennebec, a genotype known to be drought tolerant, showed less growth reduction than Superior, a genotype known to be drought sensitive, both in the root tip cutting assay (RTCA) and leaf disc assay (LDA). The linear correlation between root growth reduction (RGR) over experimental runs of the RTCA was significant ($r^2 = 0.51*$), indicating that the effects of PEG on RGR were consistent and, hence, RGR could be used to select potato genotypes for water stress. Similar results were found in leaf growth reduction (LGR) for the LDA experiments ($r^2 = $

0.75*). However, the correlation between RGR of RTCA and LGR of LDA was not significant ($r^2 = 0.01^{ns}$), suggesting that both RTCA and LDA approaches should be carried out simultaneously to select for different traits associated with water-stress tolerance.

Keywords: PEG, root tip, leaf disc, growth

5.1. Introduction

Potato (*Solanum tuberosum* L.) is well known to be very sensitive to drought stress (Ekanayake and de Jong, 1992; Vayda, 1994), due to its poor soil water extraction (Weisz *et al.*, 1994), as a result of the shallow and ineffective rooting systems (Fulton, 1970). Most potato roots are confined in the upper 30-cm soil layer (Kleinkopf, 1983; Opena and Porter, 1999). On the other hand, drought-adapted plants are characterized by a deep and vigorous root system, associated with extensive rooting depth, high root length density, and low resistance to water flow within the root (Monevaux and Belhassen, 1996). Plants experience drought by excessive transpiration and/or by a limitation of water supply (Frensch, 1997). Although drought stress reduces plant water potentials (ψ_s), it affects root and leaf growth differently (Frensch, 1997). From their glasshouse experiment, Gandar and Tanner (1976) reported that the growth of potato leaves decreased significantly with increases in water stress, and stopped completely when leaf water potential reached −0.5 MPa. Many studies show that root growth is more resistant to water deficit than is shoot growth (Frensch, 1997; Hsiao and Jing, 1987;

Hsiao and Xu, 2000; Kramer and Boyer, 1995; Sharp, 2002). Furthermore, drought stress increases both root-to- shoot ratio and root-length-to-root weight ratio (Jefferies, 1993).

Some rapid methods for screening drought-tolerance traits in potato have been established (Bansal *et al.*, 1991; Demagante *et al.*, 1995; Levy *et al.*, 1991). Canopy temperature and chlorophyll *a* fluorescence have been reported as potential tools for screening potato germplasm (Jefferies, 1992; Ranalli *et al.*, 1997; Stark *et al.*, 1991). Demagante *et al* (1995) employed apical cuttings for screening drought tolerance in raised beds. Drought stresses may be induced physically by withholding water from the plants (Gandar and Tanner, 1976; Demagante *et al.*, 1995) or chemically by applying osmotic stresses (Jia *et al.*, 2001), for example, by introducing polyethylene glycol (PEG) to the water used for watering (Michael and Kaufman, 1975; Steuter *et al.*, 1981). Bansal *et al.* (1991) established a new screening method by using the growth reduction of leaf discs floated over different concentrations of polyethylene glycol (PEG) 6000 (now PEG8000, Sigma Aldrich, 2001). When floated over water, a leaf disc will absorb water very rapidly until water potential equilibrium is reached, which takes about 2-4 hours. This rapid water uptake, known as phase I, is soon followed by slow water absorption, known as phase II, which lasts as long as the leaf stays healthy (Barrs and Wetherley, 1962; Bansal *et al.*, 1991). Phase II has been reported as the form of growth that is affected by metabolic inhibitors (Barr and Wetherley, 1962).

A root-tip assay has been used as a tool for *in vitro* screening of potato tolerance to salinity stress (Zhang and Donnelly, 1997). However, no such method has been published for screening potato drought tolerance. The objectives of this study were (1) to evaluate whether the growth reduction of root tips grown *in vitro* under drought stress can

be used as a tool for screening potato genotypes and (2) to study whether the growth reduction of leaf discs floated over water treated with PEG8000 can be used as a tool for screening potato genotypes for water-stress tolerance.

5.2. Materials and Methods

Both experiments used the same plant materials and experimental design. The plant materials were obtained from the International Potato Center (CIP), (Chagllina-INIA, E86.011, Reiche, C89.315, Tacna, and Unica) and from Dr. Feridoon Mehdizadegan, the Maine Potato Seed Board (Andover, Superior, Shepody, Kennebec, Katahdin, and Russet Burbank). Based on personal communication with a researcher at CIP, the CIP selections were presumed to be drought tolerant. While no information was available for the maturity of the CIP genotypes, Andover and Superior were known as early-mid season, Shepody and Kennebec middle season, and Katahdin and Russet Burbank late-maturing cultivars. Furthermore, Kennebec and Katahdin have been listed as drought-tolerant cultivars while Andover was reported to be drought sensitive (Barclay and Scott, 1997). From leaf disc experiments, Bansal *et al.* (1991) found Kennebec to be a drought-tolerant cultivar.

Each experiment was repeated twice and arranged in a randomized complete block design (RCBD), with two factors and five replications. The first factor was potato genotype (G), consisting of the 12 genotypes mentioned above and the second factor was two levels of PEG8000 (PEG) to induce water stress. Data were analyzed with Proc GLM (SAS Institute, Cary, NC) for the analysis of variance, followed by mean separation with

Duncan's Multiple Range Test (DMRT), in addition to a linear correlation analysis using Microsoft Excel (*r critical* = 0.58; α = 0.05; n = 12).

5.2.1. Root Tip Cutting Assay

The root-tip-cutting assay (RTCA) was carried out following Zhang and Donnelly's (1997) protocol for salinity screening. One-cm-long root-tip segments were taken from one to two week-old single-node cuttings growing in potato micro propagation medium (Zhang and Donnelly, 1997), in which a modified MS (Murashige and Skoog, 1962) basal salt solution was supplemented with inositol (100 mg l^{-1}), niacin (0.5 mg l^{-1}), pyridoxine HCl (0.5 mg l^{-1}), thiamine HCl (1.0 mg l^{-1}), Ca-pantothenate (2.0 mg l^{-1}), glycine (2.0 mg l^{-1}), 3% sucrose, without agar. The medium was adjusted to pH 5.7 prior to autoclaving at 121 $^{\circ}$C for 20 minutes. For each replication, ten pieces of root tip of each genotype were placed in a liquid potato micro propagation medium (10 ml of medium in 25 x 250 mm Pyrex glass culture tube) containing PEG8000 concentrations of 0% (control) or 8% (water stress). All cultures were incubated in the dark at 25 $^{\circ}$C, for 1 week. To create complete darkness, the cultures were wrapped with double-brown bags, then covered with a double layer of black cloth. After one week, root segments were removed, blotted and measured to the nearest 1 mm with a ruler. Root growth (RG) was calculated by subtracting the original from the final length. Root dry weight (RDW) was obtained by drying the roots at 70 $^{\circ}$C for 7 days.

5.2.2. Leaf Disc Assay

This experiment was carried out by modifying a screening method for drought tolerance developed by Bansal *et al.* (1991). Leaf discs were obtained from the third or fourth leaf from the tip of 4-week-old plants, grown in the greenhouse from microtubers. Leaves were harvested at night between 8 to 9 o'clock to avoid wilting, and put in plastic bags containing wetted paper towels. The experiments began immediately after the leaves were harvested. Discs, 10 mm in diameter, were punched out of the leaflets avoiding the midrib. For each replicate, ten discs were floated with their adaxial surface facing up in 9-cm Petri dishes on 20 ml distilled water or 10% PEG8000 solution prepared according to method of Michele and Kaufmann (1973), which produced approximately -0.22 MPa (Struter, 1981). The leaf discs were incubated under diffuse light at room temperature for 2.5 h to allow equilibration. The diffuse light was created by putting a shade (black cloth) approximately 1 m above the leaf discs. Each 10-leaf-disc replicate was blotted dry and weighed. They were then incubated in the same medium (10% PEG8000 or distilled water) in sealed petridishes in the dark at 25 °C for 15 hours, a period of incubation where the leaf disc showed the highest growth differences according to Bansal *et al.* (1991).

At the end of the experiment the leaf materials were blotted dry and weighed again to obtain their final weight. The gain in weight (as a fraction of the initial weight) for plants in contact with the PEG 8000 was compared with the control samples in contact with distilled water, and the percent differences were determined.

5.3. Results and Discussions

5.3.1. Root Tip Cutting Assay

5.3.1.1. Effect of Genotype.

Root growth (RG), which was measured by subtracting the original length (1cm) from the final length, was significantly affected by genotype in both years (Table 5.1). Reichie, Superior, and Andover, which statistically belonged to the same group, consistently showed the lowest RG value. In 2001, the highest RG was found in E86.011, Russet Burbank, and C89.315, while in 2002 it was found in Kennebec, Russet Burbank and C89.315.

The results showed that a genotype demonstrating high RG values did not necessarily produce high root dry weight (RDW) or vice versa. For example, in 2001, Russet Burbank belonged to a group of genotypes with the highest RG, but it produced the lowest RDW, while Reichie had the lowest RG and belonged to a group of genotypes with the highest RDW. Only C89.315 in 2001 and Kennebec in 2002 demonstrated a consistent relationship, in which their high RGs were reflected in high RDWs.

5.3.1.2. Effects of PEG8000.

Table 5.1 showed that 8% PEG8000 significantly affected root growth (RG), root growth reduction (RGR), root dry weight (RDW), and root dry weight reduction (RDWR) in 2001, but not in 2002. These differing values and responses between years might have been due to the changes in the microenvironment where the cultures were incubated. The experiment was carried out during the winter (February) in 2001 and

Table 5.1. The effect of potato genotypes (G), 8% polyethylene glycol (PEG) 8000, and the interactions of G x PEG on root growth (RG, cm), root growth reduction (RGR, %), root dry weight (RDW, g), and root dry weight reduction (RDWR, %). Means presented are on the basis of ten roots per sample.

Treatments	2001				2002			
	RG (cm)	RGR (%)	RDW (g)	RDWR (%)	RG (cm)	RGR (%)	RDW (g)	RDWR (%)
Genotypes (G)								
E86.011	36.5 a	-	0.065 de	-	19.2 bcd	-	0.023 cd	-
Russet Burbank	31.1 ab	-	0.021 f	-	23.8 ab	-	0.032 b	-
C89.315	30.5 ab	-	0.123 a	-	22.1 abc	-	0.017 de	-
Unica	28.2 bc	-	0.081 cd	-	17.8 bcde	-	0.020 de	-
Chagllina-INIA	26.2 bc	-	0.077 de	-	11.5 efg	-	0.014 e	-
Tacna	24.2 bc	-	0.061 e	-	15.6 cdef	-	0.033 b	-
Shepody	20.7 cd	-	0.023 f	-	15.2 def	-	0.017 de	-
Kennebec	17.1 d	-	0.009 f	-	26.7 a	-	0.075 a	-
Katahdin	16.9 d	-	0.015 f	-	16.4 cdef	-	0.034 b	-
Superior	14.8 de	-	0.098 bc	-	10.5f g	-	0.016 de	-
Andover	13.6 de	-	0.083 cd	-	10.5 fg	-	0.029 bc	-
Reichie	8.1 e	-	0.111 ab	-	5.5 g	-	0.016 de	-
Polyethylene Glycol (PEG)								
0%	25.8	0.0	0.072	0.0	16.9	0.0	0.028	0.0
8%	18.8	27.1	0.055	23.6	15.6	7.7	0.026	7.1
Analysis of variances, Pr > F								
Source of variation								
G	**	-	**	-	**	-	**	-
PEG	**	**	**	**	NS	NS	NS	NS
G x PEG	*	**	**	**	NS	**	**	**

Means within the same column followed by the same letter are not significantly different at the 0.05 level (Duncan's Multiple Range Test, DMRT). *, ** Significant at the 0.05 and 0.01 probability levels, respectively. NS is not significantly different at the 0.05 level; RGR, RDWR did not apply for genotypes.

Table 5.2. Root growth (RG, cm), root dry weight (RDW, g), reduction in root growth (RGR, %), and reduction in root dry weight (RDWR, %) of 12 potato genotypes grown at 8% PEG8000, and their ranking from the least (1) and the most (12) reduced in growth at 8% PEG8000. Means presented are on the basis of ten roots per sample.

Treatments	2001			2002			2001			2002		
	RG (cm)	RGR (%)	Rank	RG (cm)	RGR (%)	Rank	RDW (g)	RDWR (%)	Rank	RDW (g)	RDWR (%)	Rank
Genotypes (G)												
Russet Burbank	31.3 a	3.7 bc	3	22.7 b	9.7 bc	8	0.021 e	-1.7 abc	3	0.038 bc	11.5 bc	9
E86.011	30.2 ab	7.5 bc	4	17.4 bcd	8.7 bc	7	0.056 d	15.1 bcd	5	0.022 cd	-12.7 bc	5
C89.315	23.8 abc	38.6 cd	7	21.6 bc	24.4 bc	9	0.102 a	29.1 d	8	0.017 d	-2.1 bc	6
Unica	23.0 abcd	37.1 cd	6	12.6 d	58.6 c	12	0.069 bcd	27.6 cd	7	0.016 d	32.9 c	12
Kennebec	20.3 bcd	-58.8 a	1	30.7 a	-34.5	2	0.010 e	-27.9 a	1	0.088 a	-42.2 ab	2
Chagllina-INIA	20.1 bcd	47.7 cd	11	9.9 de	0.9 bc	6	0.063cd	31.0 d	10	0.015 d	-34.9 ab	3
Shepody	16.7 cd	42.2 cd	8	11.4 d	47.3 c	10	0.019 e	26.1 cd	6	0.015 d	27.6 c	10
Tacna	16.7 cd	46.2 cd	10	15.8 bcd	0.7 bc	5	0.048 d	34.1 d	11	0.032 bc	5.6 bc	8
Andover	14.6 cd	-22.8 ab	2	13.6 cd	-84.3 a	1	0.086 abc	-10.1 ab	2	0.036 b	-71.3a	1
Superior	12.8 cde	16.1 bc	5	10.6 de	-13.8 ab	3	0.091 ab	11.8 bcd	4	0.016 d	-0.1 bc	7
Katahdin	12.3 de	45.1 ab	9	18.0 bcd	-3.3 bc	4	0.013 e	29.6 d	9	0.037 b	-21.3 abc	4
Reichie	3.5 e	79.9 d	12	3.0 e	57.4 c	11	0.083 abc	40.2 d	12	0.013 d	27.7 c	11

Means within the same column followed by the same letter are not significantly different at the 0.05 level (Duncan's Multiple Range Test, DMRT).

summer (late June) in 2002. Because the room temperatures were controlled by an air conditioner, the change in macroclimate outside might have affected the microclimate of the room. Another factor that might affect the differences was the condition of the initial plant material used for producing the roots.

5.3.1.3. Interaction between Genotype and PEG8000.

Except for RG in 2002, all variables measured were significantly affected by the interaction between genotype and PEG8000 (Table 5.1). The RG values at 8% PEG8000 ranged from 3.5 to 31.3 cm in 2001 and from 3.0 to 30.7 cm in 2002 (Table 5.2). In this regard, Reichie showed the lowest RG values in both years. The highest RG was found in Russet Burbank in 2001 and Kennebec in 2002.

When exposed to water stress (8% PEG8000), Reichie consistently showed the highest root growth reduction (RGR), 79.9% in 2001 and 57.4% in 2002 (Table 5.2). Unlike Reichie, Kennebec and Andover consistently demonstrated the lowest RGR in both years (Table 5.2). The correlation between RGR in 2001 and RGR in 2002 was significant (r^2 = 0.51*), indicating that the results of this experiment were consistent. Interestingly, the RGR values of both Kennebec and Andover were negative, suggesting that Kennebec and Andover grow better at 8% PEG8000 than at 0% PEG8000. The negative RGR was also found in Superior and Katahdin in 2002. These results were contradictory to those of previous studies, in which PEG solutions were reported to reduce root growth (Verslues, 1998). In another experiment reported previously (single node cutting assay, Chapter 3), we found there was no genotype that demonstrated negative RGR. Perhaps, it might be due to the differences in the length of the incubation

Table 5.3. The effect of potato genotypes (G), 10% polyethylene glycol (PEG) 8000, and the interactions of G x PEG on the leaf growth (LG, mg) and leaf growth reduction (LGR, %) of leaf discs. Means presented are on the basis of ten leaf discs per sample.

Treatments	2002		2003	
	LG (cm)	LGR (%)	LG (cm)	LGR (%)
Genotypes (G)				
Russet Burbank	23.5 a	-	17.9 de	-
Kennebec	22.9 a	-	18.3 de	-
Tanca	21.0 ab	-	17.9 de	-
Andover	20.2 ab	-	13.0 e	-
C89.315	19.1 abc	-	13.9 e	-
E86.011	18.9 abc	-	30.9 a	-
Reiche	18.9 abc	-	27.6 ab	-
Katahdin	18.1 abc	-	16.6 de	-
Unica	17.5 bcd	-	20.3 cd	-
Chagllina-INIA	14.1 cd	-	25.0 bc	-
Shepody	14.0 cd	-	20.7 cd	-
Superior	12.8 d	-	15.9 de	-
PEG8000 (PEG)				
0%	23.5	0.0	25.0	0.0
10%	13.4	42.9	14.7	41.2
		Analysis of Variance, Pr >F		
Source of Variation				
G	*	*	-	-
PEG	*	*	*	*
G*PEG	*	NS	*	*

Mean with the same letter in the same column are not significantly different at 5% level of Duncan's Multiple Range Test. (DMRT); NS is not significantly different at 5% level, and '*' is significantly different at 5% level.

period and/or the plant materials used. In the single node-cutting assay, we used single-node plant materials, from which the root protruded and grew during a 6-week incubation period. In the recent study, we used excised root tips and a 1-week incubation period. In the former experiment, the root probably had to compete with the shoot for growth resources while in the latter experiment such competition did not exist. If the incubation period in the recent experiment had been extended, the root tips grown in 0% PEG8000 might have grown better than those in 8% PEG8000. The level of PEG8000 (8%) used in this experiment generated approximately −0.21 MPa of water potential (Steuter, 1981), which might not be high enough to inhibit root elongation of some genotypes. It is also well known that mild drought can promote root growth.

Even though the correlation analysis indicated that there was no significant linear correlation between RDWR in 2001 and RDWR in 2002 ($r^2 = 0.21^{ns}$), Kennebec and Andover consistently demonstrated the most negative RDWR values for both years (Table 5.2). The causes for negative values of RDWR might be similar to those for RGR mentioned above since the relationship between RDR and RDWR was very strong ($r^2 = 0.92^*$). In both years, Reichie again belonged to the group showing the highest growth reduction. Some of the other genotypes also had negative RDWR values in 2002 (Table 5.2), but only Kennebec and Andover demonstrated consistent results. While the Kennebec has been confirmed to be a relatively drought-tolerant genotype by Bansal *et al.* (1991), Andover is listed as a drought-sensitive genotype (Barclay and Scott, 1997). However, since no testing method has been reported for the sensitivity of Andover to drought, we tend to consider Andover to be a drought-tolerant genotype along with Kennebec. This claim is supported by the criteria of Demagante *et al.* (1995) for

screening drought-tolerant potatoes, in which a genotype showing the least reduction in growth is considered to be the most tolerant genotype.

5.3.2. Leaf Disc Assay

5.3.2.1. Effect of Genotypes.

Leaf growth (LG) was significantly affected by potato genotypes in both years, with the LG values ranging from 12.8 to 23.5 mg in 2002 and from 13.0 to 30.9 mg in 2003 (Table 5.3). Our LG values were comparatively higher than those of Desiree (drought tolerant, 12.8 mg) and Kunfri Sindhuri (drought susceptible, 8.5 mg) floated over distilled water (Bansal *et al.*, 1991) at 15 °C for 15 hours. It was not clear what caused the higher LG values in our study. It could be due to physiological and environmental differences between the studies. Furthermore, the highest and lowest LG values varied between 2002 and 2003, which might be attributed to the change in the age of microtuber seeds or micro climate in the greenhouse where the source leaf discs were grown, as indicated by Demagante *et al.* (1995).

5.3.2.2. Effect of PEG8000.

As expected, the 10% PEG8000 treatment significantly affected the growth of leaf discs (LG) and the reduction in leaf disc growth (LGR) for both years. When exposed to PEG8000, leaf growth was reduced from 23.5 to 13.4 mg (57.1%) in 2002 and from 25.0 to 14.7 mg (58.8%) in 2003 (Table 5.3). The PEG treatment was able to mimic the effect of water stress.

5.3.2.3. The Interaction between Genotypes and PEG8000.

Leaf growth (LG) was significantly affected by the interaction between PEG8000 and genotypes during 2002 and 2003 (Table 5.3). The observed LG values at 10% PEG8000 (water stress) ranged from 7.1 to 21.6 mg in 2002, and from 6.7 to 28.6 mg in 2003 (Table 5.4). This growth was greater than that reported by Bansal et al. (1991), who found 10.8 mg of growth for Desiree, a drought-tolerant genotype, and 4.1 mg for Kufri Sindhuri, a drought-susceptible genotype. However, this did not necessarily indicate that the genotypes could be categorized as drought tolerant, because the incubation conditions were different, with Bansal et al. (1991) using a temperature of 10 °C and water potential of −0.4 M.Pa, while I used a temperature of 25 °C and a water potential of −0.22 MPa (Steuter, 1981). While an increase in temperature has been reported to reduce leaf growth (Bansal et al., 1991), an increase in water potential is well known to promote leaf growth (Gandar and Tanner, 1976). Therefore, we could not draw the conclusion, from this study, that the genotypes having higher LG values than Desiree (Bansal et al., 1991) were in fact drought tolerant.

The interaction between genotype and PEG on leaf growth reduction (LGR) was significant in 2003 (Table 5.3). The LGR values were comparable to those reported by Bansal et al. (1991) for leaf disc growth reduction and by Demagante et al. (1995) for plant growth reduction. Bansal et al. (1991) examined 28 genotypes and reported that S. pheruja and Desiree, two drought-tolerant species (Doornbos et al., 1982; Levy, 1983; Mendoza and Estrada, 1979) demonstrated the lowest growth reduction, 10% and 18%, respectively which supported the validity of their protocol for screening potato genotypes

for drought tolerance. Furthermore, they found that Kennebec showed a 27% growth reduction, which was statistically not different from *S. pheruja* and Desiree, indicating that this genotype was also drought tolerant. In our study, Kennebec demonstrated 11.7% and 7.5% of growth reduction for 2002 and 2003, respectively (Table 5.4). The observed LGR values for Kennebec were twice or three times lower than that reported by Bansal *et al.* (1991). As mentioned above, we used a higher water potential and higher temperature for the assay. There were other factors that might also have contributed to the difference: the source of the seeds, the age of plant materials, and the site from which the leaf discs were taken. While our leaf materials were harvested from 4-week-old plants grown from microtubers in the greenhouse, Bansal's *et al.* (1991) were harvested from 45 to 55-day-old plants grown from normal seed tubers. No information was available whether those plants were grown in the greenhouse or in the field. Bansal *et al.* (1991) used only the middle part of the leaflet end avoiding the midrib, while we used the whole leaflet part without midrib as long as the size of the leaf disc satisfied our need (10 mm in diameter). We decided to use the whole leaflet because the plants produced very few leaves, while Bansal *et al.* (1991) reported that their plants produced many leaflets. We used younger plant materials for two reasons. First, because Bansal *et al.* (1991) reported that young leaves behaved in the same pattern as the old ones in their responses to PEG8000. Secondly, because we wanted to avoid the risk of having aphid damage that might ruin the leaflet if we waited until the plants were 7 to 8 weeks old.

Table 5.4. Leaf disc growth (LG, mg) of 12 potato genotypes at low water potential (10% PEG8000), their growth reductions (LGR, %), compared to that in distilled water, and their ranking based on the LGR performances.[1] Means presented are on the basis of ten leaf discs per samples.

Treatments	2002			2003		
	LG (mg)	LGR (%)	Rank	LG (mg)	LGR (%)	Rank
Genotypes						
Kennebec	21.6 a	11.7 a	1	17.6 b	7.5 a	1
C89.315	16.6 b	25.3 b	2	28.6 a	14.2 ab	2
Tacna	16.5 b	34.9 bcd	4	12.9 b	52.1 cdef	8
Reichie	15.6 b	27.2 bc	3	23.2 ab	31.6 bc	4
Katahdin	14.1 b	38.9 bcde	5	12.1 b	40.8 cd	6
Unica	13.5 b	40.8 cde	6	15.3 b	38.2 cd	5
Russet Burbank	13.5 b	59.1 fg	11	11.9 bc	46.9 cde	7
E86.011	12.6 b	48.9 cde	7	6.8 c	68.9 de	11
Andover	11.8 bc	57.7 fg	9	6.7 c	71.3 f	12
Shepody	9.2 bc	50.6 efg	10	12.2 b	57.5 def	9
Chagllina-INIA	8.4 bc	57.1 fg	8	20.4 ab	31.6 bc	3
Superior	7.1 c	65.1 g	12	9.1 bc	60.5 def	10

Mean with the same letter in the same column are not significantly different at 5% level of Duncan's Multiple Range Test. [1] Ranked from the least (1) to the most (12) reduced in growth.

Kennebec and C89.011 consistently demonstrated the lowest growth reduction among the genotypes tested (Table 5.4). On the other hand, the highest growth reduction was found in Superior for 2002 (65.1%) and Andover for 2003 (71.3%). Adopting Demagante's *et al.* (1995) criteria for drought-tolerant genotypes, in which a genotype showing the least reduction in growth is ranked as the most drought-tolerant, Kennebec and C89.011 were considered to be the two most drought-tolerant genotypes tested in this experiment. Reichie was statistically not different from C89.011 and ranked in the third

and fourth place for year 2002 and 2003, respectively. With the same criteria, Superior and Andover were the most sensitive genotypes to drought, while Tacna, Unica, and Katahdin were considered to be in the middle group. While there is no published information on the drought sensitivity of Superior, Andover is listed as a drought-sensitive genotype and Kennebec as drought tolerant (Barclay and Scott, 2001). In commercial practice, Superior has generally been considered to be drought sensitive and quite responsive to supplemental irrigation (Porter *et al.*, 1999).

Only Kennebec and C89.315 consistently ranked first or second for the two experiments, while others slightly changed in rank (Table 5.4). The change in the ranking might be attributed to the change in microtuber age used to produce the leaf disc, different physiological stage of the leaflet, or the change in the growing conditions. Despite the variation, most genotypes showed only slight change in position in the ranking, except for Tanca and Chagllina-INIA (Table 5.4). Slight changes in the ranking of genotypes tested for drought tolerance had been reported by Demagante *et al.* (1995) when growing apical cuttings of 10 genotypes in raised beds at different water regimes. The linear correlation between LGR over the years was significant ($r^2 = 0.75^*$), indicating that the experiments were repeatable, and hence LDA can be used to screen potato genotypes for drought tolerance.

5.3.3. Relationship between Root Tip Cutting and Leaf Disc Assay

Even though the results of excised root tip assay and leaf disc assay indicated that both methods can be used to screen potato genotypes for water stress tolerance, there was

no significant linear correlation between root growth and leaf disc reduction ($r^2 = 0.01^{ns}$). This means that they measure different physiological attributes and that both assays should be used simultaneously for screening. However, if a breeder decides to employ only one approach, he might choose the leaf disc assay over the excised root assay for several reasons. The leaf disc assay does not need an aseptic environment and, hence, there was no risk of contamination. It is also quick and inexpensive relative to the root tip method.

Unlike Kennebec, which consistently was a water-stress tolerant genotype in both assays, Andover demonstrated contradictory results over assays (Table 5.2 and Table 5.4). In the root tip cutting assay, Andover demonstrated the characteristic of a water-stress-tolerant genotype. However, it demonstrated the opposite in the leaf disc assay. This might indicate that the two assays select for different traits associated with water-stress tolerance. Further study might be needed to confirm the sensitivity of Andover to water stress.

5.4. Summary

The results of the root tip cutting assay (RTCA) indicated that the effect of PEG treatments mimicked the effects of water stress, in that the genotype known to be relatively a drought tolerant (Kennebec) showed the least reduction in growth (RGR and RDWR) when exposed to 8% PEG8000. There was a significant correlation between RGR and RDWR ($r^2 = 0.92^*$) in each experiment, as well as between RGR over experiments ($r^2 = 0.51^*$). However, no significant linear correlation was found between

RDWR over experiments. Therefore, breeders might choose RGR over RDWR as a tool to select potato genotypes for drought tolerance.

The results of the leaf disc assay (LDA) also demonstrated that PEG treatments mimicked the effects of water stress. Kennebec, a genotype known as drought tolerant in Bansal *et al.* (1991), consistently demonstrated the least growth reduction (LGR) in our experiment, indicating that this genotype was drought tolerant. The results were repeatable shown by significant linear correlation between LGR 2002 and LGR 2003. With the minor changes in the ranking of the genotypes based on their reduction in growth, we considered that our leaf disc assay could be used as a tool for screening potato for water stress.

RGR and LGR might be used together in a selection program since they probably screen for different traits related to water-stress tolerance.

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Chapter 6

SYNTHESIS

Cell growth, including leaf and root growth, is one of the first processes affected by water stress (Hsiao, 1973; Harris, 1992), simply because cellular expansion is the plant function that is most sensitive to water deficits (Gardner *et al.*, 1985). Because of this, researchers often use growth reduction as a tool to select crops for drought tolerance. For example, the growth reduction of potato cuttings grown in raised beds under different water regimes was used by Demagante *et al.* (1995) to screen potato genotypes for drought tolerance. They found that a genotype showing low growth reduction in the raised beds also demonstrated low growth reduction in the field study. Previously, Bansal *et al.* (1991) used growth reduction of leaf discs floated over water at different water potentials to select potato genotypes. They found that a genotype known to be drought tolerant demonstrated low growth reduction among the genotypes tested, while a genotype known to be drought sensitive showed a high growth reduction. In our recent study, we adopted the method of Bansal *et al.* (1991) and used it to confirm the results of other approaches employed for the study. An approach was considered to be valid to screen potato genotypes for drought tolerance if it satisfied the following criteria: First, it must be able to mimic the effect of drought stress; Secondly, it must demonstrate that a genotype known to be drought tolerant shows less growth reduction than a genotype known to be drought sensitive; Finally, the results should be relatively consistent over the repeated experiments. The best methods were also expected to have a significant linear correlation with the leaf disc assay (LDA), as LDA has been proven to be reliable for screening potatoes for drought tolerance.

Table 6.1. The coefficient of determination (r^2) between measured variables over the years of each assay with 12 potato genotypes compared.

Assay [x] (Relationship)	r^2 [y]
1. Root Tip Cutting Assay	
RGR	0.51^*
RDWR	0.21^*
2. Single Node Cutting Assay	
RLDR	0.88^*
RDWR	0.79^*
SDWR	0.0003^{ns}
RSR	0.15^{ns}
3. Microtuberization Assay	
TNR	0.59^*
TDWR	0.03^{ns}
ADWR	0.04^{ns}
4. Greenhouse Experiment	
LAR	0.0005^{ns}
SDWR	0.38^*
RDWR	0.33^*
RSR	0.15^{ns}
TNR	0.30^*
TDWR	0.21^*
ADWR	0.35^*
5. Leaf Disc Assay	
LGR	0.75^*

[x] Leaf growth reduction (LGR), root growth reduction (RGR), root dry weight reduction (RDWR), root length density reduction (RLDR), shoot dry weight reduction (SDWR), root-to-shoot ratio reduction (RSR), tuber number reduction (TNR), tuber dry weight reduction (TDWR), average tuber dry weight reduction (ADWR), leaf area reduction (LAR).

[y] '*' and NS mean significant and not significant, respectively at $\alpha = 0.05$, n = 12, and r critical of 0.58.

6. 1. Leaf Disc and Excised Root Growth

The results of the leaf disc assay (LDA) and root tip cutting assay (RTCA) showed that leaf and root behave differently in response to water stress (French, 1976). In the leaf disc assay (LDA), PEG treatment was able to mimic the effect of drought stress, shown by the reduction in leaf growth (Table 5.3; Harris, 1992; Hsiao, 1973). The least growth reduction was found in Kennebec, known to be a drought-tolerant genotype (Bansal *et al.*, 1991), with the leaf growth reduction (LGR) of 11.7% in 2002 and 7.5% in 2003 (Table 5.4). On the other hand, Superior, known to be responsive to supplemental irrigation (Opena and Porter, 1999), showed similar growth reduction to some other genotypes (Table 5.4). The linear correlation between LGR over experiments was significant, indicating that the effects were consistent. These findings led to a conclusion that LGR could be used to screen potato genotypes for water stress tolerance, confirming the previous study of Bansal *et al.* (1991).

In the root tip cutting assay (RTCA), PEG treatment caused a severe reduction in root growth of some genotypes, but it also increased the root growth of some genotypes (Table 5.1; Table 5.2). In both cases, Kennebec and Andover consistently demonstrated lower growth reduction than the rest of the genotypes tested, indicating that those two genotypes were more drought-tolerant than the others (Demagante *et al.*, 1995). Furthermore, the effects of water stress on RDWR and RGR showed a low consistency (Table 6.1). Also, there was no linear correlation between LGR of leaf disc assay and RDWR or RGR of root tip cutting assay (Table 6.2). This indicates that the assays select for different traits.

Table 6.2. The results of correlation analysis between leaf growth reduction (LGR) of leaf disc assays (LDA) and growth reduction of other dependent variables from other assays (α = 0.05, n = 12, r critical value of 0.58).

Assay [x] (Relationship)	r^2 [y]
1. Root Tip Cutting Assay	
LGR vs RGR	0.03^{ns}
LGR vs RDWR	0.01^{ns}
2. Single Node Cutting Assay	
LGR vs RLDR	0.36^{*}
LGR vs RDWR	0.40^{*}
LGR vs SDWR	0.01^{ns}
LGR vs RSR	0.58^{*}
3. Microtuberization Assay	
LGR vs TNR	0.001^{ns}
LGR vs TDWR	0.002^{ns}
LGR vs ADWR	0.006^{ns}
4. Greenhouse Experiment	
LGR vs LAR	0.01^{ns}
LGR vs SDWR	0.08^{ns}
LGR vs RDWR	0.50^{*}
LGR vs TNR	0.03^{ns}
LGR vs TDWR	0.001^{ns}
LGR vs ADWR	0.03^{ns}

[x] Leaf growth reduction (LGR), root growth reduction (RGR), root dry weight reduction (RDWR), root length density reduction (RLDR), shoot dry weight reduction (SDWR), root-to-shoot ratio reduction (RSR), tuber number reduction (TNR), tuber dry weight reduction (TDWR), average tuber dry weight reduction (ADWR), leaf area reduction (LAR).

6.2. Shoot and Root Growth

Two approaches, including a single-node cutting assay (SNCA) and a greenhouse experiment (GE), were employed to evaluate if shoot and root growth reduction can be used to select potato genotypes for drought tolerance. A reduction in root and shoot growth as well as an increase in RS, the typical responses of plants exposed to drought stress (Hsiao, 1973; Harris, 1992; Jefferies, 1983), were also observed both in the SNCA and GE (Table 3.1 and Table 3.4). However, the responses of the genotypes to water stress were not similar. In the SNCA, Kennebec consistently showed superior growth in any variable measured, compared to the other the genotypes tested (Table 3.2 and Table 3.3). On the other hand, Reichie tended to be more drought resistant than the other genotypes in the greenhouse (Table 3.5; Table 3.6). This difference might be due to the differences in the plant materials used to start the experiments, the media, and the environments where the plants were grown, as discussed in Chapter 3. Furthermore, the r^2 values of RLDR and RDWR in the single-node-cutting assay suggested that these two variables were more consistent than those of SDWR and RSR of single node cutting assay and LAR, RDWR, SDWR, and RSR of the greenhouse experiment (GE) (Table 6.1). These findings led to a conclusion that RLDR and RDWR could be used to select potato genotypes grown *in vitro* for water-stress tolerance.

The linear correlation between LGR of leaf disc assay (LDA) and RLDR, RDWR, or RSR of single node cutting assay (SNCA) was significant (Table 6.2). However, due to the low coefficient of determination (r^2) between LDA and SNCA, it may be advisable that both approaches (SNCA and LDA) be carried out simultaneously when screening potato genotypes for water-stress tolerance. A genotype showing low growth reduction

when exposed to water stress in the SNCA and low growth reduction in the LDA would be ideal.

6.3. Tuberization

PEG treatment was able to mimic the effect of drought stress, shown by the reduction in tuber number, tuber dry weight, and average tuber dry weight of potato genotypes grown either *in vitro* or in the greenhouse (Table 4.1 and Table 4.2). These findings confirmed a previous report of Lynch and Tai (1989). The effect of water stress on tuber number reduction (TNR) of *in vitro* experiments (microtuberization assay or MA) was significant, with moderate correlation (r^2 = 0.59*; Table 6.1), indicating moderate consistency of the effects. Reichie (thought to be drought tolerant) consistently demonstrated the lowest TNR (-10.4% in 2002 and 10.6% in 2003) over the years, confirming the claim by CIP that this genotype was drought tolerant. In contrast, the TNR of Kennebec, known to be drought tolerant (Bansal *et al.*, 1991), was statistically not different from TNR of Superior (Table 4.3), known to be responsive to irrigation (Opena and Porter, 1999). These findings raised a question whether TNR might be useful to select potato genotype grown *in vitro* for drought tolerance, because Kennebec was supposed to demonstrate better performance than Superior when exposed to water stress. Also, there was no significant correlation between LGR of the leaf disc assay and TNR of the microtuberization assay.

Although most genotypes showed a reduction in tuber number (Table 4.4), the effect of water stress on TNR of greenhouse experiment (GE) was even less consistent over the years (Table 4.2 and Table 6.1). Kennebec showed the least reduction in tuber

number in 2002, but it was not significantly different from most genotypes tested. In the following year, Kennebec had higher TNR than most genotypes tested even though they were not statistically different. Moreover, the linear correlation between LGR and TNR of greenhouse experiment (GE) was not significant (Table 6.2). These data suggested that one might not use TNR of potato grown in the greenhouse for screening drought tolerance.

PEG treatments were able to mimic the effect of drought stress on tuber dry weight reduction (TDWR) and average tuber dry weight reduction (ADWR) in the microtuber assay (MA) and the greenhouse experiment (GE) (Table 4.1 and Table 4.2). However, the effect was not consistent over the years in the MA (Table 6.1). In the GE, there was a low degree of consistency with r^2 values of 0.21 and 0.35 (Table 6.1). The fact that Kennebec did not statistically differ from Superior or Russet Burbank in both studies (Table 4.3 and Table 4.4) raised a concern whether TDWR and ADWR of potato grown either *in vitro* or in the greenhouse could be used to screen potato genotypes for drought tolerance. This concern was supported by the results of correlation analysis between LGR of LDA and the measured variables of MA and between LGR of MA and the measured variable of GE (Table 6.2).

6.4. Summary and Recommendation

It had been demonstrated in these studies that PEG8000 treatments mimicked the effect of water stress. PEG significantly reduced root and shoot growth, as well as the tuberization of potato genotypes. These studies led to the following conclusions and recommendations.

Leaf growth reduction (LGR) of potato leaf disc floated over PEG8000 solution could be used to screen potato genotypes for water-stress tolerance. Root growth reduction (RGR) and root dry weight reduction (RDWR) of excised root tip grown *in vitro* could be also used to screen potato genotypes for water-stress tolerance simultaneously with LGR of leaf disc floated over PEG8000 solutions. It is likely that these assays screen for different traits that would be desirable in a water-stress-tolerant potato variety.

Root length density reduction (RLDR), root dry weight reduction (RDWR), and root-to-shoot ratio reduction (RSR) of single node potato grown *in vitro* could be used to screen potato genotypes for water-stress tolerance simultaneously with LGR of leaf disc floated over PEG8000 solutions. Due to the inconsistency in the response to water stress, RDWR, shoot dry weight reduction (SDWR), leaf area reduction (LAR) and root-to-shoot ratio reduction (RSR) of potato genotypes grown in the greenhouse from microtuber seeds might not be used to select potato genotypes for water-stress tolerance. In contrast, the results of the greenhouse study were not conclusive. The results of the *in vitro* and greenhouse study for tuber number reduction (TNR), total tuber dry weight reduction (TDWR), and average dry weight reduction (ADWR) were not conclusive. The technique for the greenhouse assay should be refined. For example, screening might be carried out in a growth chamber to minimize the effects of uncontrolled environmental factors.

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