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MICROTUBERIZATION AND DORMANCY BREAKING IN POTATO (*SOLANUM TUBEROSUM* L.)

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

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ABSTRACT

This thesis describes experiments designed to improve microtuberization efficiency, and to evaluate a range of dormancy-breaking agents for microtubers and minitubers. Provision of continuous darkness, agitation to cultures, mechanical resistance to stolons, or lower levels of medium nitrogen did not improve microtuberization. The 16/8 h d/n cycle at step 1 of microtuberization (layering) was significantly better than 12/12 or 8/16 h d/n cycles based on microtuber yield (number and fresh weight). Cultures exposed to prolonged step 2 (microtuber induction and growth) (60-65 d) or 2 successive harvests, rather than a single harvest at 30-35 d, had significantly improved microtuber yield (number, number ≥ 500 mg, fresh weight). In a series of chemical and mechanical treatments applied to microtubers and minitubers, with or without variable periods of cold storage, 500 mg l^{-1} GA_3 was the most efficient in breaking-dormancy and inducing precocious sprouting. GA_3 was the only agent that was able to break dormancy of minitubers that had not been cold stored. After 2 weeks of cold storage, minitubers treated with GA_3 also broke dormancy, while Signal was less effective in promoting sprouting. After 3 weeks of cold storage, microtubers treated with anti-ABA alone, or in combination with GA_3 sprouted, but Signal combined with GA_3 was less effective. On minitubers cold-stored for 3 weeks, GA_3, Signal, or combinations were equally effective in breaking dormancy but fewer sprouts occurred after Signal than GA_3 treatment and no sprouting occurred in the controls. After 8 weeks of cold storage, microtubers and minitubers sprouted readily when exposed to a range of dormancy-breaking agents. Minitubers that were cold-stored for 8 weeks, and treated with dormancy-breaking agents before planting showed yield variations that were attributed to these agents. While GA_3 induced more sprouts than the other treatments, this resulted in the formation of a larger number of tubers that had reduced fresh weights than the control. Tuber number was not affected but the mean fresh weight of tubers was significantly increased above the control in the Signal treatment.

MICROTUBÉRISATION ET LEVÉR DE LA DORMANCE DE LA POMME DE TERRE (*SOLANUM TUBEROSUM* L.)

RÉSUMÉ

Cette thèse a pour but d'améliorer l'efficacité des techniques de microtubérisation et d'évaluer une gamme de produits susceptibles de lever la dormance des microtubercules et des minitubercules. Le maintien à l'obscurité, l'agitation des cultures, la résistance mécanique des stolons, ainsi que les niveaux réduits d'azote n'ont pas amelioré la microtubérisation. La photopériode de 16/8 h (j/n) pendant la première étape de microtubérisation a entrainé une augmentation significative du rendement des microtubercules (nombre et poids frais) comparativement aux régimes de 12/12 et de 8/16 h (j/n). Une prolongation de la durée de la deuxième étape de microtubérisation (60-65 j), ainsi que deux récoltes successives au lieu d'une seule (30-35 j), ont amelioré la production des microtubercules (nombre, nombre ≥ 500 mg et poids frais). Parmi plusieurs traitements chimiques ou mécaniques, avec différentes périodes de stockage au froid, l'application de 500 mg l^{-1} GA_3 était la plus efficace pour la levée de la dormance et l'induction du développement précoce des germes dans les microtubercules et les minitubercules. L'application du GA_3 était la seule méthode suceptible de lever la dormance des minitubercules non soumis au froid.Après deux semaines d'entreposage au froid, la dormance des minitubercules a été levée par GA_3 mais pas par le traitement Signal. La dormance des microtubercules gardés au froid pendant 3 semaines a été levée davantage par les anti-ABA en combinaison avec GA_3, que par GA_3 combiné a la solution Signal. Cependant, la dormance des minitubercules a été aussi bien levée par les traitements de GA_3 et de Signal que par leurs combinaisons. Les témoins n'ont pas été affectés par ces traitements. Après huit semaines d'entreposage au froid, les mt et les minitubercules ont germé quelque soit le traitement appliqué. Le rendement des minitubercules gardés au froid pendant huit semaines puis traités avec des agents avant la plantation, a varié suivant le traitement appliqué. Tandis que le traitement de GA_3 a augmenté le nombre des germes plus que les autres traitements, il a entrainé la reduction du poids d'un grand nombre de tubercules, comparativement aux témoins. Le traitement Signal n'a pas affecté le nombre total des tubercules, mais a entrainé une augmentation significative du poids frais moyen.

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The Author

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LIST OF ABBREVIATIONS USED IN THE THESIS

Abbreviation	Full description
ABA	abscisic acid
AC	alternating current
BAP	6-benzylaminopurine
BE	bromoethane
^0C	degree Centegrade (Celcius)
CCC	chlorocolinechloride
CIP	International Potato Center (Peru)
cm	centimetre
CRD	complete randomized design
cv.	cultivar
DC	direct current
d/n	day/night
FAO	Food and Agricultural Organization of the United Nations
Fig.	figure
g	gram
GA_3	gibberellic acid
h	hour
IAA	indole-3-acetic acid
Kcal	kilocalorie
Kn	kinetin
KPa	kiloPascal
l	litre
LD	long day
$\mu l l^{-1}$	micro litre per litre
$\mu mol\ m^{-2}s^{-1}$	micro mole per square metre per second
mg	milligram

mgl^{-1}	milligram per litre
ml	millilitre
mll^{-1}	millilitre per litre
mm	millimetre
mM	millimole
mo	month
MS	Murashige-Skoog basal medium (1962)
NAA	naphthalene acetic acid
P	probability
PBI	Plant Biotechnology Institute (Saskatoon)
SD	short day
step-1 microtuberization	layering step
step-2 microtuberization	microtuber induction and growth step
T	ton
WHO	World Health Organization
wk	week

CHAPTER 1 *GENERAL INTRODUCTION*

Potato (*Solanum tuberosum* L.) is an integral part of the diet of a large proportion of the world's population. It is ranked fourth in the list of major world food crops following rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) and its production represents roughly half of the world's annual output of all roots and tubers (CIP and WHO, 1995). It is an excellent food source for people of areas with high population density because it provides more nutritious food from less land, in less time, and often under more adverse conditions, than most other crops (CIP, 1984). Potatoes are consumed by people, fed to animals, and used for starch and alcohol production. The major reasons for rapid increase in global potato production are its adaptability to a wide range of growing conditions (Woolfe, 1987) and its excellent nutritional value. Potato contains high quality protein and substantial amount of minerals, vitamins and trace elements (CIP, 1984). The high lysine content of potato has made it advantageous over the cereal staples. Its protein content is comparable, on a dry weight basis, with that of the cereals and on a cooked basis, with that of boiled rice or of cereals cooked as porridges (Woolfe, 1987). The energy content of raw potato is 344 KJ (80 Kcal) 100 g^{-1} (Toma *et al.*, 1978). A small quantity (100 g) of boiled potato supplies up to 13% of the FAO/WHO recommended daily allowance of protein for children and up to 7% of the adult allowance (CIP, 1984).

Global potato production was about 135 x 10^6 T at the beginning of the 20^{th} century, 250 x 10^6 T in the 1950s and 200 x 10^6 T in the late 1980's (Horton and Anderson, 1992). About 75% of the world's potatoes are grown in developed countries (40% in Europe) with 25% in developing countries (Horton and Anderson, 1992). Potato is grown in 140 countries, more than 100 of which are located in the tropical and sub-tropical zones (Beukema and Van der Zaag, 1990). Potato is considered one of the most efficient crops for converting natural resources, labour, and capital into a high quality food (Horton, 1981).

Microtuberization refers to the *in vitro* production of miniature potato tubers (microtubers) either directly from axillary buds or on stolons that grow from leaf axils of micropropagated potato plantlets. *In vitro* microtuberization was first reported by Barker (1953). Potatoes can be micropropagated rapidly on a large scale by meristem and shoot tip culture (Roca *et al.*, 1978; Goodwin *et al.*, 1980; Escalada and Garcia, 1982) or by proliferation of axillary shoots developed from single-node cuttings (Espinoza *et al.*, 1984 (cited in Espinoza *et al.*, 1986); Hussey and Stacey, 1984).

In vitro produced microtubers have advantages compared with conventional field-grown tubers; they are small, light, and aseptic. They resemble field-grown tubers in shape and colour and the electrophoretic patterns of their storage proteins are identical (Espinoza *et al.*, 1986). Plantlets derived from microtubers in the field grow normally under field conditions (Espinoza *et al.*, 1986; Leclerc *et al.*, 1990).

Both micropropagated potato plantlets and microtubers can be mass-produced in culture under high density growing conditions independent of seasonal fluctuations. They are used to increase valuable specific pathogen tested potato stocks. Plantlets are more vulnerable to damage during storage, shipping, and transplant than microtubers. Microtubers are more easily utilized in automated commercial propagation and for large-scale mechanized planting, compared with plantlets (McCown and Joyce, 1991). Both are used to produce greenhouse minitubers for nuclear stock. Minitubers are larger than microtubers and are field-planted in the first of the elite levels of certified seed tube production.

Microtuberization efficiency is increased when a 2-step procedure is used that involves first layering and then microtuber induction (Leclerc *et al.*, 1994). Plantlets with their shoot tips and roots removed are layered in liquid medium to allow them to develop axillary shoots that can contribute to more efficient stolon production. Once these axillary shoots have grown microtubers are induced. Several factors are involved in induction of microtubers including short photoperiod (Ewing, 1978; Wang and Hu, 1982; Slimmon *et*

al., 1989; Seabrook *et al.*, 1995), low temperature (Bushnell, 1925; Marinus and Bodlaender, 1975; Wang and Hu, 1982; Garner and Blake, 1989; Snyder and Ewing, 1989; Khuri and Moorby, 1996a), osmotic concentrations (Lawrence and Barker, 1963; Wang and Hu, 1982; Hussey and Stacey, 1984), and level of nutrients, primarily nitrogen in the medium (Krauss and Marschner, 1976; Moorby, 1978; Krauss, 1985; Cutter, 1978; Dobranszki and Mandi, 1993; Hoque *et al.*, 1996; Zarrabeitia *et al.*, 1997), presence or absence of growth regulators (Harmey *et al.*, 1966; Wareing and Jennings; 1978; Garner and Blake, 1989; Hussey and Stacey, 1984; Leclerc *et al.*, 1994; Khuri and Moorby, 1996b), flux density (Garner and Blake, 1989; Avila *et al.*, 1996; Kane, 1996; Seabrook *et al.*, 1995) and gaseous environment in the culture containers (Paterson, 1970; Angel *et al.*, 1974). Microtuber induction and yield may be manipulated by changing one or more of these factors.

Freshly harvested microtubers and minitubers, like field-grown tubers, exhibit a period of dormancy; they do not readily sprout even if environmental conditions are favourable, including optimum temperature and humidity. While it is sometimes convenient to store them, it is frequently desirable to plant them immediately. Dormancy is important while storage is desired, but lack of control over microtuber dormancy duration may detract from their immediate efficient use at any time of the year. The length of the dormancy period is cultivar-specific; varying from 5 to 10 weeks (Emillson, 1949) and appears to be analogous for field-grown tubers as for microtubers (Leclerc *et al.*, 1995). The problem of efficient use of minitubers, and control of dormancy duration for immediate use when wanted is identical to that of microtubers. Dormancy is thought to be regulated mainly by endogenous ABA content present in tubers (Rappaport *et al.*, 1957; Hemberg, 1985; Van den Berg *et al.*, 1991; Suttle and Hultstrand, 1994).

The objectives of this research were to try to improve the microtuber production system used in our laboratory (Leclerc *et al.*, 1994) by experimenting with aspects of the incubation environment (variable photoperiodic cycles i.e.16/8, 12/12, 8/16 h d/n cycles

and continuous darkness; agitation, mechanical resistance) or medium (different nitrogen levels) during the microtuberization layering (step 1) or induction (step 2) phases, and to develop method(s) of limiting microtuber and minituber dormancy using a range of dormancy-breaking agents.

CHAPTER 2 *LITERATURE REVIEW*

Plant tissue culture has played a very important role in the improvement of cultivated potato. For many years tissue culture techniques have been applied to improve potato production by means of thermotherapy and meristem or shoot tip culture for pathogen elimination, micropropagation, microtuberization, and germplasm conservation.

Micropropagation of potato is usually done by cutting single-node segments and subculturing them on a monthly basis, onto fresh medium, to promote new plantlet development. Efficient *in vitro* production of potato microtubers involves a two step procedure consisting of layering (step 1) and minituber induction (step 2) (Leclerc *et al.*, 1994). In layering (step 1), shoot tips are cut off from micropropagated plantlets to break apical dominance and the roots are removed. Then shoots are layered horizontally during culture to promote axillary buds to develop into new shoots and stolons. In step 2, cultures are incubated at low temperature (15°C) and shorter photoperiod (8/16 d/n) to induce microtuberization.

The physical status of the culture medium has a great influence on micropropagation and microtuberization. The basal medium mostly used for potato micropropagation is Murashige and Skoog (MS; 1962) basal salt medium with vitamins, myo-inositol and sucrose, solidified with agar (Goodwin *et al.*, 1980; Hussey and Stacey, 1981; Wang and Hu, 1982; Estrada *et al.*, 1986; Akita and Takayama, 1988; Garner and Blake, 1989; Leclerc *et al.*, 1994; Bizarri *et al.*, 1995; Hoque *et al.*, 1996; Khuri and Moorby, 1996a,b; Kane, 1996; Avila *et al.*, 1996; Hulscher *et al.*, 1996; and Ziv and Shemesh, 1996). Improvement of growth and yield in liquid medium is caused by greater availability of water (Bouniols, 1974; Debergh, 1983) and of nutrients (Debergh, 1983) compared with solidified medium, due to lower resistance to diffusion and closer contact between the plant and the culture medium (Singha, 1982; Pierik, 1990).

Although microtubers develop from single-node segments in MS solid medium microtuberization was greatly favoured by liquid medium (Rosell *et al.*, 1987; Leclerc *et al.*, 1994; Avila *et al.*, 1996). Layered shoots microtuberized more rapidly and produced significantly larger microtubers than conventionally used nodal segments (Leclerc *et al.*, 1994). Layering saves both time and labour. *In vitro* grown plantlets or microtubers are transplanted into greenhouses for production of ex *vitro* minitubers. These microtubers are used as the first level (nuclear) in seed certification programs.

For microtuber induction several factors are considered to be influential. The major ones are photoperiod, temperature, osmotic concentration, illumination (flux density), and growth regulators.

Proper photoperiodic induction is one of the main factors for microtuberization because under proper photoperiod tuberization stimulus is perceived by the cultured plants. For potato micropropagation and microtuberization different photoperiods and temperatures are required. Cultured nodes produce single stemed shoots at 20-25°C temperature for 16 h (Hussey and Stacey, 1984; Leclerc *et al.*, 1994; Avila *et al.*, 1996). For microtuberization, relatively lower temperature and photoperiod i.e. 15-18°C for 8 h are applied (Wang and Hu, 1982; Garner and Blake, 1989; Slimmon *et al.*, 1989; Khuri and Moorby, 1996b). According to Hussey and Stacy (1984) long photoperiod is good for vegetative growth because vigorous leafy shoots are produced in long days. Short photoperiod affects micropropagated shoots; (1) leaflets get larger (Bodlaender, 1963), (2) stems get shorter (Schick, 1931; Edmundson, 1941), (3) the dry weight ratio of leaves to stems increases (Driver and Hawkes, 1943), (4) axillary branches get suppressed (Demagante and Van der Zaag, 1988), (5) root dry weight decreases (Steward *et al.*, 1981), and (6) stolon growth is replaced by tuber growth (Rasumov, 1931). Partitioning of assimilates usually shifted toward tubers, as photoperiod was shortened (Wolf *et al.*, 1990). Leafbud cuttings taken from short day (SD) and long day (LD) potato plants showed that SD plant leaves were much more strongly induced to tuberize than LD plant

leaves (Lorenzen and Ewing, 1990). Previous investigation showed that greenhouse potato plants derived from tissue cultured plantlets exposed to 12 h photoperiod increased the number, dry weight, and specific gravity of microtubers compared with plantlets micropropagated using 16 h photoperiod (Seabrook *et al.*, 1995). The tuberization stimulus was favored by photoperiods shorter than a critical photoperiod i.e.15 h or more (Ewing, 1978). Long photoperiodic treatment gave an overall reduction in tuberization (Snyder and Ewing, 1989; Seabrook *et al.*, 1995). During potato micropropagation prior to culturing the plantlets for tuberization, a regime of 16 h photoperiod was most commonly applied. It was reported that during a period of 120 d *in vitro* culture, inversion from LD (16h) to SD (8h) at 50 d increased the number of microtubers (Vecchio *et al.*, 1994). Microtubers produced under 8 h photoperiod usually weigh more than those produced under a 16 h photoperiod (Kane, 1996). On the contrary, acceleration of microtuberization was reported to occur in darkness (Akita and Takayama, 1988; Slimmon *et al.*, 1989; Levy *et al.*, 1993; Dobranszki *et al.*, 1993; Zarrabeitia *et al.*, 1997) in different systems.

Temperature is a major determining factor for microtuberization. Traditionally, potato has been classified as a cool season crop and Bushnell (1925) reported that 17°C was the optimal mean temperature for good yield. The optimal temperature for net photosynthesis has been estimated as extending from 15-20°C (Winkler, 1961) or 16-25°C (Ku and Edwards, 1976). Higher temperature can have adverse effects on potato yields (Bodlaender, 1963; Ewing, 1981; Menzel, 1983; Nowak and Colborne, 1989). At high temperatures (28-30°C) potato plants show considerable stem elongation at the expense of tuber yields. The leaf/stem ratio at high temperature decreases (Warner, 1934; Gregory, 1954; Bodlaender, 1963). Higher temperature and longer photoperiod delay the onset of tuber growth and linear tuber bulking (Benkhedher and Ewing, 1985; Vandam *et al.*, 1996). The cultivars Kennebec and Arran Pilot did not form tubers when high temperature was combined with LD or low light intensity, respectively (Gregory, 1954;

Borah and Milthorpe, 1962). Although SD and low temperature were inductive to tuberization, raising the temperature to 30°C caused about 50% reduction in tuber dry weight in the cv. Katahden (Snyder and Ewing, 1989). High temperature favoured tuber yield in the wild potato species *S. commersonii* (Davis, 1941). It is generally agreed that low temperature and short photoperiod restrict haulm growth and promote the accumulation of dry matter in the tubers (Menzel, 1985; Struik, 1989). It is well accepted that tuber yield of *S. tuberosum* cultivars is favoured by relatively low temperature (Marinus and Bodlaender, 1975).

The concentration of sucrose, the main carbohydrate source in culture media, is thought to be one of the controlling agents for microtuberization. Micropropagation of nodal segments occur in 2-3% sucrose, but the shoots require higher levels of sucrose for microtuberization to occur. No tubers were formed on nodal stem pieces cultured on a medium containing 2% sucrose (Harmey *et al.*, 1966). Several researchers have reported that 6% sucrose was optimal for microtuberization when medium was supplemented with 6-benzylaminopurine (BAP) and chlorocholine chloride (CCC) (Hussey and Stacey, 1984; Abbott and Belcher, 1986) but later on the optimal level of sucrose was standardized at 80-90 gl^{-1} (Lawrence and Barker, 1963; Harmey *et al.*, 1966; Wang and Hu, 1982) when no growth regulators were used.

Researchers have applied different illumination sources like cool white fluorescent, agrolite fluorescent, grolux fluorescent etc. during the micropropagation phase but few workers have explored the application of different coloured light. The colour of light may play an influential role on the growth of cultured materials. It was reported that blue light was essential for overall normal development of plants (McLachlan, 1993). Potato stem length was increased using yellow light (Seabrook *et al.*, 1996). Multiplication of potato plantlets requires internodes, which are of approximately 1.0 cm length. This allows for sufficient space in between the leaves to maneuver the dissecting blade around the delicate leaves. Seabrook's work was with slow growing

potato cultivars that exhibited short internodes. The variation of the amount of light (μ mol $m^{-2}s^{-1}$) does have an effect on the growth of potato stems since photosynthesis is greatly influenced by the light intensity. Some researchers used 30 (Kane, 1996), some used 47.5 (Avila *et al.*, 1996), 85 (Garner and Blake, 1989; Leclerc *et al.*, 1994), or 160 μ mol $m^{-2}s^{-1}$ (Abbott and Belcher, 1986).

The effects of growth regulators on microtuberization were investigated by several researchers (Harmey *et al.*, 1966; Wareing and Jennings, 1979; Garner and Blake, 1989). The different growth regulating substances they worked with are cytokinins (Kinetin (Kn) and BAP), gibberellic acid (GA_3), abscisic acid (ABA), indole-3-acetic acid (IAA), maleic hydrazide (MH), CCC, and coumarin. Some of these (i.e. Kn, BAP, CCC) were inductive to tuberization while some (i.e. ABA, GA_3) were inhibitory. The promotion of tuberization on isolated stolons by exogenous kinetin was first demonstrated by Palmer and Smith (1969). Later on, it was found that cytokinins were not directly responsible for the onset of tuberization but they do play an important role in tuber growth (Sattelmacher and Marschner, 1978). Kinetin-induced tuberization on isolated stolons was inhibited by ABA application (Palmer and Smith, 1969). Exogenous GA inhibited tuberization in potato whether applied to whole plants or to stem cuttings (Tizio, 1971). Ethrel (2-chloroethyl-phosphonic acid) or ethylene was thought to induce tuber initiation. But later on it was demonstrated that although ethylene or ethrel promoted swelling in the subapical region of stolons these did not contain starch (Catchpole and Hillman, 1969). They then concluded that ethylene may play a part in the early stage of tuber initiation and that starch deposition may occur at a separate stage of development. Maleic hydrazide did not induce tuberization but increased the tuber size (Harmey *et al.*, 1966). Since Garner and Blake (1989) were able to induce microtuberization in media free of growth regulators, the use of these growth regulators for microtuberization became of little use. This is because the commercial production cost using media free of growth regulators will undoubtedly be lower. Now the work of

Akita and Takayama (1988), Leclerc *et al.* (1994), Hulscher *et al.* (1996) and Khuri and Moorby (1996b) have established and confirmed the non-essentiality of growth regulators during microtuberization.

Incubation period was positively correlated with increased microtuber fresh weight. The incubation period for microtuberization ranges from 4-10 wk depending on the type of cultivar. Early cultivars like Norland and Norchip required 4-5 wk of incubation although, as incubation period was increased up to 7 wk, the microtuber number and fresh weight increased (Leclerc *et al.*, 1994).

Techniques of plant tissue culture provide obvious advantages for investigating the responses of the potato plant to nutrition and other environmental conditions. The amount of nitrogen had an effect on microtuberization (Cutter, 1978; Moorby, 1978). A reduction in the availability of nitrogen can initiate rapid tuberization (Krauss and Marschner, 1976; Sattelmacher and Marschner, 1978, Krauss, 1985, Ewing and Struik, 1992). Hoque *et al.*, (1996) reported that higher number of microtubers can be produced in MS medium by reducing KNO_3 by half of the total amount but they could not conclude whether it was due to the reduction of nitrogen or potassium. Reduction in the amount of NH_4NO_3 to 1/8th of its amount present in MS medium, initiated early and higher microtuberization after 4 wk of culture in cv. Jaerla (Zarrabeitia *et al.*, 1997). Low level of N (5 mM) was also reported to produce better minitubers (Teixeira and Pinto, 1991).

The gaseous environment in the culture containers is also a factor for efficient microtuber production. It is reported that a constant flow of carbon dioxide increases microtuber yield (Angel *et al.*, 1974; Paterson, 1970). Potato shoots cultured on sucrose-free medium exhibited up to a 9-fold dry weight increase at CO_2 levels of 10,000 to 50,000 $\mu l\ l^{-1}$ CO_2 using a passive diffusion flow system (Cournac *et al.*, 1991; Buddendorf-Joosten and Woltering, 1996).

Mechanical resistance encountered by stolons is thought to induce and enhance microtuberization. Developing stolons that fail to encounter sufficient mechanical

resistance had extremely vigorous stolon-growth and delayed tuberization (Lugt *et al.*, 1964; Cary, 1986) and secondary stolons and numerous small tubers (Gray, 1973, Cary, 1986). Similar phenomena were observed by Vreugdenhil and Struik (1989). But it is still unknown whether mechanical resistance has any positive impact on tuber induction. It is not known whether still higher yields could be obtained by providing more mechanical resistance (Ewing and Struik, 1990).

It is important to break tuber dormancy for seed potato multiplication, rapid post-harvest disease testing, and early production in the field or greenhouse. Tubers are considered dormant if they are unable to sprout at a favourable temperature (Coleman, 1987). Dormancy of potato tuber buds is difficult to control. The period of dormancy depends on the type of variety, the maturity of the tubers, soil and weather conditions during growth and storage conditions (Beukema and Van der Zaag, 1990). Immediately after harvest, field-grown potato tubers or *in vitro* microtubers or greenhouse-grown minitubers can not be induced to sprout even under optimal environmental conditions. This is usually termed as the rest period and it is of 5-10 wks duration in different cultivars (Emillson, 1949; Cho *et al.*, 1983). The bud rest in potato tubers has been reviewed by Hemberg (1985). In the so-called rest period tuber buds fail to grow even under optimum temperature and light conditions and there appears to be an internal 'clock' which triggers cellular events leading to sprouting (Rappaport and Wolf, 1968, 1969). According to others the tubers may be unable to supply the buds with metabolites essential for growth (Madec and Perennec, 1969). When the rest period is terminated then the buds of the tuber resume growth and begin to sprout out. The optimum temperature for sprout growth is 16-20°C (Beukema and Van der Zaag, 1990). For many years researchers have been trying to break the tuber bud dormancy or to shorten the resting period, but no appreciable results were achieved before the 1980's, after which Coleman (1983) started working extensively on the breaking of tuber dormancy. To shorten the rest period different chemicals have been applied. Treating dormant potato tubers with ABA (abscisic acid),

MH (maleic hydrazide), NAA (naphthalene acetic acid) and ethylene did not accelerate dormancy breaking (Stallknecht, 1983), rather they all were proved to be inhibitory. Ethylene and thiourea inhibited growth of apical sprouts but led to outgrowth of swollen sprouts from the lateral buds (Minato *et al.*, 1979 and Stallknecht, 1983). GA_3 (gibberellic acid) is reported to shorten the rest period of freshly harvested potatoes, to hasten sprouting and emergence (Rappaport, 1957; Rappaport *et al.*, 1957; Choudhury and Ghose, 1963) and under certain conditions, to increase tuber yield (Timm *et al.*, 1960). Gibberellin-like substances have been found in peelings of potato tubers (Smith and Rappaport, 1961; Okazawa, 1959). They showed that the level of endogenous gibberellins remained low during the rest period and increased towards the end of this time. Boo (1962) found an increase in endogenous gibberellin-like substances during the last part of the rest period. The most interesting finding was that gibberellin-like substances increased markedly following wounding of the tuber tissues. The amount of such substances produced by an excised plug of tuber tissue would be sufficient to trigger changes in the buds associated with termination of dormancy (Rappaport and Sachs, 1967). Treatments with GA_3 proved that GA_3 at a concentration of 500 mg l^{-1} was effective in breaking dormancy when the tubers were soaked for 90 min. (Rappaport *et al.*, 1957). Rappaport dealt with field grown potatoes. In the case of microtubers, dormancy was influenced by cultivar and microtuber size (Leclerc *et al.*, 1995). According to them the dormant periods of microtuber were positively correlated with the relative dormant periods of field-grown tubers. They reported that cultivars having tubers with short dormancy *in vivo* also had microtubers with short dormancy periods *ex vitro*. Microtuber dormancy is also influenced by the time of harvest. Microtubers had longer dormancy when harvested after 28 d as compared with 56 d of incubation (Leclerc *et al.*, 1994). Gibberellins A3, A4, A5, and A7 stimulated sprouting; NAA and IAA both promoted sprouting slightly at low concentrations (4×10^{-8} M) but inhibited sprouting at higher concentration i.e. 4×10^{-5} M (Rappaport *et al.*, 1957). Several researchers worked

extensively with a chemical compound named bromoethane (BE); treating potato tubers with BE vapour (0.2 ml l^{-1}) shortened dormancy and initiated sprouting (Coleman, 1983, 1987; Coleman and Coleman, 1986; Coleman *et al.*, 1992; Leclerc *et al.*, 1996). BE at 0.2 ml l^{-1} was effective on freshly harvested field-grown potato tubers (cvs. Bintje, Kennebec, Red Pontiac, Russet Burbank) and 0.1 ml l^{-1} was only effective on tubers that had already met their dormancy requirement (Coleman, 1983). He obtained 50% sprouting in freshly harvested tubers in less than 8 d. Kocacaliskan (1990) reported that electric currents could help in breaking dormancy associated with potato seed tubers. He found that 50 V of alternating current (AC) or direct current (DC) could induce tuber sprouting, but it was not as efficient as BE. BE was the most effective for high sprout numbers and for short emergence time of sprouts.

ABA plays a pivotal role in the regulation of plant dormancy (Addicott and Cairns, 1983). At an early stage of potato tuber dormancy there is a synthesis of substantial amounts of endogenous ABA in tubers and this declines as sprouting commences (Suttle and Hultstrand, 1994; Korableva *et al.*, 1980; Coleman and King, 1984; Van den Berg *et al.*, 1991). The levels of ABA in fresh microtubers were assayed by Suttle and Hultstrand (1994). After 3 wk of microtuber development *in vitro*, the ABA content of microtubers was 214 ± 9 pmol g^{-1} fresh weight. They also reported that microtubers developed in the presence of fluridone had a reduced amount of endogenous ABA (23 ± 1 pmol g^{-1} fresh weight) and it caused precocious sprouting. There was no evidence of a threshold concentration of ABA below which sprouting would occur (Coleman and King, 1984). ABA prolonged the dormancy of tubers and retarded the growth of buds.

A chemical called ABA-mimic or racemic-ABA or anti-ABA was effective in shortening the cold treatment and bringing about early flowering in canola (*Brassica campestris*) (PBI Bulletin, 1995). An acetylenic ABA analog known as PBI-51, had physiological effects identified as antagonistic to ABA in a variety of biological systems

(Abram and Gusta, 1989; Reaney *et al.*, 1990; Walker-Simmons *et al.*, 1992). The acetylenic analog of ABA reversed the inhibitory effects of ABA on cress seed germination (Reaney *et al.*, 1990). The biological activity of PBI-51 was measured using microspore-derived embryos of *Brassica napus* as the biological material and their response to ABA in the expression of genes encoding storage protein (Wilen *et al.*, 1993). Possible explanations for the inhibitory effects of PBI-51 were that it somehow promoted the degradation of endogenous ABA to less active metabolites like phaseic and dihydrophaseic acids (Wilen *et al.*, 1993). This chemical has never been used before against endogenous ABA found in the potato tubers/minitubers/microtubers during dormancy. Signal solution, a fermentation tank product, was reported to be involved in facilitating nodulation of soybean (*Glycine max* L.) seed under cool soil conditions (Bios, Montreal, QC. Canada). This solution was also used in the present experiments to see if it had any effect on dormancy breaking.

CHAPTER 3 *MICROTUBER PRODUCTION*

3.1 Introduction

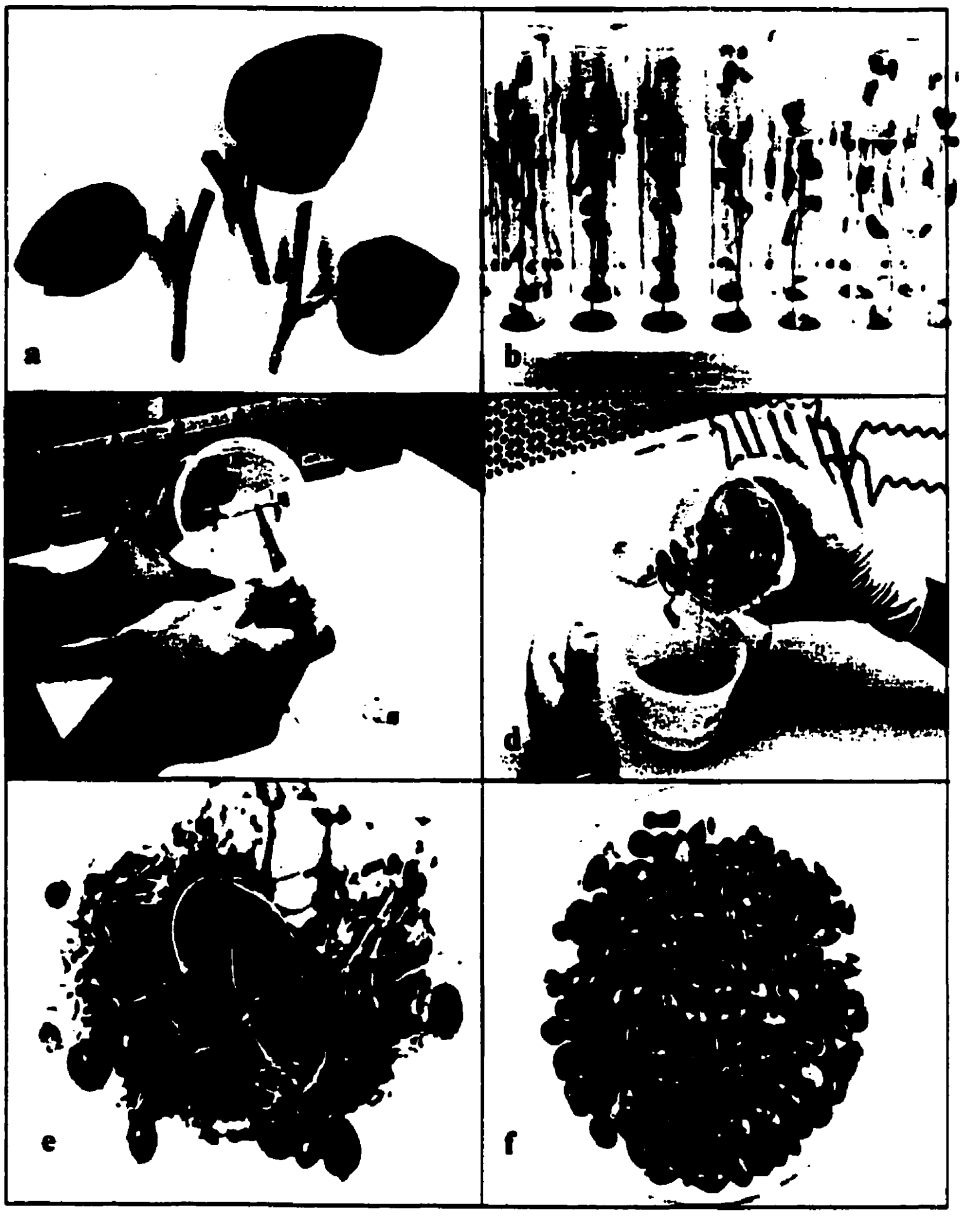
Microtuberization is the process of microtuber development in vitro. For several years this method has been practiced to produce microtubers for the production of greenhouse minitubers that serve as the primary source of nuclear seed stock. Several environmental and nutritional factors act together to induce microtuberization and a change in one or more of these factors may affect the efficiency of microtuber production. In these investigations, experiments were designed to investigate aspects of the physical environment (i.e. light, agitation, mechanical resistance, and photoperiodic cycles), nutritional level (mainly N), and incubation duration, with a view to improving the efficiency of microtuber production.

3.2 Materials and Methods

Plant Materials and The Establishment of Aseptic Cultures

The potato cvs. Norland and Onaway were obtained as in vitro plantlets from the Plant Propagation Centre, Department of Agriculture and Rural Development, New Brunswick, Canada. Cultured plantlets were increased through single-node divisions (Fig. 1a) at 1-mo intervals in 10 ml micropropagation medium in 24 x 150 mm glass test tubes. Plantlets 1-mo-old (Fig. 1b) were used for microtuberization which involved the 2-step procedure described by Leclerc *et* al. (1994). In step 1 (layering) plantlets with root and shoot tip removed (5-nodes long) were layered in 50 ml of liquid layering medium in 400 ml Better Plastics containers (Kissammee FL, U.S.A.) (Fig. 1c). During step 1 axillary shoots and stolons developed. After 4 wk, the step-1 medium was drained-off (Fig. 1d) and replaced with 50 ml of step 2 induction medium. During step 2 microtuber induction and growth occurred (Fig. 1e). Harvest occurred after 5 wk (Fig. 1f).

Figure 1. Micropropagation and microtuberization in potato. a) Nodal segments; b) 1 month-old plantlets ready for step 1 layering; c) Layering of a single plantlet with removed roots and shoot tip; d) Cultures for microtuber induction at step 2; e) Microtubers developed on layered shoots, and f) Harvested microtubers.


a
b
d
e
f

Media Preparation

Micropropagation medium was Murashige and Skoog (MS; 1962) basal salt formulation with MS vitamins and glycine as well as 2.0 mg l^{-1} Ca-pantothenate. It also included 100 mgl^{-1} myo-inositol, 30 g l^{-1} sucrose, and 7 g l^{-1} agar (Anachemia, Montreal, QC, Canada). Layering medium was similar except that the sucrose level was 20 g l^{-1}, no agar was used, and the growth regulators added were BAP at 0.5 mg l^{-1} and GA_3 at 0.4 mg l^{-1}. Induction medium was similar except that sucrose level was 80 g l^{-1} and there were no growth regulators or agar. The pH for all media was adjusted to 5.7±1. Media were autoclaved for 20 min (micropropagation medium) or 25 min (layering and induction media) at 121°C, 103.46 KPa.

Incubation Conditions -Micropropagation and Layering (Step 1 Microtuberization)

Micropropagated and layered cultures were incubated at 20°C under a long day (16/8 h d/n cycle) with 45 μ mol $m^{-2}s^{-1}$ photon flux density (cool white fluorescent lamps) in a walk-in growth room.

3.3 Experiment 1- Effects of Light and Dark With or Without Agitation on Microtuber Yield

Micropropagation and the layering step of potato microtuberization, are usually carried out under long days (16/8 d/n cycle) in stationary cultures (Leclerc *et al.*, 1994). The exception to this involves the conditions applied to jar fermentor systems where continuous dark and agitation using shakers (125 rpm; Akita and Takayama, 1988) or impelled air (Hulscher *et al.*, 1996) improved microtuber yields. Changes of photoperiod from long days to darkness (Slimmon *et al.*, 1989; Levy *et al.*, 1993) and from short days to continuous darkness (Dobranszki and Mandi, 1993) promoted or accelerated

microtuberization in different systems. In Experiment 1, an investigation was carried out to determine if similar changes in the physical environment applied during the induction phase (step 2) of potato microtuberization (± light ± agitation) would affect microtuber yields.

Materials and Methods

Induction stage conditions involved four treatments, with eight replicates per treatment, and each treatment randomly positioned in the growth chamber (Fig. 2A). The treatments included short days (8/16 h d/n cycle) at 80 μ mol $m^{-2}s^{-1}$ or continuous dark (wrapped cultures), with or without agitation on rotary shakers (Lab Line, Model No. 3520, LabLine Instrument Inc.) used at 130 rpm, and located inside the growth cabinet. One rotary shaker was used for dark-agitation and the other for light-agitation. Stationary cultures were placed on wooden platforms at the same height as the shakers and enclosed in boxes in which ventilation holes were made (Fig. 2B). Cultures were incubated at 15°C for 5 wk in a growth chamber. After 5 wk, microtubers were harvested and yield (mean number, mean number ≥ 500 mg, and mean total fresh weight per container) determined. Statistical analysis was performed using ANOVA (SAS, Cary, NC, 1989).

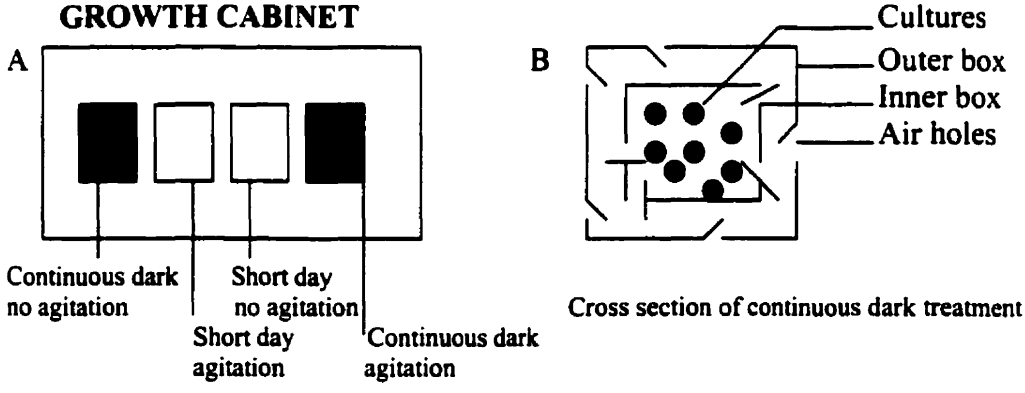


Fig.2. A. Set-up of treatments in the growth cabinet; B. Technique of providing continuous darkness.

Results

Microtuber number per container was similar in all treatments but the number of microtuber ≥ 500 mg was greater in cultures incubated under short days than in continuous darkness (Table 3-1). Microtuber FW per container was greater in cultures incubated under short days than in continuous darkness. Microtubers that developed in the short day treatments formed submerged in the medium and were reddish-green in colour. In contrast, microtubers that formed in the dark were borne aerially above the medium and were purple in colour.

In conclusion, microtuber yields (number, number ≥ 500 mg, and fresh weight per container) were best in cultures incubated under short day conditions (8/16 h d/n), rather than in continuous darkness, and yields were not improved by agitation on a rotary shaker. These results are clearly in contrast with the results described for bioreactors (Akita and Takayama, 1988; Hulscher *et al.*, 1996) and for stationary *in vitro* cultures in the dark (Slimmon *et al.*, 1989; Levy *et al.*, 1993; Dobranszki and Mandi, 1993). However, others have found that short days were stimulatory to microtuberization compared with continuous darkness (Garner and Blake, 1989) and shaking would not have been beneficial if air was not limited inside the culture containers.

Table 3-1.The effect of short days (8/16 h d/n cycle) or continuous darkness, with or without agitation (130 rpm) on microtuber yield (mean number, number ≥ 500 mg, and total fresh weight per container).

Treatments	Number		Fresh weight
	Mean	≥ 500 mg	mg
Short days No agitation	12.75 a	2.37 a	29.31 a
Short days Agitation	12.00 a	2.12 a	29.04 a
Continuous dark No agitation	13.00 a	0.62 b	17.66 b
Continuous dark Agitation	10.50 a	0.12 c	13.43 b

Numbers within each column represented by the same letters are not significantly different at the 0.05 level.

3.4 Experiment 2 - Effects of Reduced Medium Nitrogen on Microtuber Yield

Nitrogen is one of the major nutrient elements that regulate plant growth and development. The form of nitrogen supplied to plants may influence the uptake of other nutrients (Schmitz and Lorz, 1990). Potato preferentially take up nitrate over ammonium (Davis *et al.*, 1989). A low level of ammonium supplied in combination with nitrate is favourable to tuber growth, but a higher level of ammonium supply is detrimental (Garner and Blake, 1989). Nitrogen fertilization levels are routinely decreased in the field (Payton, 1989) and in greenhouses (Teixeira and Pinto, 1991) at the time of tuber induction. The concentration of salts in MS basal salt medium for optimum growth in different crops, including potato, was too high for optimal growth (Pierik, 1987; Pennell, 1987). In MS basal salt medium the two main sources of nitrogen (N) are ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3). It is the ammonium ion (NH_4^+) which is

thought to be detrimental to potato tuber yield if nitrate:ammonium ratio is greater than 2:1 (Garner and Blake, 1989). Reduction of KNO_3 by 50% increased microtuber production in four cultivars namely, Diamant, Cardinal, Kufri Sindhuri, and Ajiba (Hoque *et al.*, 1996). Reduction of NH_4NO_3 by 90% increased minituber yield in cv. Jaerla (Zarrabeitia *et al.*, 1997). The effects of reduced nitrogen on the growth of potato plantlets (not on microtuberization) were investigated by Evans (1993). He reduced the amount of NH_4NO_3 to different extents (40 or 20 mM) and concluded that for certain potato genotypes, N in MS medium was too high for producing desirable plantlets in terms of leaf area and shoot length. In Experiment 2, an investigation was carried out to determine if reduced NH_4NO_3 or KNO_3 levels in MS basal salt medium during the induction phase (step 2) would affect microtuber yield.

Materials and Methods

In the experiments, total N in the medium was reduced from 60 mM (MS medium control level) by decreasing medium: (1) NH_4NO_3 concentration to 50, 40, or 30 mM or (2) KNO_3 concentration to 50 mM. There were five replicates per treatment, randomly distributed in a growth cabinet maintained at 15°C under 8/16 h d/n cycle with 65 μ mol $m^{-2}s^{-1}$ flux density for 5 wk. Cultures were randomized every 5 d to ensure equal exposure to the light. Harvests were taken after 5 wk, and data included microtuber number, number ≥ 500 mg, and total fresh weight. Data were subjected to ANOVA and means were compared by t-test.

Results

The amount of nitrogen present in control MS medium was not inhibitory to microtuber production (Tables 3-2). Microtuber yields were not improved when total medium N was reduced by decreasing either medium (1) NH_4NO_3 or (2) KNO_3, in contrast to results of Hoque *et* al. (1996) and Zarrabeitia *et* al., (1997) who advocated reduction of KNO_3 and/or Pierik (1987) and Pennell (1987) who suggested that MS medium contained higher levels of salts, particularly N which were "too high for optimal growth". However,

there were no significant differences in microtuber yields (number, number ≥ 500 mg, or total fresh weight) when NH_4NO_3 or KNO_3 were reduced. At 30 mM of total N, the minituber yield was almost the same as at the 60 mM control MS level, with respect to mean number, number ≥ 500 mg, and total fresh weight. Therefore, it would be more cost-effective to use 30 mM of total nitrogen in microtuber production systems.

Table 3-2. Effects of reduced medium nitrogen on microtuber yield.

N source (mM)			Total N	Number		Fresh weight
NH_4NO_3	KNO_3	NH_4NO_3:KNO_3	mM	Mean	≥ 500 mg	(mg)
41.2	18.8	2.19:1	60	11.80 a	2.4	3095.4 a
31.2	18.8	1.65:1	50	12.40 a	2.2	2777.8 a
21.2	18.8	1.10:1	40	11.44 a	2.0	2934.0 a
11.2	18.8	0.59:1	30	11.60 a	2.0	2980.2 a
41.2	8.8	4.68:1	50	10.60 a	2.0	2817.6 a

Numbers represented by the same letter within each column are not significantly different ($P < 0.05$).

3.5 Experiment 3 - Effects of Mechanical Resistance on Microtuber Yield

Axillary buds of a potato plant have the capacity to grow out as stolons but only buds subjected to a certain level of apical dominance will do so (Booth, 1963). Longitudinal growth of a stolon will continue as long as the conditions are unfavorable for tuberization. Long day conditions favor stolon elongation, whereas, short days result in cessation of stolon growth (Chapman, 1958). Extremely vigorous growth and a delay of tuberization occurred when the stolon environment did not provide enough mechanical resistance (Lugt *et al.*, 1964). There is evidence that ethylene favours tuberization in the

case of modified roots (Biran *et al.*, 1972). Ethylene produced by friction between soil particles and the growing stolon tip might stop extension growth of the stolons, thereby exerting indirect effect on tuberization (Vreugdenhil and Van Dijik, 1989; Vreugdenhil and Struik, 1989, 1990). Ethylene does not play a similar role in the case of potato tuberization (Vreugdenhil and Struik, 1990). Apparently, ethylene stimulates gibberellin levels, which inhibit tuberization. Ethylene application inhibited potato tuberization *in vitro* and addition of an ethylene antagonist promoted tuberization (Vreugdenhil and Struik, 1990). The role of mechanical resistance encountered by potato stolons for tuberization is still obscure. In Experiment 4, an investigation was carried out to determine if mechanical resistance, applied during step 2, would affect microtuber yield.

Materials and Methods

Step 2 cultures of cvs. Russet Burbank and Onaway were incubated in a growth cabinet under 8/16 h d/n cycle at 65 μ mol $m^{-2}s^{-1}$ cool white fluorescent light and in liquid induction medium with or without 150 g pebbles (1-1.2 mm diameter). Pebbles were used so that stolons faced mechanical barriers on their way to growth further. There were six replicates per treatment for cv. Onaway, and only three for cv. Russet Burbank because it lost three replicates due to contamination. Cultures were arranged in a complete randomized design. Microtubers were harvested after 5 wk and microtuber number and fresh weight were noted. Statistical analyses were performed using the t-test.

Results

Microtuber fresh weight was greater for both cvs. in culture containers without compared to with pebbles (Table 3-3). For cv. Russet Burank, microtuber number was not different between the treatments but for Onaway there were significantly more microtubers in the culture containers without than with pebbles.

Mechanical resistance encountered by stolons during step 2 appeared to reduce, rather than increase microtuber yield, in contrast to speculations made by Ewing and Struik (1992). The stolons sometimes grew into the pebble particles and formed

microtubers but most of them grew above the pebble surface. The pebbles did provide mechanical resistance so the reason for decreased microtuber number was not clear. Friction of stolons with pebbles might have produced ethylene that resulted in reduced number of microtubers (Vreugdenhil and Struik, 1990). Since the results of mechanical resistance given to stolons were not promising in terms of microtuber yield this does not appear to be worth pursuing.

Table 3-3. Effects of mechanical resistance on microtuber yield (number and fresh weight) for cvs. Russet Burbank and Onaway in medium ± pebbles.

Cultivar	Number of replicates	Fresh weight (mg) - pebbles	Fresh weight (mg) + pebbles	Number - pebbles	Number + pebbles
Russet Burbank	3	2.39 a	2.15 a	19.66 a	15.66 a
Onaway	6	3.31 a	2.82 a	17.83 a	10.00 b

Numbers represented by the same letter within each column are not significantly different at ($P < 0.05$).

3.6 Experiment 4 - Effect of d/n Cycle on Microtuber Yield

Photoperiod plays an important role in the growth and development of plants. Long days (16/8 h d/n cycle) are optimum for *in vitro* micropropagation (Hussey and Stacey, 1989; Avila *et al.*, 1996) and short days (8/16 h d/n cycle) are generally recommended for the induction step of microtuberization (Garner and Blake, 1989; Slimmon *et al.*, 1989). Shortened day length (12/12 h) during micropropagation was shown to be beneficial to minituber yield from greenhouse-grown plantlets (Seabrook *et al.*, 1995). However, the effects of shorter days during the layering phase (step 1) have not been assessed as a means of increasing microtuber yield during step 2. Microtuber yields were increased by

extending the duration of incubation during step 2 and also by more than one harvest (successive harvests) per culture container (Lommen *et al.*, 1990; Haverkort and Marinus, 1995). In Experiment 4 an investigation was carried out to determine if reduced day length during step 1, successive harvests, or extended duration of step 2, would effect microtuber yields.

Materials and Methods

During the layering phase, 3 photoperiod treatments included 16/8, 12/12, and 8/16 h d/n cycles.cultures were grown under 40 μ mol $m^{-2}s^{-1}$ and incubation occurred at 20°C in a walk-in growth room for 4 wk. There were four replicates in each treatment. Cultures were randomly distributed on the shelves and were rearranged every 1 wk to equalize light exposure.

Incubation in step 2 occurred at 15°C under short days (8/16 h d/n) with 65 μ mol $m^{-2}s^{-1}$ flux density. Single harvests were after 10 wk and successive harvests occurred at 5 and 10 wk. Data were collected on microtuber mean number, number ≥ 500 mg, and total fresh weight, and analyzed using ANOVA (SAS, Cary, NC).

Results

Mictotuber yields were affected by the d/n cycles during step 1 (Table 3-4). These differences were evident both in terms of 1st and 2nd harvests. Mean microtuber yields for 1st and 2nd harvests were greatest in cultures incubated under long days (16/8 h) and declined as the light period lessened. Although the total number of microtuber ≥ 500 mg was not significantly different between 16/8 and 12/12 h cycles, this number was greatly reduced by a 8/16 h cycle. There were no significant differences in microtuber yields from successive compared with single harvests over all (Fig. 3). However, successive harvests yielded more than a single harvest in the 8/16 h treatment. The number of larger microtubers declined and the number of smaller microtubers (200-300 mg) increased, in the 2nd, compared with the 1st harvest (Table 3-5). It is also important to notice that the

total number of larger microtubers was greater in single compared with successive harvests.

Table 3-4. Effects of different d/n cycles applied at step1 of microtuberization, on microtuber yield (number, number ≥ 500 mg, total fresh weight) in successive harvests and overall.

d/n cycle (h)	1st harvest mean fresh weight	2nd harvest mean fresh weight	Total fresh weight (mg)	Total microtuber number ≥ 500 mg	
				1st harv.	2nd harv.
16/8	3822.25	3701.75	7524.00 a	6	3
12/12	2432.75	3220.00	5652.75 b	5	5
8/16	2607.25	1568.75	4176.00 c	2	1

Numbers designated by different letters within each column are significantly different ($P < 0.05$)

Table 3-5. The fresh weight distribution (mg) of microtubers comparing successive and single harvests obtained from 16/8 d/n cycles.

Harvests	Microtuber fresh weight				Total
	200-300	300-500	500-1000	>1000	
Successive harvest (1st)	9	12	5	1	27
Successive harvest (2nd)	17	6	2	1	26
Single harvest	13	12	14	4	43

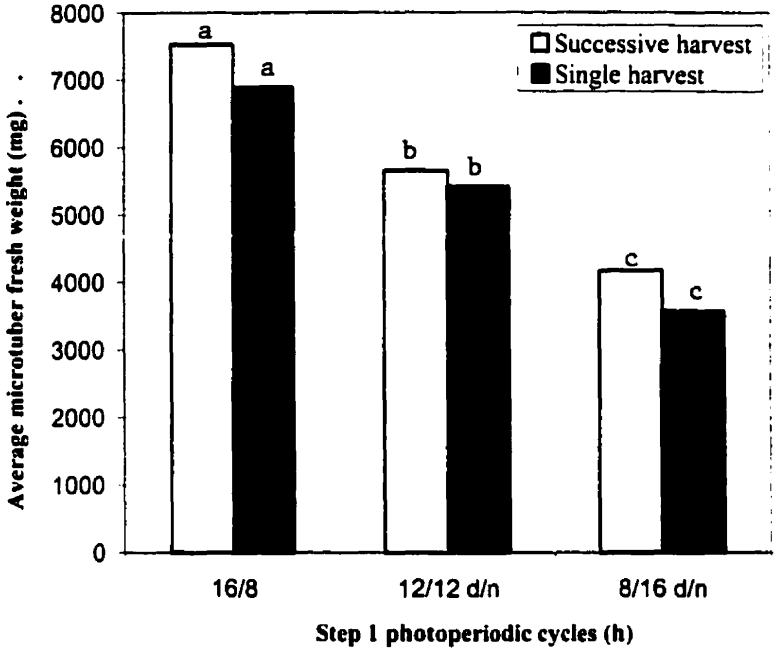


Fig. 3. Difference in microtuber yields (fresh weight), being highest at 16/8, moderate at 12/12, and lowest at 8/16 h d/n cycle for both successive and single harvests.

The influence of different light cycles at step 1 of microtuberization was very distinct. The 16/8 h cycle was best in terms of microtuber yield. The number of larger microtuber was less in the 2nd harvest which confirms the findings of Lommen *et al.* (1990). Clearly, there is a large yield advantage to be gained from maintaining step-2 cultures for longer intervals beyond the standard 4-5 wk, especially since no fresh medium incorporation is required during the prolonged culture period (Abdulnour, 1999). A single harvest procedure after a prolonged microtuberization period was better compared with successive harvests since the number and the number $\geq$ 500 mg were greater than the number obtained from successive harvests. Moreover, successive harvests were labour-

intensive and increased the possibility of contamination, which may lead to reduction in overall yields after the 2nd harvest.

3.7 Discussion

Experiments to improve the microtuberization efficiency by changing the physical environment, nutrition, or harvest pattern were carried out, but only prolonging incubation time prior to harvest increased microtuber fresh weight. The 8/16 h d/n cycle was better than continuous darkness probably due to the fact in addition to medium carbohydrate, potato plantlets benefit from photosynthesis to some extent. Microtuber yield was better in non-agitated small containers than agitated ones, unlike results from bioreactors. The expectation that agitation would increase gaseous exchange or nutritional availability in culture containers and so increase microtuber yield was not fulfilled, probably because these factors were not limiting culture productivity.

Although N is a major element that regulates plant growth and development, withdrawal of N generally favoured tuberization (Krauss and Marschner, 1976; Krauss, 1985; Ewing and Struik, 1992). Reduction in medium N level (KNO_3 by Hoque et al., 1996; NH_4NO_3 by Zarrabeitia *et al.*, 1997) increased microtuber yield and very low medium N (5 mM) promoted the greatest number of microtubers (Teixeira and Pinto, 1991). Treatments with reduced levels of NH_4NO_3 and KNO_3 did not bring about any significant improvement in yield (microtuber number or fresh weight). Similar yields were obtained at half the total medium N (30 mM) that is usually used in full strength MS medium. Zarrabeitia *et al.* (1997) reported that out of four cultivars he used, namely Jaerla, Spunta, Turia, and Baraka, only cv. Jaerla yielded more microtuber than control when N was reduced. So it may be concluded that response to medium N is cultivar-specific and in the present case, cv. Norland might not be one of those potato cvs. that yields better at low N level.

The idea adopted from Lugt *et al.* (1964) that mechanical resistance would restrict stolon elongation, brings about early tuberization, and thus would increase yield, did not work. The resistance provided in the experiment may not have been sufficient to exert adequate mechanical resistance or mechanical resistance may not be useful to increase yield.

Microtuberization efficiency was greatly influenced by the step 1 photoperiodic cycles and harvest times. A d/n cycle of 16/8 h was significantly better than 12/12 and 8/16 h d/n cycles in terms of microtuber fresh weight although there was no significant differences between 16/8 and 12/12 h d/n cycles with respect to microtuber number or number ≥ 500 mg and fresh weight. The reason for better microtuberization in the 16/8 h d/n cycle treatment was probably due to the fact that potato plantlets grew better compared with 12/12 or 8/16 h d/n cycles. Prolongation of harvesting time and maintaining cultures for two successive harvests on the same medium were efficient methods for greater microtuber production. Prolonged culture (single harvest after 60-65 d) yielded considerably more microtuber (number ≥ 500 mg) than successive harvests (30-32 d for 1st harvest and 60-65 d for 2nd harvest). It is therefore recommended that the single harvest technique be used with prolonged incubation.

CHAPTER 4 *DORMANCY BREAKING IN MICROTUBERS AND MINITUBERS*

4.1 Introduction

Potato microtubers are an alternative to micropropagated plantlets for minituber production. Microtubers are an increasingly important component of healthy seed tuber production systems. This is particularly true in hot, humid tropical countries where true seed development has failed and conventional cut tuber pieces facilitate opportunities for microbial infection (Van der Zaag, 1990). Microtubers can be produced year 'round without seasonal constraints. Microtubers are a cost effective method for storing and shipping healthy germplasm (Coleman and Coleman, 1986) that may be easier to handle than in vitro-grown plantlets. Microtubers are generally considered to be too small to be efficiently used as seed tubers (Haverkort *et al.*, 1991), although current opinion is mixed, rather they are used in greenhouses or screenhouses for the production of minitubers to generate nuclear stock. Potato microtubers or minitubers exhibit varying periods of cultivar-dependent dormancy. Field grown potato tubers do not tend to sprout readily after harvest even if they are provided with conditions of ideal temperature (20-28°C) and moisture (> 85% RH) for efficient sprouting (Beukema and Van der Zaag, 1990). This phenomenon is also true for microtubers (Leclerc *et al.*, 1993). This dormancy poses a challenge to the economical utilization of microtubers in seed tuber production systems since they must be cold-stored and cannot be immediately planted.

A limited number of workers have succeeded in breaking microtuber dormancy (Rappaport *et al.*, 1957; Choudhury and Ghose, 1960; Goodwin, 1966; Coleman, 1983; Coleman and Coleman; 1986, Coleman 1987; Coleman *et al.*, 1992; Nasiruddin and Blake, 1997). Success has been achieved but the information on impact of dormancy-release treatments on subsequent yield is meager (Choudhury and Ghose, 1960; Slomnicki and Rylski, 1964). Forcing potato tubers to break dormancy and to sprout early did not lead to better yield upon cultivation (Choudhury and Ghose, 1960;

Slomnicki and Rylski, 1964). The type of dormancy-breaking agents used are those utilized for stored, field-grown tubers, including GA_3 (Rappaport *et al.*, 1957; Choudhury and Ghose, 1960), BE (Coleman, 1983; 1987), rendite (deBokx, 1990; Nasiruddin and Blake, 1997; Mcdonald and Coleman, 1984) and thiourea (Ramanujam *et al.*, 1957). Other dormancy-breaking agents which have not yet been tried for potato, but which may have promise include agents implicated in breaking dormancy in seeds, such as anti-ABA which promoted germination in canola seeds (PBI Bulletin, 1995) and a natural extract (Signal solution) which promoted nodulation of soybean in cold soils (Bios, Montreal, QC, Canada). Anti-ABA, an ABA analog, is effective as an ABA antagonist (Wilen *et al.*, 1993).

The objective of these experiments was to evaluate a range of dormancy-breaking treatments for potato microtubers and minitubers.

4.2 Materials and Methods

The cv. Norland was used in all experiments. The microtubers were obtained from *in vitro* cultures grown under a 16/8 h d/n cycle. The minitubers were obtained from *ex vitro* plantlets grown in a rockwool-based hydroponic set-up in the greenhouse (Lowe, 1999). Some microtubers and minitubers were used right after harvest and were not cold-stored, while some microtubers were cold-stored in sealed petri dishes in the fridge (4-5°C) and some minitubers were stored in paper bags in a walk-in cold room (3°C) for 2, 3, 4, or 8 wk.

The dormancy-breaking treatments involved soaking tubers (300 ml for minituber and 50 ml for microtuber) in solutions for 24 h. The treatment solutions included (mg l^{-1}): GA_3 (500), ABA (500), anti-ABA (250, 500), Signal, GA_3 (250, 500) + Signal, GA_3 (500) + anti-ABA (500), GA_3 (250) + anti-ABA (250). Some of the treatments were done in mixtures of two agents and some were done as successive treatments with two agents. The agents were dissolved in a few drops of ethanol (95%) and the solutions made to

volume with double distilled water. Control treatments included soaking in double distilled water (with no ethanol) or no soaking.

A gassing treatment with BE (reagent grade, Aldrich Chemical Co. Milwaukee, WI, USA) at 0.2 ml l^{-1} for 24 h was also done. BE is volatile at room temperature so it was stored as a liquid in the fridge (4-5°C). To prepare the BE solution, double distilled water of fridge temperature (5°C) was used to dilute the liquid BE, and mixed in a 1 l plastic container in the fume hood. A folded filter paper was dipped into the solution as a wick to promote better evaporation. The tubers to be treated were enclosed with the 1 l container holding the 0.2 ml l^{-1} BE in a 5 l plastic container. The 5 l container was tightly sealed with Parafilm for 24 h. Mechanical treatments were also done to break dormancy. Mechanical injury treatments were made either by cutting tubers into halves or by microwaving them at full power for 10 sec in a microwave (Citizen, Model No. JM-5522W, AC 120V 60Mz 1300W).

Tubers that had been treated in the dark at room temperature (20°C) were kept in continuous darkness at 20°C for sprouting. For adequate moisture supply, 3 ml of sterile double distilled water was added on a regular basis to filter papers placed on the inside of petri dishes for microtubers and 400 ml Better Plastics containers for minitubers. Observations on sprouting were made by examining each tuber under a lens (Luxo Magnifier, Luxo Lamp Ltd. Canada) on a weekly basis. Microtubers or minitubers were considered to be sprouted when the length of the germinated sprouts was ≥ 1 mm.

4.3 Experiment 1 - The Effective Concentration of GA_3 for Breaking Dormancy

Exogenous GA_3 was used for terminating potato tuber dormancy by several workers (Rappaport *et al.*, 1957; Lippert *et al.*, 1958; Choudhury and Ghose, 1960). The exact role of GA_3 in breaking dormancy is not well understood (Coleman, 1987). GA_3 may stimulate reducing sugar formation in recently harvested tubers (Clegg and Rappaport, 1970) but this has been questioned by others (Baily *et al.*, 1978).

Materials and Methods

To determine the most effective level of GA_3, a concentration series was prepared: 25, 50, 100, 150, 250, and 500 mg l^{-1} and treatments were applied to microtuber of 250-500 mg fresh weight that had been cold-stored at 4-5°C for 4 wk. Sprouting was monitored weekly. For the experiment, a sample size of 10 microtubers (250-500 mg) was used for each treatment.

Results

GA_3 at a concentration of 500 mg l^{-1} was found to be the most effective in breaking dormancy; after 1, 2 and 3 wk 30, 70, and 90% of microtubers had sprouted, respectively (Table 4-6). With 250 mg l^{-1} GA_3 , after 1, 2, and, 3 wk only 10, 50, and 70% of microtubers had sprouted,respectively. Microtubers exposed to lower concentrations of GA_3 exhibited less sprouting.

Table. 4-6. The number of sprouted microtubers (cold stored for 4 wk at 5°C prior to treatment) observed 1, 2, or 3 weeks after 24 h soak in GA_3 concentrations.

GA3	Number of microtubers		
mg l^{-1}	1st wk	2nd wk	3rd wk
25	0 b	0 c	20 d
50	0 b	10 c	30 cd
100	0 b	30 bc	40 cd
150	0 b	30 bc	50 bc
250	10 b	50 ab	70 ab
500	30 a	70 a	90 a
LSD	19.97	31.58	28.25

Numbers represented by the same letter within each column are not significantly different ($P < 0.05$)

4.4 Experiment 2 - Effects of Light and Dark Incubation on the Effectiveness of GA_3 Solutions For Breaking Dormancy

The dormancy-breaking treatments of Choudhury and Ghose (1960) and Slomnicki and Rylski (1964), using GA_3, did not state whether treatments were followed by incubation in light or in darkness. The objective of this experiment was to determine whether light or dark incubation was best for sprout induction following dormancy-breaking treatment with GA_3.

Materials and Methods

A sample size of 40 microtuber (150-300 mg) per treatment were soaked in 500 mgl^{-1} GA_3 for 24 h in the light, and then placed either (1) under 16/8 h d/n cycle (lighted phase 40 μ mol $m^{-2}s^{-1}$ cool-white fluorescent light) or (2) in complete darkness.

Results

The numbers of sprouted microtuber were 50% (20/40) and 65.5% (27/40) respectively under 16/8 h d/n cycle and continuous dark treatments in the 1st wk. But after 2 wk, these numbers were increased to 87.5% (35/40) and 75% (30/40) respectively (Fig. 4). This finding indicated that after soaking in 500 mg l^{-1} GA_3 extending the dark incubation interval helped to increase the number of sprouted microtubers. The 16/8 h d/n cycle favoured sprouting in the first wk, but as the incubation period was extended, the frequency of sprouting slowed down. The % of sprouting in the dark was greater than in the light, although differences were not that large (87.5-75.0 =12.5%). The trend towards improved sprouting in the dark was consistent with what is known regarding the light sensitivity of GA_3.

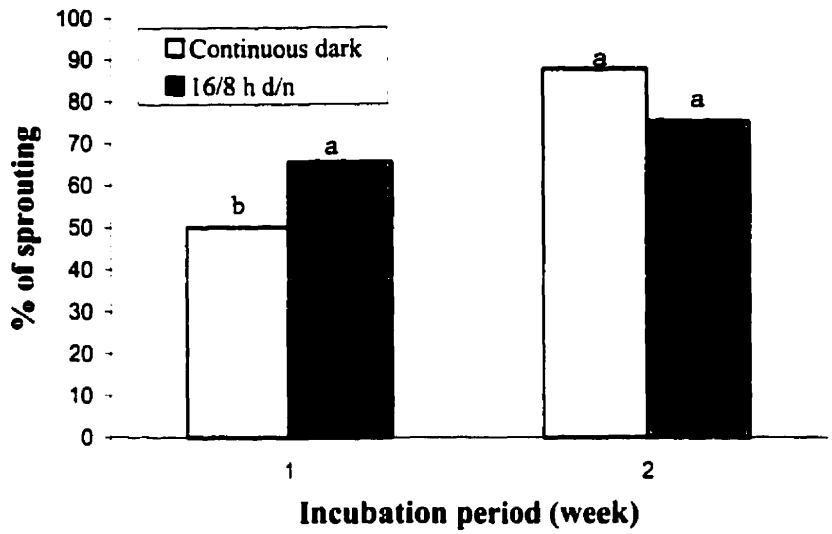


Fig.4. Effects on microtuber sprouting of 16/8 h d/n cycle and continuous darkness following GA_3 treatment.

4.5 Experiment 3 - Effectiveness of Signal Solution for Breaking Dormancy

Signal solution is a bacterial fermentation tank product, containing natural plant substances, such as flavone genistein, and is reported to be involved in facilitating nodulation of soybean seeds under cool soil conditions (Bios, Montreal, QC, Canada). These substances may affect plant growth by acting as signal molecules between plants and bacteria or other organisms in their environment (Zhang and Smith, 1995; Verma *et al.* 1997). While the stimulatory effect of Signal solution in the soybean-*Bradyrhizobium japonicum* complex has been described (Zhang and Smith, 1995; Zhang *et al.* 1996), the effects of these plant substances in other plant species and their associated rhizospheres' organisms have not been investigated.

Materials and Methods

Treatments occurred on microtubers (200-400 mg) that had been cold-stored for 8 wk to determine their effect on breaking dormancy. Signal solution was used at full strength (100%) or diluted to 20% (as for soybean), 12%, or 6% of full strength. GA_3 (500 mg l^{-1}), water soaking, and control treatments were performed for comparison purposes. Microtuber soaking treatments lasted 24 h and then incubation occurred either in the light (40 μ mol $m^{-2}s^{-1}$ cool-white fluorescent) or in the dark. Five microtubers were used in each treatment. Observations for sprouted microtubers were made at 1 and 2 wk.

Results

One hundred % Signal was as effective as GA_3 (500 mg l^{-1}) when evaluated after 1 wk with respect to the number of sprouted microtubers (Table 4-7). One hundred % Signal induced multiple sprouts and dark incubation favoured sprouting as compared with the light regime after 1 wk of incubation. The exact cause of dark incubation favouring 100% Signal solution was not understood. One hundred % Signal solution was more effective than diluted Signal when numbers of sprouted microtuber were counted after 1 wk. After 2 wk of incubation all treatments were equally effective in causing sprouting but the Signal and GA_3 solutions were most promotive of multiple sprouting which did not occur in the water soaking treatment and only in the control treatment incubated in the dark.

Table 4-7. Effects of gibberellic acid (GA_3) and Signal solutions on dormancy breaking of microtubers (200-450 mg) that had been cold-stored at 5 °C for 8 weeks and evaluated after treatment and incubation with or without light for 1 and 2 weeks, for number of sprouts and for number with multiple sprouts (> 1) at 2 weeks.

Treatments	Number of sprouted microtubers		Number of multiple sprouted microtubers	Mean number of sprouts ±
	1 wk	2wk	(2wk)	SE
GA_3 500 mgl^{-1} + light	1/5	5/5	2/5	2.5 ± 0.5
GA_3 500 mgl^{-1} - light	5/5	5/5	4/5	2.5 ± 0.3
100 % Signal + light	0/5	5/5	2/5	2.5 ± 0.5
100 % Signal - light	5/5	5/5	3/5	2.3 ± 0.3
20 % Signal + light	0/5	4/5	0	0
20 % Signal - light	2/5	5/5	2/5	2.0 ± 0
12 % Signal + light	0/5	4/5	1/4	2.0 ± 0
12 % Signal - light	2/5	5/5	2/5	2.0 ± 0
6 % Signal + light	0/5	4/5	2/4	2.0 ± 0
6 % Signal - light	1/5	5/5	2/5	2.0 ± 0
Water + light	0/5	4/5	0	0
Water - light	2/5	5/5	0	0
Control + light	0/5	5/5	0	0
Control - light	1/5	5/5	1/5	2.0 ± 0

4.6 Experiment 4 - Effectiveness of Combinations of GA_3 and 100% Signal Solution in Breaking Dormancy

Experiment 3 suggested that the 100% Signal solution was effective in breaking microtuber dormancy. However, the microtubers used in the trial had been cold-stored for 8 wk. In this trial, the effects of Signal were evaluated in combination with GA_3 on minitubers with only 3 wk of cold storage. It was also investigated whether the effect of 100% Signal solution might be synergistic if used with GA_3 (500 mg l^{-1}).

Materials and Methods

Minitubers (20-30 g) with 3 wk cold storage treatment were soaked for 24 h in 500 mg l^{-1} GA_3, 100% Signal, or a mixture of the two. Another treatment involved successive soaking for 12 h each, in first GA_3, and then Signal. A control treatment without soaking was also performed. There were 8 minitubers used per treatment. Treatments occurred at room temperature (20°C). Microtubers were observed after 2 wk and the number of sprouted minitubers and the number with multiple sprouts were counted.

Results

All treatments were able to break minituber dormancy except the control (Table 4-8). The 500 mg l^{-1} GA_3 treatment alone or together with 100% Signal for 24 h both caused 100% sprouting, and significantly more multiple sprout formation than the other treatments. The 100% Signal alone or in combination with 500 mg l^{-1} GA_3 were as effective as 500 mgl^{-1} GA_3 alone for breaking dormancy within 2 wk. However, less multiple sprouting occurred with 100% Signal alone, or following 12 h GA_3 treatment, compared with the GA_3 treatment alone or the combined GA_3 and Signal treatments. There were no clear synergistic effects of 100% Signal solution in combinations with 500 mg l^{-1} GA_3.

Table 4-8. Individual and combined effects of GA_3 and 100% Signal solution on dormancy breaking of minitubers that had been cold-stored for 3 weeks, evaluated over a 2 week period.

Treatments	Number of sprouted minituber	Number of minituber with multiple sprouts	Mean number of sprouts ± SE
GA_3 500 mg l^{-1} 24 h	8/8	6/8	3.37 ± 0.62
100% Signal soln. 24 h	7/8	2/7	1.33 ± 0.42
GA_3 500 mg l^{-1} + 100% Signal Soln. 12 h + 12 h	8/8	6/8	2.37 ± 0.41
GA_3 500 mg l^{-1} + 100% Signal Soln. Combination 24 h	8/8	6/8	3.75 ± 0.67
Control	0/8	0/8	0/8

4.7 Experiment 5 - Effectiveness of GA_3 and Signal solution Compared with Bromoethane and Mechanical Injuries in Breaking Dormancy

Bromoethane was reported to break potato tuber dormancy when applied as a fumigant and it was found that BE at a concentration of 0.2 ml l^{-1} was the most effective (Coleman, 1983). Conventionally, large potato tubers are cut into small pieces, each containing an eye, to be used as seed pieces. To obtain quick and uniform sprout emergence, potato tubers should be cut at least 2 wk before planting (Slomnicki and Rylski, 1964). Mechanical injury contributes to sprout induction. The objective of this experiment was to determine the effects of BE and mechanical injury on microtubers and minitubers compared with other dormancy-breaking treatments.

Materials and Methods

Microtubers (200-500 mg) cold-stored for 8 wk and minitubers (20-35 g) cold-stored for 0, 2, or 8 wk were used for these experiments. BE (0.2 ml l^{-1}) and mechanical injury (cutting in half, microwaving at full power for 10 sec) were compared with GA_3 (500 mgl^{-1}), 100% Signal, water soaking, and control treatments. Six microtubers or minitubers were used per treatment. Observations were made at 1, 2, 3, and 4 wk intervals and the number of sprouted tubers were counted. The evaluation period was extended because tubers with little or no cold storage treatment took longer to sprout.

Results

GA_3 was the only agent which was able to break dormancy of minitubers that had not been cold-stored; 0/6 at 2 wk but 4/6 by 4 wk (minituber, 0 wk storage; Table 4-9). Minitubers with 2 wk cold storage that were treated with GA_3 also broke-dormancy; 0/6 at 2 wk but 5/6 by 4 wk with 2/6 showing multiple shoots. Signal treatment of minitubers cold-stored for 2 wk caused 1/6 (with multiple shoots) minitubers to break dormancy after 4 wk. For minitubers cold-stored for 8 wk, all treatments (including water soaking and control), except the microwaving, showed some sprouting the first week and multiple sprouting was evident in the GA_3 (6/6), 100% Signal (4/6), and BE (1/6) treatments by 2 wk. Cutting caused sprouting in 9/12 cut halves by 2 wk but only a few multiple shoots (2/12) were evident by 3 wk.

With microtubers cold-stored for 8 wk, dormancy-breaking occurred within the first week in all treatments except microwaving. After 2 wk, sprouting had progressed in all treatments; water (4/6) was similar to GA_3 solution (5/6), BE (4/6), Signal (4/6), and cutting (8/12). Only GA_3 solution (3/6 at 2 wk, 6/6 at 4 wk) and Signal (1/6 at 4 wk) caused multiple sprout formation.

Signal solution (100%) was effective in causing sprouting in minitubers with 2 or 8 wk cold storage and microtubers with 8 wk cold storage but it was ineffective on minitubers that had not been cold-stored. GA_3 and 100 % Signal solution induced

multiple sprouts from different eye-points (Fig. 5b, c, e, f) unlike the control (Fig. 5a, d), BE, or cutting treatments, that induced single sprouts only from the rose end. BE worked well in inducing single sprouts in minitubers and microtubers with 8 wk cold-storage but was not as effective as GA_3 for minitubers that had not been cold-stored.

Cutting minitubers or microtubers in half after 8 wk cold storage induced single sprouts on each cut half. This occurred quite efficiently in minitubers (9/12 in 2 wk, 12/12 in 4 wk) and somewhat less efficiently with microtubers (8/12 in 2 wk, 10/12 in 4 wk). Cutting was not effective in breaking dormancy in minituber without cold storage and worked poorly in minitubers that had been cold stored for only 2 wk (0/12 after 2 wk, 1/12 after 4wk). Sprouting from two halves was good, in the sense that by cutting minitubers or microtubers in half, two propagules, each with one sprout were derived although a very insignificant number of minituber halves (2/12) showed multiple sprouting. However, cutting was risky in that this sometimes provided opportunities for fungal or bacterial infection. Microwaving induced limited sprouting but only in minitubers or microtubers that had been cold-stored for 8 wk, and not in the minitubers with 0 or 2 wk cold storage. Microwaving caused some tuber damage that may account for the reduced sprouting observed.

Table 4-9. Sprouting and (multiple sprouting) performance on minitubers and microtubers cold-stored for 0,2,4, or 8 weeks and evaluated each week for 4 weeks after exposure to Bromoethane (0.2 ml l^{-1}), mechanical injury (cutting in half or microwaving), GA_3 (500 mg l^{-1}), 100% Signal, water soaking, and control treatments.

Treatments	Minituber (CS8W)				Minituber (CS2W)				Minituber (NCS)				Microtuber (CS8W)			
	1wk	2wk	3wk	4wk	1wk	2wk	3wk	4wk	1wk	2wk	3wk	4wk	1wk	2wk	3wk	4wk
GA_3 500mg l^{-1}	5/6	**6/6 (6)**	**6/6 (6)**	**6/6 (6)**	0	0	3/6	**5/6 (2)**	0	0	3/6	4/6	4/6	**5/6 (3)**	**6/6 (3)**	**6/6 (6)**
100% Signal solution	4/6	**6/6 (4)**	**6/6 (4)**	**6/6 (4)**	0	0	0	**1/6 (1)**	0	0	0	0	1/6	2/6	4/6/	**6/6 (1)**
Bromoethane 0.2 ml l^{-1}	3/6	**4/6 (1)**	**6/6 (4)**	**6/6 (4)**	0	0	0	2/6	0	0	0	0	2/6	4/6	4/6	6/6
Cutting into halves	7/12	9/12	**12/12 (2)**	**12/12 (2)**	0	0	0	1/12	0	0	0	0	5/12	8/12	9/12	10/12
Microwaving	0	0	1/6	2/6	0	0	0	0	0	0	0	0	0	0	0	1/6
Water	4/6	5/6	**6/6 (1)**	**6/6 (1)**	0	0	0	0	0	0	0	0	2/6	4/6	6/6	6/6
Control	2/6	4/6	6/6	6/6	0	0	0	0	0	0	0	0	3/6	4/6	6/6	6/6

'CS' stands for cold storage; 'W' for week; N for no
'( )'denotes number of tubers with multiple sprouts

Figure 5. Sprouting in minitubers (photographed 1 week following first appearance of sprouts) and microtubers (photographed 2.5 weeks following first appearance of sprouts). a) Control minitubers; b) Control microtubers; c) GA_3-treated minitubers; d) GA_3-treated microtubers; e) 100% Signal-treated minitubers, and f) 100% Signal-treated microtubers.

a
d
c
f

4.8 Experiment 6 - Effectiveness of Anti-ABA Compared with Other Dormancy Breaking Treatments

Anti-Abscisic acid (anti-ABA), the acetylenic analog of ABA, has never been used to induce sprouting in dormant tubers since it was first shown to be an ABA antagonist (Wilen *et al.*, 1993), although it has been used to terminate dormancy in canola seeds (PBI Bulletin, 1995). The objective of this experiment was to test anti-ABA for breaking dormancy in potato minitubers and compare its efficacy with other dormancy-breaking treatments.

Materials and Methods

Microtubers (200-600 mg) were cold-stored for 3 wk prior to the experiment. Seven microtubers were used per treatment. Treatments included 24 h soaks in anti-ABA or GA_3 (500 and 250 mg l^{-1}) applied alone or in combination, GA_3 (500 mg l^{-1} in combination with 100% Signal, and water. Bromoethane (0.2 ml l^{-1}) and control treatments were also performed. Observations were made after 2 wk in the dark at room temperature (20°C). Data included number of sprouted microtubers and number of multiple sprouts. Means of sprout number were calculated only from microtubers that had sprouted

Results

Anti-ABA alone and in successive treatments or in combination with GA_3 was effective in breaking microtuber dormancy (Table 4-10). Among the different treatments using anti-ABA and GA_3 the greatest mean number of sprouts (1.8 ± 0.48) occurred when microtubers were soaked in a mixed solution of 500 mg l^{-1} GA_3 and 500 mg l^{-1} anti-ABA for 24 h but it was not significantly different from the 500 mg l^{-1} GA_3 treatment (1.71± 0.28). The combined Signal and GA_3 was not more effective than GA_3 alone and was less effective than any GA_3 and anti-ABA treatment in breaking dormancy.

The overall results with anti-ABA underline its importance as a potential dormancy-releasing agent, as much so as GA_3. Anti-ABA and GA_3 both induced multiple sprouts

but sprouts were longer after GA_3 than anti-ABA treatment. Both agents caused sprouts to emerge at various eyes over the tuber surface. However, the GA_3 -induced multiple sprouts were profusely branched; a group of sprouts protruded from each eye, while the anti-ABA-induced sprouts were singles. The mechanism of dormancy breaking by anti-ABA and GA_3 therefore was similar, but GA_3 appeared stronger. These agents should be tested on an equimolar basis in the future.

Table 4-10. Sprouting performance on microtubers cold-stored for 3 weeks and evaluated at 2 weeks after exposure to dormancy-breaking agents.

Treatments	No. sprouted microtubers	Mean No. sprouts ± SE
GA_3 500 mg l^{-1} 24 h	7/7	1.71 ± 0.28
GA_3 500 mg l^{-1} + 100% Signal Sol. 24 h	3/7	1.33 ± 0.29
Anti-ABA 500 mg l^{-1} 24 h	5/7	1.4 ± 0.24
Anti-ABA 250 mg l^{-1} 24 h	5/7	1.2 ± 0.20
GA_3 500 mg l^{-1} 12 h + Anti-ABA 500 mg l^{-1} 12 h	6/7	1.33 ± 0.21
GA_3 250 mg l^{-1} 12 h + Anti-ABA 250 mg l^{-1} 12 h	7/7	1.57 ± 0.29
GA_3 500 mg l^{-1} + Anti-ABA 500 mg l^{-1} Combination 24 h	5/7	1.8 ± 0.48
GA_3 250 mg l^{-1} + Anti-ABA 250 mg l^{-1} Combination 24 h	5/7	1.6 ± 0.24
Bromoethane	1/7	1.0 ± 0
Water	1/7	1.0 ± 0
Control (no treatment)	0/7	0

4.9 Experiment 7 - Harvests from Minitubers Sprouted Using a Range of Dormancy-Breaking Treatments

There is only limited information on the relative yield performance of potato tubers that were treated with dormancy-breaking agents (Choudhury and Ghose, 1960; Slomnicki and Rylski, 1964). Yields from potato tubers that were treated with GA_3 at 25-100 mg l^{-1} (Choudhuri and Ghose, 1960) or 5-40 mg l^{-1} (Slomnicki and Rylski, 1964) were reduced compared with untreated controls. The objective of this experiment was to evaluate the effect of dormancy-breaking agents on subsequent yield in greenhouse pot trials.

Materials and Methods

Minitubers (20-35 g) that were cold-stored for 8 wk were given dormancy-releasing treatments including 24 h soaking in GA_3 (500 mg l^{-1}), 100% Signal, or water. Other treatments included BE (0.2m l^{-1}), cutting in half, and the control. All minituber were observed at 3 wk following treatment and the number of sprouts were noted at the time of planting. Five minitubers per treatment were individually planted into 11 x 12 cm plastic pots in the greenhouse. The potting mixture was 2:1 peat:perlite without fertilizer added. The pots were arranged in a complete randomized design and watered equally every alternate day. Harvest occurred after 60 d and tuber yields (number and fresh weight) were recorded.

Results

GA_3 caused significantly more sprouts per minituber (4.2 ± 0.37) than the other treatments, with 100% Signal (2.0 ± 0.31) and BE (1.8 ± 0.2) giving intermediate values, and water-soaking and cutting similar to the control (Table 4-11). The average number of tubers per plant was greatest in the GA_3 treatment (3.6); almost double than of other treatments that were not different from the control. The mean fresh weight of tubers (per replicate i.e. pot basis) harvested from minitubers exposed to the 100% Signal treatment was the greatest (34.97 g); greater than the control fresh weight and three times more than

the GA_3 treatment. The size and shape of tubers harvested from the GA_3 treatment were small and more elongated than that of the control and other treatments. Yields from BE treated minitubers were significantly lower compared with controls. The cut halves each yielded almost the same as uncut controls and had similar fresh weight to control (28.41 vs 27.16). Two cut halves of each minituber together would effectively double control yield and bring the mean number of tubers into the GA_3 treatment range. However, cutting into halves posed a problem of infection and decomposition at the cut surfaces.

Table 4-11. Harvests after 60 d from minitubers that were forced to break dormancy by different methods.

Treatments	Mean number sprouts at planting ± SE	Mean number of tubers produced per replicate	Mean fresh weight (g)
GA_3	4.2 ± 0.37	3.6*	11.13 d
Cutting into halves (1/2 minituber)	1.2 ± 0.20	2.0	28.41 b
Bromoethane	1.8 ± 0.20	2.0	14.01d
100% Signal soln.	2.0 ± 0.31	1.8	34.97 a
Water	1.2 ± 0.20	1.0	17.41 c
Control	1.0 ± 0	2.0	27.16 b

Numbers represented by the same letter are not significantly different at the 0.05 level.

*4.10 **Discussion***

Dormancy of in vitro grown microtubers is positively correlated with the relative dormant periods of field-grown tubers (Leclerc *et al.*, 1995). This dormancy prevents the immediate planting of microtubers after harvest and as such restricts their immediate use

in seed certification programs. An efficient method of controlling tuber dormancy would greatly increase their utilization and possibly reduce the storage cost. Dormancy is thought to be regulated by ABA (Addicott and Cairns, 1983; Suttle and Hultstrand, 1994). The ability of GA_3 to break dormancy was reported by many researchers (Rappaport, 1957; Dooren Bos, 1958; Lippert *et al.* 1958; Tsukamoto *et al.*, 1961; Matkur, 1961). The most effective concentration of GA_3 for dormancy-breaking in field-grown tubers was 500 mg l^{-1} (Rappaport *et al.*, 1957). In the previous experiments, GA_3 at 500 mg l^{-1} was found to be the best among many other dormancy-releasing compounds. It was the only chemical that was found to have a positive effect in releasing dormancy in microtubers and minitubers cold-stored for different durations and in minitubers without any cold storage (Table 4-9). The sprouts developed by GA_3 treatments were very long and slender. Shoots developed from the sprouts were many per minituber and not upright but creeping. Though the number of shoots was many the overall yield in terms of fresh weight was not encouraging (Table 4-10). While the number of minitubers formed from minitubers exposed to the GA_3 treatment was the highest, these were invariably smaller. The results support the findings of Choudhury and Ghose (1960). It was observed that a high concentration of GA_3 posed abnormalities in growth and development of both plants and tubers formed. This phenomenon was perhaps due to a large number of stems per plant grown from sprouted minitubers because "this is symptomatic of physiologically older seed potatoes" (Slomnicki and Rylski, 1964).

The 100% Signal solution was reported to break dormancy and increase percentage of germination in soybean seeds (Bios, Montreal, QC, Canada). From different treatments with or without GA_3, it showed a positive response in shortening dormancy in potato minitubers and microtubers (Table 4-8). The combination effect of GA_3 and 100% Signal solution was thought to improve the phenomenon of dormancy release. Though the results were better than those of 100% Signal solution alone they were not as good as

those of GA_3 and did not show additive effects when applied with GA_3 (Table 4-8, 4-10).

BE and mechanical injuries did not improve dormancy breaking in minitubers with 0 or 2 wk cold storage but induced multiple sprouting in minitubers cold-stored for 8 wk (Table 4-9). The cut-tuber pieces of minitubers that had not been cold stored did not have sprout (Table 4-9). Cutting provided chances for microbial infection. Microwaving at full power for 10 sec. was not good since it sometimes burnt the outer tissues. Microwaving at low power may be applied for less mechanical injury.

The effects of anti-ABA on dormancy-breaking were promising. It has not been used before to sprout potato tubers. Its only success has been so far reported on the early germination of canola seeds (PBI Bulletin, 1995). But it really worked on potato microtubers (Table 4-10). The present investigations demonstrated that anti-ABA might have uses as a potential dormancy-releasing agent (Table 4-10).

The effects of precocious dormancy breaking by different agents on subsequent yield were interesting. Though GA_3 was the best for dormancy breaking, it reduced individual tuber fresh weight since there were more tubers. Signal was no more effective than the control treatments in breaking dormancy but it appeared to stimulate tuber bulking in an important way.

CHAPTER 5 *SUMMARY AND CONCLUSION*

Investigations were carried out to develop techniques for more productive microtuberization and breaking of microtuber and minituber post harvest dormancy. Continuous dark condition and agitation did not improve microtuber yield (number and fresh weight), neither did mechanical resistance or lower level of medium N. One positive effect of lower medium N level was that almost equal microtuber yield (number and fresh weight) could be obtained with half the total medium N as compared with full N level. Photoperiodic effects during step 1 on microtuber induction and growth during step 2 had never been investigated before. Step 1 photoperiod greatly influenced the process of microtuberization. Step 1 plantlets produced more microtuber yield (number and fresh weight) under long day photoperiod (16/8 h d/n) than 12/12 h or 8/16 h d/n cycles. So long day photoperiod (16/8 h d/n) may be retained for efficient microtuberization. Culturing for a prolonged period (60-65 d) was an efficient technique for having better yield, both in terms of microtuber fresh weight and number ≥ 500 mg, as compared with a single harvest after 30-35 d or successive harvests (1st harvest at 30-35 d, 2nd harvest at 60-65 d). Both single harvest from prolonged culture, and successive harvest, took longer than the usual single harvest period (30-35 d), but they were more efficient with medium and labour (for the resultant yield) compared with conventional microtuberization practices. It may be recommended that prolonged step 2 culture be performed for maximizing microtuber yield.

Post harvest dormancy of potato tubers has always posed a problem for their immediate use when wanted. The nature of dormancy of minitubers or microtubers was consistent with field grown potato tubers. A range of chemical treatments along with a couple of mechanical injury-treatments was applied on microtubers and minitubers. GA_3 was the most effective, anti-ABA and Signal solution being less, and BE being the least, among the chemical treatments. Development of early multiple sprouts with profuse secondary branches was the common feature of GA_3 treatment. Mechanical injuries such

as cutting minitubers or microtubers into halves or microwaving at full power for 10 sec. Presented infection risks or excessive injury (microwaving).

Minitubers that were forced to sprout precociously, before the end of the natural dormant period, using chemical agents such as GA_3, Signal, or BE showed significant differences harvest when compared with untreated controls. GA_3 was the most active compound for early as well as multiple sprouting; it produced the highest number of tubers with lowest total fresh weight compared with the other treatments. Signal solution was far less effective than GA_3 in releasing dormancy, but it produced the highest yield and indeed, the yield was almost double the harvest of GA_3-treated minitubers and better than the control. Clearly, precocious germination with various agents has a "carry over" effect on subsequent yield. While it is difficult to make recommendations at this point, some combination of GA_3 and Signal may increase tuber number with increased bulking and fresh weight productivity.

5.1 *Work to be done*

The experiments carried out so far were to assess microtuberisation efficiency and what chemical agents might break dormancy and thus bring about precocious sprouting. Microtuberization efficiency has been increased by prolongation of harvesting period but still, there are lots of opportunities to improve the process with changes in physical and chemical environmental factors. One factor might be experimenting with maximal photosynthetic photon flux applied during micropropagation and microtuberization for optimal microtuber production. The precise concentrations of dormancy breaking agents at which optimal sprouting would occur in least period without any adverse "carry over" effect on harvests, have not been studied yet in detail owing to time limitations. Since some chemical compounds have been found effective against dormancy in potato tubers (minituber/microtuber) there is enough area for future research.

Primary questions that must be addressed include:

i. What are the internal hormonal (and other) changes triggered in dormant tubers by GA_3, and other dormancy-breaking agents, that help to induce early sprouting?
ii. What are the main constraints, hormonally and otherwise, that limit yield when dormancy is broken prematurely by GA_3 and other dormancy-breaking agents? How can these be counteracted so that yield-impairment is not a natural consequence of premature dormancy breaking?
iii. How can physiological age be quantified in tubers, and applied for the more successful application of dormancy-breaking technology?

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