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ORIGINAL ARTICLES

Yield Components and Chemical Compositions of Some Potato Cultivars: *In Vitro* Microtubers Production and Field Performance

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ABSTRACT

Potato (*Solanum tuberosum* L.) microtubers are a valuable source for germplasm conservation, disease-free germplasm exchange and nutritional values. Microtubers induction potential of three potato cultivars were tested under *in vitro* conditions. The experimental design, complete randomized with three replications was applied. The results indicate that the effect of cultivar, hormone had influence on whole traits. MS medium supplemented with NAA at 2.0 mg/l and BAP at 0.5 mg/l resulted in the longest main shoots with highest node number. BAP at 0.1 mg/l were produced microtubers from 3 potato cultivars. Microtubers stored at 5^{o} C for one year and planted in soil. It was observed that tubers retained their viability under low temperature when tuber diameter was more than 5 mm. Such tubers produced healthy plantlets upon transfer to soil. The study showed that no significant differences of the chemical components of microtuber production *in vitro* compared with field tuber plants. The highest values in total protein and starch content were recorded in case of cv. Spunta, while the highest total sugars were obtained in case of cv. Agria. Moreover, the cv. Mondial ranks between in all assayed organic constituents. The expression of a genotype for microtuber production *in vitro* is different from that of tuber production under field situation i.e. the performance of a genotype *in vitro* is not a measure of its field performance.

Key words: Potato, tissue culture, hormonal combination, tuberization, conservation, chemical constituents.

Introduction

Potato (*Solanum tuberosum* L.) is one of the majority important field crops grown in all over the world. It is considered to be one of the fourth important energy vegetable crops of the world. In Egypt it is cultivated not only to local consumption but also to exportation as well as processing. It is economically important vegetable and has a great potential to provide nutritious food in diversity of environments for increasing hungry world and ranks after wheat, rice and maize as the fourth most important crops for human consumption (Ewing, 1997).

Potatoes can be propagated vegetatively from seed tubers or sexually from true potato seed. Commercial production has been based on seed tubers because of ease of planting, fast and vigorous early growth, and high yield potential. However, commercial potato production in developing countries has been constrained by high cost of imported seed tubers and the difficulties associated with the storage and handling of large quantities of seed tubers under hot humid conditions. One of the most factors resulting in the low productivity is the use of low quality seeds. The seeds are frequently obtained from previous harvest, which are commonly not virus free (Meulemans *et al*., 1986; Le, 1999; Ghavidel *et al*., 2012).

Most of the world potato producers use micropropagation techniques to achieve healthy tuber seed (Marinus, 1985; Jones, 1988; Hagman, 1990). The *in vitro* micropropagated potato plants can produce virus free microtubers seeds to increase potato productivity (Goodwin *et al*., 1980; Wang and Hu, 1982; Uyen and Zaag, 1985). These materials can be planted in a greenhouse or can be directly planted in the field. The use of *in vitro* growth of plants for production of microtuber has the advantage of higher control of different factors that might affect the tuber formation compared to plants grown in soil (Roca *et al*., 1978; Ziv and Shemesh, 1996; Veramendi *et al*., 1999). Furthermore, by using microtubers it is possible to maintain gene bank accessions in a much smaller space, and to remove virus-infection in asexually propagated species (Epinoza *et al*., 1989; Coleman and Coleman, 2000; Zobayed *et al*., 2001). Potato (*S. tuberosum* L.) microtubers offer several advantages over *in vitro* propagated plants, since they can be stored and transplanted directly into the field without an acclimatization stage. Also handling and shipping are easier, thus facilitating commercialization and international exchange of germplasm (Prematilake and Mendis, 1999; Miller et al., 1985; Jimenez-Gonzales, 2005).

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Tuberization in potato under *in vitro* condition is a highly complex developmental process, which may be modified in various ways. Growth regulators and genotypic differences influence potato tuberization (Hussey and Stacey, 1984; Leclerc *et al*., 1994; Villafranca *et al*., 1998; Anjum and Villiers, 1997; Silva *et al*., 2001; Hils and Pieters, 2005). Plant hormones have been studied for decades, but the interactions that take place between them are still being discovered (Naik and Chandra, 1993; Ross and O'Neill, 2001). Hormones play a crucial role in the control of potato tuberization (Vreugdenhil and Struik, 1989; Badoni and Chauhan, 2010), and the effect of exogenous plant growth regulators are commercially significant for the inducing of potato tuberization (Zhang *et al*., 2005). For inducing tuberization *in vitro*, much attention has so far been focused on the use of cytokinins such as BAP (Lentini and Earle, 1991), N6-(2-isopentenyl) adenine (Levy *et al*., 1993), kinetin (Pelacho and Mingo-Castel, 1991), and zeatin (Koda and Okazawa, 1983). In spite of the fact that the auxins indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at lower concentrations induce tuberization (Mangat *et al*., 1984), surprisingly little attention has been given to these hormones. Cytokinins play an important role in creating the sink during plant development, and through regulating the expression of a gene involved in the partition of assimilates towards the stolons as observed in potato (Prat, 2004). Longest main shoot and highest node numbers are reported to be obtained in medium containing NAA and BAP (Yousef *et al*., 1997).

In seed potato production programmes, the use of microtubers depends on their yielding ability under field conditions. Agronomic factors controlling field performance of potato microtubers have been extensively studied (Falconer, 1989; Ranalli *et al*., 1994a; Ranalli *et al* 1994b). However, no information exists on parameters affecting potato microtuber production and their field performance. Genotypic differences for microtuber production have been observed in a number of studies (Estrada *et al.*, 1986: Kumar and Verma, 1988; Ranalli *et al.* l994a). It has also been reported that plants forming more microtubers *in vitro* produced fewer microtubers in the soil (Ahloowalia, 1994). The relationship between corresponding *in vitro* and field yield components may allow effective use of this technology in seed tuber production programmes. In addition, this may allow the breeder to predict the relative yielding ability of genotypes under field conditions (Naik *et al.*, 1998; Struik and Lommen, 1999).

Within this context, studies were performed to find out the microtuber induction potential of 3 potato cultivars, investigate the role of 6-benzylaminopurine (BAP) and Naphthalene acetic acid (NAA) *in vitro* tuberization, use microtubers as a source for germplasm conservation and to investigate the relationship between the yield components of microtubers and corresponding yield components during field performance.

Materials And Methods

In vitro propagation:

Three *in vitro* ready stocks of plantlets of potato (*Solanum tuberosum* L.) genotypes were used in the present study. These genotypes (Spunta, Mondial and Agria) were provided from the plant production department, College of Food and Agricultural Sciences, King Saud University, Saudi Arabia. The number of plantlets was increased by sub-culturing via nodal cutting of about 2-4 cm at 3 weeks interval. The multiplication medium contained standard salts and vitamins of MS (Murashige and Skoog, 1962) supplemented with sucrose 30 g/l and 8.0 g/l agar. The pH of the media was adjusted to 5.7 before autoclaving. The nodal cutting as explants was cultured in MS medium consisting three different growth regulators combinations i.e. NAA and BAP (2.0 mg/l NAA+ 0.5 mg/l BAP, 2.0 mg/l NAA + 1.0 mg/l BAP and 2.0 mg/l NAA + 2.0 mg/l BAP). Cultures were incubated at 23 ± 1°C with 16 hours of fluorescent light (40 µEs-1m-2) to promote plantlets development. At the end of the each growing period, the single-stem plantlets were cut into single-node explants and placed on a fresh MS medium for further multiplication. The process was repeated until the required number of plantlets for *in vitro* tuberization experiments and *in vitro* storage experiments were achieved.

In vitro microtuberization:

Microtubers were produced using sub-cultured axillary shoot cuttings on tuberization induction medium. The basal induction medium was MS (Murashige and Skoog, 1962) supplemented with 0.1 mg/l BAP and 30 g /l sucrose. The microtuber induction cultures were incubated at 23 ± 1^{0} C under complete darkness for 75-90 days depending on the growth of microtubers. Observations were recorded on days to microtuber formation, average tuber diameter (cm), average of tuber weight (mg) and number of tubers per plant. The experiment was conducted in a randomised complete block design with three replications.

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Microtubers conservation:

After harvesting, microtubers were washed with sterilized distilled water and wiped dry with pre-autoclaved filter papers. Microtubers were put in Petri dishes sealed with Parafilm and stored under dark conditions at 5°C in a refrigerator for one year to field experiment performance. Fresh microtubers of 3 potato cultivars were used to determine the chemical constituents.

Field performance:

One year later, tubers were transferred to ambient temperature (25 ± 1^0C). One week afterwards, these microtubers were planted in a sandy loam field in a randomised complete block design with three replications. Each genotype was represented by three rows of 10 plants each at a spacing of 20 cm in rows 50 cm apart per replication. Fertiliser was applied at the rate of 150 kg N, 100 kg P_2O_5 and 100 kg K_2O per feddan at planting time, and at first earthing up. Plots were irrigated by furrow irrigation throughout the growing period according to evapotranspiration requirements to avoid drought stress during crop growth. After 80 days of growth, data on average tuber weight, number of tubers per plant and tuber diameter per plant were recorded.

Chemical constituents:

To determinate the chemical constituents of plant foliage of all 3 cultivars under study, total nitrogen (N) of dried plant foliage produced *in vitro* as well as open field plants were determined according to the method described by Pregl, 1945 using the microkjeldahl aparatus. Phosphorus (P) was determined calorimetrically according to Murphy and Riely, 1962 as modified by John, 1970. Potassium (K) was evaluated flame photometrically as mentioned by Brown and Lilleland, 1946. Total sugars in tubers were determined according to the method described in A.O.A.C., 1975. Starch content was measured in dry matter of tubers according to the method mentioned in A.O.A.C., 1990. In addition, total protein content in tubers was calculated by using the conversion factor (N x 6.25) as described by Pregl, 1945.

Experimental design and statistical analysis:

Experiments were arranged in a completely randomized design with three replications. Data were estimated as the average value of the different traits. The collected data were analyzed with the help of computer using Microsoft Excel 2010 program.

Results:

In vitro propagation:

In the present study, explants excised from nodal stem segments of aseptically grown potato plants were investigated for their *in vitro* regeneration efficiency and to get more nodes for future propagation. After 4 weeks of incubation on propagation media, shoot induction were observed for all potato cultivars studied. Mean of shoot height and number of nodes varied with the levels of NAA and BAP incorporated with the MS solidified media. In general, cultivar Spunta resulted in highest node number and shoot height comparing with cvs. Mondial and Agria. Shoots of cv Spunta in MS medium containing (2.0 mg/l NAA and 0.5 mg/l BAP) reached 20.0 cm with 22.0 nodes (Table-1). Combinations (2.0 mg/l NAA and 1.0 mg/l BAP) and (2.0 mg/l NAA and 2.0 mg/l BAP) respectively responded the least mean shoot height and number of nodes (Table 1).

Table 1: Effect of NAA and BAP concentrations with MS media on average of shoot height and node number of three potato cultivars

Cultivars / Growth Regulators (mg/l)		Spunta		Mondial		Agria	
NAA	BAP	Shoot height (cm)	Node number	Shoot height (cm)	Node number	Shoot height (cm)	Node number
2.0	0.5	20	22	11	13	10	12
2.0	1.0	17	18	10	11	9	11
2.0	2.0	15	17	9	10	8	10

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In vitro microtuberization:

In vitro tuberization was obtained after proliferating the culture in pre-tuberization medium where cultures grew previously and sub cultured on MS medium supplemented with 30 mg/l sucrose in presence of 0.1 mg/ BAP. It is quite evident from table 2 that all potato cultivars tested induced microtubers under *in vitro* conditions. Spunta cultivars produced the highest number of microtuber per plant (Figure 1). Average number of microtubers varied between 2.0-6.0 per plant. Average number of days to microtuber formation ranged from 60.0-85.0 days. Tuber size ranged from 5.0 mm to 8.0 mm in diameter whereas tuber weight was between 250.0-350.0 mg/tuber. The results suggested that all tested cultivars of potato have microtuber size and number varied within and between cultivars. Microtubers (more than 5 mm) obtained from all 3 cultivars subjected to low temperature storage 5^{o} C one year under complete darkness for germplasm conservation which germinated quickly when transferred to soil and produced normal and healthy plants (Figure 1).

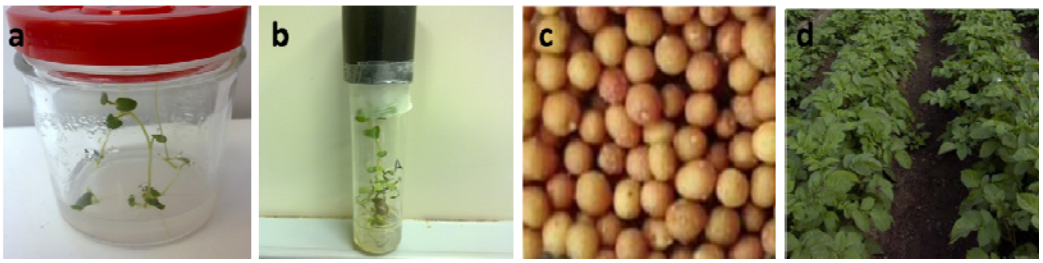

Fig. 1: Induction of microtubers of potato (cvs.Mondial) under *in vitro* conditions. (a) Shoot proliferation, (b) In vitro tuberization, (c) *In vitro* microtuber production, (d) Open field plants produced from microtuber.

Table 2: Characteristics of microtubers of tested potato cultivars.

Characteristics / Cultivars	Days to microtuber formation	Number of tubers/plant	Tuber diameter (mm)	Tuber weight (mg)
Spunta	75	4	6	250
Mondial	80	3	5	300
Agria	90	2	8	350

Characteristics of vegetative growth and physical tuber quality of tested potato cultivars under field:

Data presented in Table 3 indicate clearly that there were differences among the tested cultivars in all measured growth traits expressed as plant height, average number of nodes, average of tuber diameter and weight. In this respect, the highest values in all recorded growth aspects were obtained in case of cv. Spunta followed by cvs Mondial and Agria.

Table 3: Effect of potato cultivars on vegetative growth characteristics at 80 days after planting.

Characteristics / Cultivars	Plant height (cm)	Node Number	Tubers per plant	Tuber diameter (mm)	Average fresh weight of tuber (g)
Spunta	41	6	6	3647	81
Mondial	35	5	5	3560	83
Agria	31	4	5	3534	86

Chemical composition of plants produced in vitro and open field:

Data recorded in figure 2 indicate that there were differences among the tested cultivars in all assessed chemical constituents in plant leaves. The highest values (%) in total nitrogen, phosphorus and potassium percentage as well as protein content were recorded in open field plants comparing with *in vitro* plants for all tested cultivars. Spunta cultivar was the highest values followed by Mondial and Agria (Figure 2). The total N of open field plants was 4.38, 4.32 and 4.31%, while *in vitro* plants was 3.1, 3.0 and 2.7% of cultivars Spunta, Agria and Mondial, respectively. The total P of open field plants was 0.4, 0.39 and 0.38%, while it was estimated in *in vitro* plants as 0.28, 0.22 and 0.20% of cultivars Spunta, Agria and Mondial, respectively. In addition, the total K in dry foliage weight of open field plants was 4.66, 4.49 and 4.39% comparing to 4.0, 3.91 and 3.8% *in vitro* plants of Spunta, Agria and Mondial cultivars, respectively. Moreover, the total protein content was calculated in open field plants as 27.38, 27.36 and 27.11%, while it was estimated in in vitro plants as 24.45, 23.36 and 22.12% of Spunta, Agria and Mondial cultivars, respectively (Figure 2).

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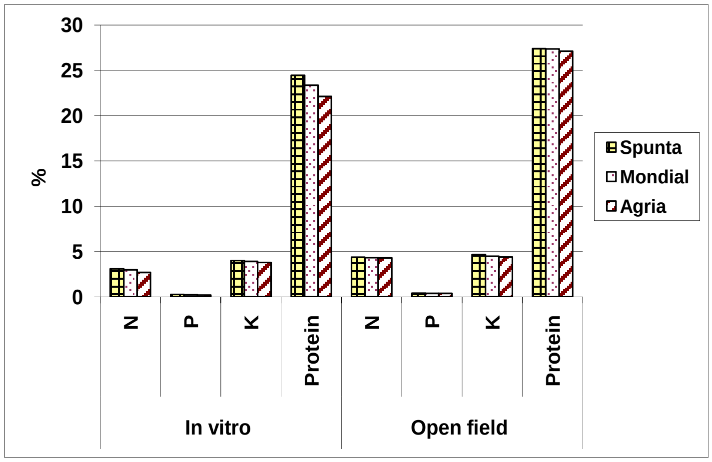

Fig. 2: Comparison of chemical composition of plant foliage obtained from *in vitro* and open field of three potato cultivars (N, P, K and Protein content were recorded as percentage).

Figure 3 reflects that organic constituents i.e., protein, total sugars as well as starch content of produced tuber were differences among *in vitro* and open field for all cultivars under current study. The highest values in total protein and starch content were recorded in case of cv. Spunta, while the highest total sugars were obtained in case of cv. Agria. Moreover, the cv. Mondial ranks between in all assayed organic constituents. The total protein in tubers of open field plants was 16.36, 16.13 and 15.98%, while *in vitro* plants was 13.14, 13.12 and 12.97% of cultivars Spunta, Agria and Mondial, respectively. The total sugar content of open field plants was 80.59, 79.48 and 82.87 mg/ 100 g dry tubers weight, while it was estimated in *in vitro* plants as 77.67, 76.34 and 77.79 mg/ 100 g of cultivars Spunta, Agria and Mondial, respectively. In addition, the total starch content in tubers of open field plants was 72.4, 72.36 and 71.67% comparing to 70.0, 69.67 and 69.0% *in vitro* plants of Spunta, Agria and Mondial cultivars, respectively (Figure 3).

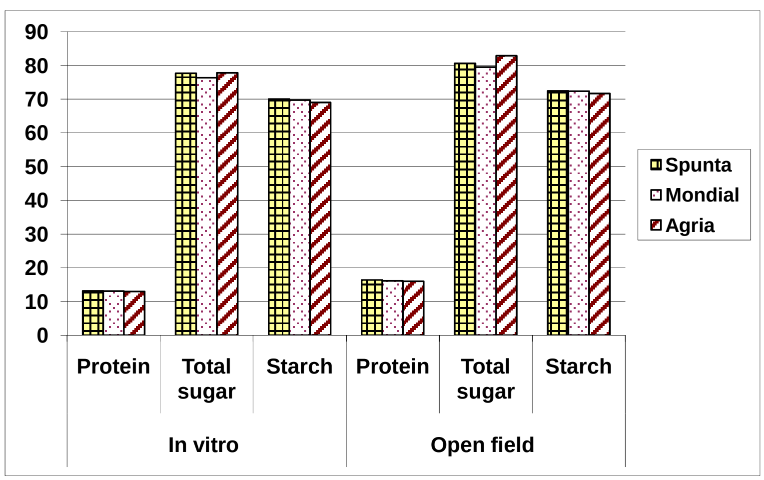

Fig. 3: Effect of potato cultivars on organic constituents in tubers obtained from *in vitro* and from open field (Chemical constituents were estimated in the dry matter of tubers. Protein and starch content were recorded as percentage and total sugar estimated in mg/100g dry weight).

Discussion:

Over the last three decades, micropropagation technology became an inseparable part of the seed potato programmes around the world, providing disease-free propagules in the early phases of mass multiplication. *In vitro* produced plantlet is the base in the elite seed potato production systems, in the majority of the programmes. This investigation outlines improvements to micropropagation methods commonly used in commercial laboratories, with a special emphasis on the production of microtubers (small tubers produced *in vitro).* It is a common believe that these propagules are underutilized in the commercial seed potato production due to their small size (0.02 - 0.70 g or 3 – 10 mm in diameter) and lack of uniformity (Hussey and Stacey, 1984; Estrada *et al.*, 1986; Struik and Lommen, 1990; Levy *et al*., 1993; Le, 1999). Moreover, it is difficult to obtain more than one microtuber per plantlet or nodal explant (Struik and Lommen, 1990; Ahloowalia, 1994; Struik and Wiersema, 1999). The lack of uniformity in the microtuber induction and the bulking processes directly affects the length of their dormancy period as well as their physiological age (Leclerc *et* al., 1995; Tabori *et al.,* 1999; Coleman and Coleman, 2000). Consequently, not all microtubers uniformly sprout and produce vigorous plants after planting.

Genotypic differences:

There are numerous varieties of the potato plant. More than 4,000 have been described in detail (Hils and Pieterse, 2005). These varieties differ in foliage maturity type, earliness and intensity of tuber formation, their response to abiotic environmental conditions both during the field phase and during storage, in their tolerance to or resistance against many diseases and pests, rate of multiplication in different systems of rapid in vitro or field multiplication, quality for various uses and in many other aspects (including their response to cultural practice).

Three commercial varieties were tested in the current investigation. It was found that all three responded positively to *in vitro* tuberization conditions and can be recommended for commercial production. Mean comparison showed that Spunta cultivar is superior for most traits than Mondial and Agria (Tables 1, and 2). This cultivar was superior in terms of shoot height, node number, microtubers number, while Agria Cultivar was superior regarding to microtuber diameter and fresh weight. The varietal differences in responses to *in vitro* tuberization have been reported earlier (Estrada *et al.*, 1986; Kumar and Verma, 1988; Nowak and Asiedu, 1992; Pruski *et al.,* 1993; Leclerc *et al*., 1994; Ranalli *et al*., 1994a; Ziv and Shemesh, 1996; Anjum and Villiers, 1997; Struik and Wiersema, 1999; Ghavidel et al., 2012).

Growth regulators:

In addition to genotype effect, growth regulators would effect on microtuber production in potato. Growth regulators are substances modifying plant growth and development and are widely used in *in vitro* multiplication to change the behaviour of the propagules or plants, thus optimizing the quality or quantity of the produce obtained. Also in potato, growth regulators are widely used to change the behaviour of the crop (manipulating tuber number, secondary growth, sprouting behaviour of tubers produced), change the behaviour of the stored seed or ware tubers (influence duration of dormancy and sprouting) or during *in vitro* plantlets or microtubers production (e.g. to create a more sturdy *in vitro* plantlets to obtain stronger transplants, induce tuberization or increase the number or size of the propagules) (Struik and Wiersema, 1999: Ross and O' Neil, 2001).

Results of preliminary studies with BAP alone (0.5, 1.0 and 2.0 mg/l) or in combination with NAA (0.1 mg /l) revealed callus formation at 2.0 mg/l of BAP only (Novak *et al.*, 1980). Nevertheless, callus proliferation from the tissues of most dicotyledonous plants is usually thought to require the presence of both an auxin and a cytokinin in the growth medium (George and Sherrington, 1984). However, Hagman, (1990) reported that the absence of callus is desirable to avoid any genetic variations through adventitious *in vitro* shoot development. Epinoza *et al*., (1989) reported that 10-20 fold increase in the number of potato nodes cultured for 2-3 weeks on MS liquid medium supplemented with 0.5 mg /l BAP, 0.01 mg /l NAA and 0.4 mg /l GA_3. In current trial, usage of plant growth regulators BAP 0.5 mg/l and NAA 2.0 mg/l in medium led to a significant increase in shoot height and node number (Table 1).

Different responses by potato cultivars to various growth regulators *in vitro* condition has been reported by many researchers (Koda and Okazawa, 1983; Mangat *et al*., 1984; Miller *et al*., 1985; Naik and Chandra, 1993; Prat 2004; Zhang *et al*., 2005; Ghavidel *et al*., 2012).

Several workers (Wang and Hu, 1982; Hussey and Stacey, 1984; Meulemans *et al*., 1986; Lillo, 1989; Harvey, 1990; Zhang *et al*., 2005; Badoni and Chauhan, 2010; Ghavidel *et al*., 2012) reported on the role of cytokinins of the microtuberization process *in vitro.* Present results showed that 30 g /l sucrose in presence of 0.1 mg /L BAP gave optimal microtuber number per plant in media cultures. At the end of the season, the number increased to 6 per *in vitro* rooted shoot after 20 weeks of incubation (Table 2). This result was strongly agreement with Yousef *et al*., 1997. On the other, this finding disagrees with the results of Wang and Hu, 1982; Badoni and Chauhan, 2010 who reported that 10.0 mg /l BAP were optimal for inducing highest number of microtubers.

Microtubers conservation:

Storage ability of microtubers at low temperature simplifies germplasm conservation of potato. This method of storing is further attractive as it is safer than field maintenance and since no subculturing is involved (as *in vitro* plant conservation), more simple and less time consuming. Since all cultivars behaved in a similar manner under darkness at 4^o C, this protocol is suitable for establishment of a potato germplasm gene bank with microtubers. It is reported that under these conditions, varietal characteristics of potato retained the ability to regenerate genetically identical plants (Uyen and Zaag, 1985; Villafranca *et al*., 1998; Prematilake and Mendis, 1999). In addition, all tested potato cultivars suggested that these tubers have the potential to behave as an alternative to seed potatoes. Small sizes of these tubers make them particularly attractive in germplasm exchange programmes where storage and transportation of bulky quantities of potato tubers or delicate in vitro plantlets is difficult. During transport microtubers can withstand more prolonged dark periods, rougher handling and wider range of temperatures compared to in vitro shoot cultures. On the other hand, microtubers can be produced in the laboratory year around and stored until request is made. The *in vitro* phase was not studied here as such, nor was the low-temperature storage of the *in vitro* stock material, since the procedures had already been developed (Goodwin *et al.*, 1980; Hussey and Stacey, 1981), optimized (Marinus, 1985) and successfully applied (AAFC, 1996) in the commercial production of *in vitro* plantlets and in conventional conservation of plant germplasm (Bessembinder *et al.,* 1993; Withers and Engelmann, 1998; Zobayed *et al*., 2001).

Field performance:

In microtubers, the size of the propagule is of essence for their behaviour during storage and after field planting (Ranalli *et al*., 1994b; Struik and Lommen, 1990; Struik and Lommen, 1999). Larger sizes give better emergence and a better early vigour, and produce a higher yield and more tubers per plant. Some of these effects can also be found in ranges that are common for normal seed tubers (Struik and Wiersema, 1999), although these effects depend on the variety used. Usually, larger seed sizes have more eyes per seed tuber, a higher proportion of eyes producing a sprout, a larger proportion of the sprouts producing a stem, a larger number of tubers per stem and often also a larger individual weight of the progeny tubers (Struik and Wiersema, 1999). In the experiment reported here, all microtubers used in field plantings were produced. The present study showed that there was variation in the estimated values of various parameters for vegetative growth and yield components of microtuber production *in vitro* and their field performance. The relative importance of the components of microtuber production *in vitro* differed from that of the corresponding yield components in the field (tables 2, 3 and figures 2 and 3). The estimated values of these parameters were greatest for vegetative growth and yield components (plant height, number of nodes, average microtuber number, weight of microtuber, total nitrogen and protein in plant leaves and tubers) of field plants comparing to *in vitro* plant characteristics. The present finding is in conformity with the earlier findings of Naik *et al*., 1998; Veramendi *et al*., 1999. The divergent variation in the two systems could be due to the fact that the parameters constitute properties not only of a specific trait, but also of the environmental circumstances to which the individuals are subjected (Falconer, 1989). On the other hand, there were no significant differences of the K content in leaves as well as total sugar and starch content in tuber derived from *in vitro* and field plants. Starch content of potato tuber as a source of energy is more important than other components. This makes the important in determining microtuber yield quality and field tuber yield, respectively. In conventional seed potato systems, farmers use seed potato tubers for the multiplication and production. This method has some major disadvantages (Beukema and Van der Zaag, 1990; Struik and Wiersema, 1999). Over the last three decades rapid multiplication systems became an important technique to provide disease-free propagules. These techniques yield in vitro plantlets, transplants, microtubers and minitubers, which are used in the initial phases of a seed tuber production scheme (Murashige, 1974; Roca *et al*., 1978; Hussey and Stacey, 1981; Wang and Hu, 1982; Jones, 1988).

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