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Efficient microtuber production of potato in modified nutrient spray bioreactor system

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ABSTRACT

A laboratory scale bioreactor system has been developed using nutrient spray technology for in vitro mass production of potato microtubers. Its effectiveness on the production of microtubers was investigated and compared with conventional liquid and semi-solid culture systems through bioreactor. Optimal culture conditions such as spray intervals, varying concentrations of 6-benzylaminopurine (BAP) and explants density were determined for the NSB. In order to determine optimal spray intervals, liquid medium was sprayed inside the NSB at different intervals (½, 1–4 h) of which the 1 h interval resulted in the highest number of shoots (3.47) and length (8.99 cm). Number of microtubers produced (5.13) was highest with 1 h intervals and fresh weight of microtubers (0.90 g) was highest for ½ h interval. Different concentrations of BAP (0.5, 1.0 and 1.5 mg/L) were used to evaluate its effect on microtuberization. It was observed that number and diameter of microtubers were increased (5.31 and 0.96 cm) when 0.5 mg/L BAP was supplemented in MS medium. We found fresh weight of microtubers (0.97 g) was increased when 1.0 mg/L BAP were added to the medium. In order to determine suitable explants density, single nodes grouped into five categories e.g 30, 45, 60, 75, and 90 and placed in the NSB system. A density of 60 explants resulted in increases in shoot length (17.5 cm) number of internodes (12.5) and with highest amount of chlorophyll (40.2 mg/g) as well as with highest number and fresh weight of microtubers (4.43 and 0.89 g, respectively). Out of the three culture systems, the NSB performed best where 1.5–2.0 fold increases in shoot growth and microtuberization without hyperhydration. The NSB also produced the highest number (4.67), fresh weight (0.86 g) and diameter (0.78 cm) of microtubers. From this study we may conclude that the NSB system has good potential for commercial mass production of potato micro-tuber.

1. Introduction

Potato (*Solanum tuberosum* L.) is grown in a wide range of climatic conditions and no other crops in the world can match the potato in energy value (Asghari-Zakaria et al., 2009). In vitro grown microtubers are genetically identical, high quality, and pathogen-free small-sized seed potatoes (about 0.5–1.5 cm diameter) which do not need acclimatization similar to the field-grown seed tubers (Srivastava et al., 2012). Potato plants derived from microtubers are normal and strong, and can be used in the production of original seed (Perez et al., 2007). The handling and shipping of microtubers is also more convenient which facilitate its commercialization (Imani et al., 2010). In a commercial laboratory, microtubers are produced round the year in a conventional semi-solid nutrient medium. However, this method usually produces 1.0–1.5 microtubers per plantlet, with an average diameter of <0.5 mm, thus limiting the success rate of direct plant to field conditions (Struik and Wiersema, 1999).

Bioreactor systems are used mostly for secondary metabolite production from cell and root cultures in order to achieve rapid and efficient growth and multiplication of high quality plant propagules at low costs (Ziv, 2005). Various bioreactor systems such as recipient with automatized temporary immersion (RITA), bubble column bioreactor (BCB), and balloon type bubble bioreactor (BTBB) have been developed for plant micropropagation (Takayama 1991;

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Alvard et al., 1993; Shohael and Paek, 2013). They offer several benefits including better control of culture conditions, optimal supply of nutrients and growth regulators, renewal of culture atmosphere, changing of the medium during the culture period according to developmental stage, filtration of the medium for exudates, contamination control and production of clusters of buds or somatic embryos (Ziv, 2005).

Several studies have reported the potential of different bioreactor systems for the mass propagation of potato microtubers (Donnelly et al., 2003). Liquid cultures together with various temporary immersion techniques, ebb and flood, Rita™ system, twin-flask system, plastic bag cultivations as well as tilting rocker system have also been evaluated for the production of potato microtubers (Estrada et al., 1986; Akita and Takayama, 1994; Teisson and Alvard, 1999; Jimenez et al., 1999; Grigoriadou and Leventakis, 2003; Piao et al., 2003; Kämäräinen-Karppinen et al., 2010). Most of these techniques, however, involve the periodical immersion of plant propagules in liquid media. However, Weathers and Giles (1988) suggested that a different bioreactor system, wherein plant materials were cultured in nutrient mist created by ultrasonic transducers, might be more effective for plant micropropagation. They cultured various plants in nutrient mist bioreactor systems and reported improved growth (Hao et al., 1998). As plant propagules were directly exposed to the nutrient mist, composed of medium micro-particles and gas, both nutrient absorption and gas exchange of the plant tissue improved, resulting in enhanced plant growth (Hao et al., 1998). Although nutrient mist bioreactors are mainly used for hairy root cultures, other plant propagules may also be cultured in such systems (Weathers et al., 2008). Hao et al. (1998) and Kurata et al. (1991) reported improved shoot growth and microtuberization of potato in a nutrient mist bioreactor. A nutrient spray bioreactor (NSB) functions on a principle similar to that of a nutrient mist bioreactor, except that in the former the liquid medium is sprayed over the plant materials by a spray nozzle rather than by ultrasonic transducers.

In recent years, the farming of crops particularly seed-potato development through tissue culture has silently revolutionized in agricultural sector of Bangladesh and also in the world. Quality seeds of different crops including potato are produced in commercial laboratories in Bangladesh using conventional micropropagation techniques such as semi-solid and liquid cultures. These laboratories have standardized suitable protocols of their own with the standard potato cultivars of the country by incorporating different factors like culture media, light, temperature, explants, etc for maximizing microtuber production (Hossain, 2005; Hoque, 2010). However, little work has been done on microtuberization in bioreactor systems. In this study a nutrient spray bioreactor (NSB) was developed at the laboratory scale and its potential for application in potato micropropagation was evaluated.

The aim of this study was to develop and optimize a suitable system for potato shoot growth and microtuberization by investigating the effect of different nutrient spray intervals, BAP concentrations and inoculation densities on various aspects of microtuber production. It also aimed to compare microtuber productivity of the NSB system with conventional semi-solid and liquid culture systems.

2. Materials and methods

2.1. Plant materials

Nodal cuttings (with one leaf) of potato plantlets were cultured in 0.6% agar solidified MS (Murashige and Skoog, 1962) medium containing 30 g/L sucrose (pH 5.8); incubated at 25 °C and 50–55% relative humidity, and subjected to 50 μ mol m^{-2} s^{-1} light intensity with 16 h photoperiod. After 3 weeks of culture, each shoot was divided into nodal segments, each containing one node, and subcultured in a similar medium.

2.2. Culture systems

The nutrient spray bioreactor (NSB): The NSB system has developed by us consists of two vessels, each constructed from 1 L glass reagent bottles – an upper culture chamber, and a lower medium reservoir (Fig. 1a). The culture chamber is 12 cm in height with 9.5 cm inner diameter, and has 2.5 cm wide rims at the top and bottom. It has a lid (diameter: 14.5 cm) with a brass spray nozzle (diameter: 0.8 cm, length: 3.7 cm, spray orifice: 0.5 mm) attached. The medium reservoir is 10 cm in height and 9.5 cm in diameter, and has a 2.5 cm wide rim at the top, an air inlet, and a medium outlet, which is connected to the spray nozzle by a silicone tube (ID 0.6 mm). Nutrient spray was created by releasing compressed air into the medium reservoir via a hydrophobic membrane filter (0.22 μm) connected to the air inlet. There is a one-way check valve at the bottom of the culture chamber, which allows the sprayed liquid to return to the medium reservoir but prevents reverse flow of liquid or air from the medium reservoir to the culture chamber.

The semi-solid and liquid culture systems: For the semi-solid culture system (Fig. 1b) the protocol described in Piao et al. (2003) was followed. For the liquid culture system (Fig. 1c) a combined support of stainless steel net and filter paper (Whatman No. 1) was used and for sterile air exchange the caps of the culture vessels were fitted with 0.22 μm hydrophobic filters.

2.3. Culture medium and growth conditions

Liquid MS medium was used in the NSB and liquid culture systems. For the study on shoot growth 30 g/L sucrose solution was added to the medium and the pH was adjusted to 5.8 before autoclaving (121 °C, 15 psi). Then, the culture systems were incubated under 50 μmol m^{-2} s^{-1} light intensity for 3 weeks at 25 ± 1 °C in 16/8 h day/night cycles. For the microtuber development study fully grown plantlets were transferred into similar culture vessels in which the MS medium was supplemented with 80 g/L sucrose solution and various concentrations of BAP (0.5, 1.0 and 1.5 mg/L). Cultures were incubated in the dark at 20 ± 1 °C for 10 weeks.

2.4. Optimization of nutrient spray interval, concentration of BAP and explants density in NSB systems

In order to determine the optimum nutrient spray interval, liquid nutrient medium was sprayed at five different intervals e.g ½, 1–4 h and controlled by a solenoid valve, regulated by an electronic timer. Compressed air was supplied at 0.4 MPa, which created a liquid nutrient spray at the rate of 0.2 L/min in the NSB system. For the study investigating the effect of growth regulators on microtuberization, the MS medium was supplemented with different concentrations of BAP (0.5, 1.0 and 1.5 mg/L). Sixty explants were inoculated in each culture vessel and nutrient medium was sprayed at 1 h intervals. Then the NSB system was incubated in darkness at 20 ± 1 °C for 10 weeks. To optimize explants density in the NSB, explants (single nodes) were grouped into five categories e.g 30, 45, 60, 75, and 90 and placed in the culture vessel (9.5 cm in diameter) with 250 mL of liquid medium in the reservoir. Nutrient medium was sprayed at 1 h intervals and the NSB system was incubated for shoot growth and microtuber induction in the above-described culture conditions.

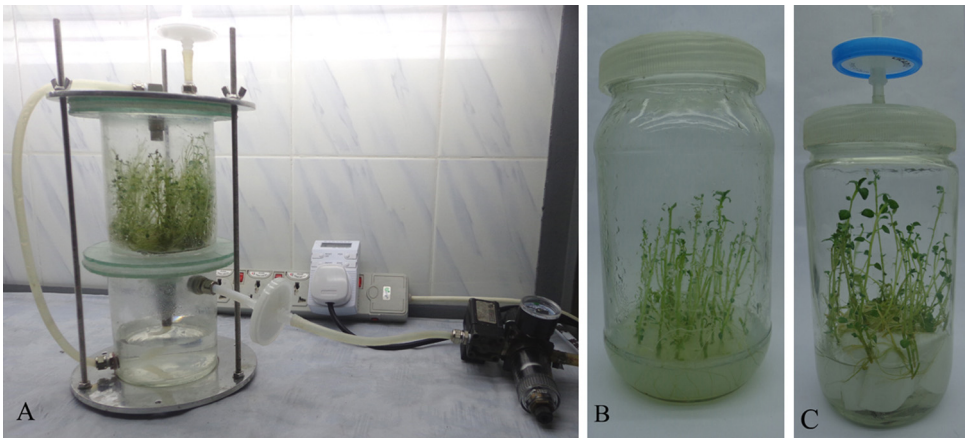

Fig. 1. Three different culture systems – (a) Nutrient Spray Bioreactor, (b) Semi-solid culture and (c) Liquid culture.

Table 1
The effect of 6-BAP on microtuberization of potato in NSB system after 10 weeks of culture.

Parameters	BAP (mg/L)		
	0.5 ±SE	1.0 ±SE	1.5 ±SE
Number of microtubers per plant	5.31 ± 0.16a	3.08 ± 0.19b	4.22 ± 0.2b
Diameter of microtubers (cm)	0.96 ± 0.09a	0.81 ± 0.12b	0.79 ± 0.05b
Fresh weight of microtubers (g)	0.83 ± 0.11b	0.97 ± 0.08a	0.80 ± 0.16b

Means with the same letter in a row has no significant difference and are separated using Duncan's multiple range test (DMRT)

2.5. Comparative studies between different culture systems

Each NSB was inoculated with 60 explants. Total of 250 mL of liquid MS medium was sprayed at 1 h intervals. On the other hand the semi-solid and liquid culture systems contained 50 mL of liquid MS medium, and 12 explants in each vessel. The medium and culture conditions for the semi-solid and liquid culture systems were the same as those described above for the NSB system.

2.6. Growth of shoots and microtubers

After three weeks, shoot growth was studied by measuring (1) number of shoots, (2) length of shoots and (3) number of internodes. The total chlorophyll content of fully developed leaves was measured by a UV–vis spectrophotometer at 645 and 663 nm following the method of Hiscox and Israelstam (1979) and results are presented in Table 2. Occurrence of hyperhydricity was detected by visible symptoms including thick, broad, translucent leaves that were wrinkled or curled, or both, and brittle (Table 3). Data on microtuberization was recorded after 10 weeks of culture; the number and fresh weight of microtubers was measured as an index of their quality (Fig. 3).

2.7. Statistical analysis

Experiment was arranged as factorial based on CRD (completely randomized design) with three replications (each NSB considered as a replicate). All characteristics (except occurrence of hyperhydricity) were compared across experimental treatments using analysis of variance (ANOVA) with a p value of 0.05. Statistical significance between mean values of the replicates was assessed using a Duncan's multiple range test (DMRT). Statistical analysis was performed using SPSS.

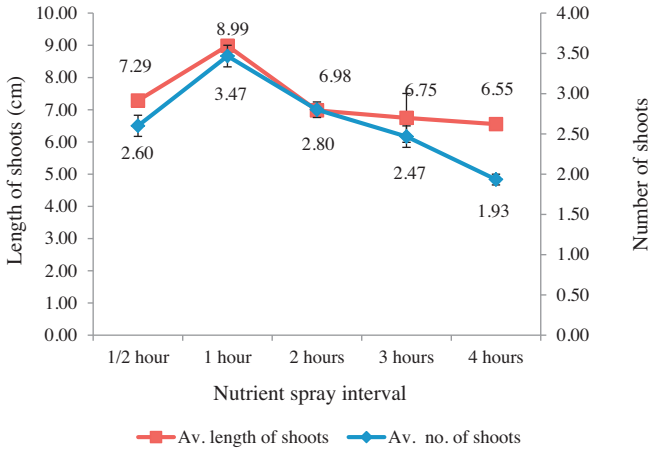

Fig. 2. Effect of nutrient spray interval on number and length of potato shoots cultured in nutrient spray bioreactor system. Each point is the mean value with three replicates.

3. Results

The results of the nutrient spray interval experiment showed that in the NSB system, the highest growth of potato shoots occurred when the nutrient medium was sprayed at 1 h intervals (Fig. 2). Compared to shorter (½ h) and longer (2–4 h) spray intervals, the 1hr interval produced the highest number and greatest length of potato shoots (3.47 and 8.99 cm, respectively). Shorter intervals produced hyperhydrated shoots (with the glassy appearance of water-logged tissue, short nodes and abnormal leaves) whereas longer intervals resulted in reduced shoot growth.

Nutrient spray interval in the NSB system had a pronounced effect on potato microtuberization (Fig. 3). Among the five nutrient

Table 2
Effect of explants density on shoot growth and microtuberization of potato in NSB system after 3 weeks of culture.

No of explants	No. of internodes/ plantlet	Length of shoot (cm)	Total chlorophyll (mg/g)	No. of microtubers per plantlet	Fresh weight of microtubers (g)
30	12.2 ± 0.06a	16.7 ± 0.2a	39.2 ± 0.25a	3.92 ± 0.29b	0.86 ± 0.17a
45	12.3 ± 0.1a	16.9 ± 0.13a	39.7 ± 0.35a	3.94 ± 0.43a	0.85 ± 0.38a
60	12.5 ± 0.05a	17.5 ± 0.14a	40.2 ± 0.2a	4.43 ± 0.36a	0.89 ± 0.17a
75	9.7 ± 0.17b	16.8 ± 1.2b	31.2 ± 0.38b	2.97 ± 0.11c	0.71 ± 0.26b
90	8.5 ± 0.23c	16.3 ± 1.7c	30.8 ± 0.31b	2.41 ± 1.4c	0.64 ± 0.31b

Means with the same letter in a column has no significant difference and are separated using Duncan's multiple range test (DMRT).

spray intervals, the highest number of microtubers was obtained at intervals of ½ h (5.13 g) followed by 1 h (4.79 g). However, the differences between the intervals were not significant. Furthermore, the fresh weight of microtubers was significantly higher at 1 h nutrient spray intervals (0.90 g) than others.

The experiment investigating the effect of different doses of BAP on potato microtuberization in the NSB system revealed that the MS + 0.5 mg/L BAP medium induced the highest number (5.31) of microtubers as well as largest (0.96 cm diameter) when compared to the other concentrations (1.0 and 1.5 mg/L; Table 1). However, 1.0 mg/L BAP increased results in the highest fresh weight of microtubers (0.97 g) compared with lower doses (0.5 mg/L) or more (1.5 mg/L) of the plant growth regulators (Table 1).

The results of explants density experiment showed that from 30 to 60 explants per bioreactor has no significant difference in the number of internodes per plantlet, length of shoots and total chlorophyll content as well as the number and fresh weight of microtubers (Table 2). In all cases, plant morphology was similar in the color, vigor, or quality of the shoots or microtubers. But further increase to higher densities (75 and 90 explants per bioreactor) resulted in thin, light green shoots with fewer microtubers. Furthermore, calli of watery consistency were formed at the base of shoots during microtuberization (Fig. 4a).

To evaluate the effectiveness on shoot growth and microtuberization, the developed NSB system was compared with micropropagation using semi-solid and liquid culture (Table 3). The experimental comparison of shoot growth between the NSB and the semi-solid and liquid cultures showed that the NSB system produced potato explants with the highest number of shoots (3.33), the longest shoots (8.69 cm) and the highest number of internodes (7.03; Fig. 4b). Hyperhydricity was observed in the liquid culture (17%) but not in the NSB or semi-solid cultures. Microtuberization also varied significantly across the three types of cultures with higher numbers as well as fresh weights and diameters of microtubers (4.67, 0.86 g and 0.78 cm, respectively) obtained from the NSB system compared to those obtained from liquid and conventional semi-solid cultures (Fig. 4a).

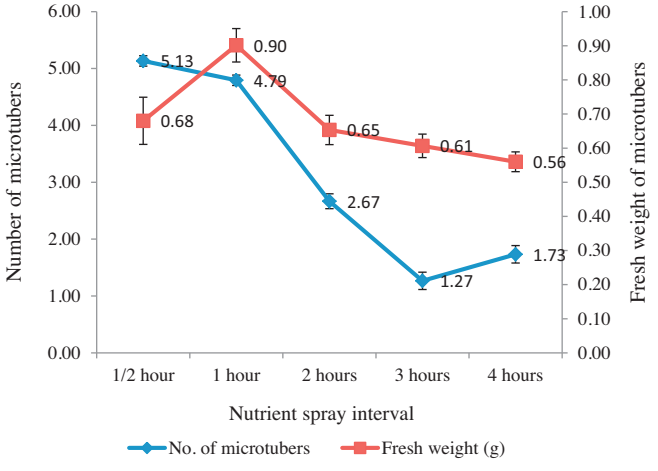

Fig. 3. Effect of nutrient spray interval on number and fresh weight of microtubers of potato cultured in nutrient spray bioreactor system. Each point is the mean value with three replicates.

4. Discussion

Seed production technique of potato can be designed with in vitro multiplication through either plantlet regeneration or microtuber production. But microtuber method has tremendous advantages over plantlet regeneration. Plantlets are more vulnerable to damage during storage, shipping and transplant than microtubers. Production of microtuber can also be mechanized and they can be stored for long time (Hoque, 2010). Microtubers can be directly sown into the field too, since they do not require time consuming hardening period and have higher survival rate than plantlets (McCown and Joyce, 1991).

The optimization of nutrient supply frequency in bioreactor systems is very important because it determines the occurrence of hyperhydricity and the delivery of nutrients (Etienne and Berthouly, 2002; Wang and Qi, 2010). Studies have reported varying optimum frequencies depending on the system used. Hao et al. (1998) reported that an optimum nutrient mist supply frequency of 5 min on/2 h off for shoot growth and microtuberization in a nutrient mist bioreactor. On the other hand in a temporary immersion system, Jiménez et al. (1999) observed that the best growth of potato shoots at an immersion frequency of 5 min for every 3 h. Etienne and Berthouly (2002) reported that 1 h of immersion per 6 h interval was useful for microtuberization of potato. In our NSB system, a nutrient spray frequency of once per hour was optimum for shoot growth and microtuberization.

It is well established that continuous or prolonged contact of plant tissue with a liquid medium is the main cause of hyperhydricity (Albarrán et al., 2005). In our study, hyperhydricity occurred at shorter intervals; other authors reported that hyperhydricity due to different long/short immersion intervals in bioreactor cultures (Kevers et al., 2004; Chakrabarty et al., 2007; Welander et al., 2007). Additionally, in our study, whereas shorter intervals in nutrient supply resulted in hyperhydricity, longer intervals (2–4 h) led to decreased growth in potato shoots and microtubers. Akula et al. (2000) reported that for tea (*Camellia sinensis*) cultured in a temporary immersion bioreactor, longer intervals resulted in slower growth. It is likely that longer intervals limit the uptake of nutrients thus resulting in the slow growth of plant materials cultured in bioreactors (Preil and Hempfling, 2002).

In our NSB system, a low concentration of BAP (0.5 mg/L) worked well for potato microtuberization. Piao et al. (2003) observed that in a temporary immersion bioreactor, a concentration of 2.0 mg/L BAP stimulated microtuberization, whereas in a nutrient mist bioreactor 10 mg/L BAP worked well (Hao et al., 1998). Growth regulators, when supplied in specific balanced ratios, act as morphogenic signals and control the development of propagules. The direct contact of explants with the medium may make the availability of growth

Table 3
In vitro shoot growth and microtuberization of potato by using different culture systems.

Culture systems	Shoot growth (after 3 weeks)				Microtuberization (after 10 weeks)		
	Number of shoots	Length of shoots (cm)	Number of internodes	Occurrence of hyperhydricity (%)	Number of microtubers	Fresh weight (g)	Diameter (cm)
Semi-solid culture	1.07 ± 0.07b	3.16 ± 0.13b	3.60 ± 0.13b	0	1.47 ± 0.13b	0.14 ± 0.03b	0.29 ± 0.02b
NSB culture	3.33 ± 0.13a	8.69 ± 0.14a	7.03 ± 0.10a	0	4.67 ± 0.09a	0.86 ± 0.09a	0.78 ± 0.05a
Liquid culture	2.37 ± 0.12b	5.66 ± 0.08b	5.09 ± 0.13b	17%	1.79 ± 0.11b	0.25 ± 0.11b	0.31 ± 0.03b
p value	<0.05	<0.05	<0.05		<0.05	<0.05	<0.05

Means with the same letter in a column has no significant difference and are separated using Duncan's multiple range test (DMRT).

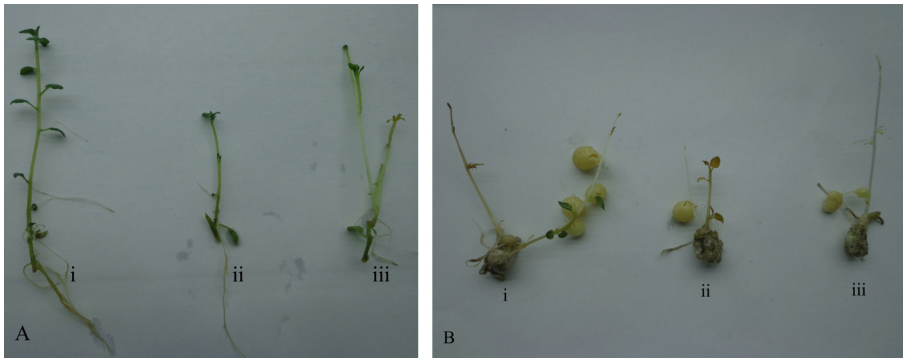

Fig. 4. Microtubers (a) grown in – (i) NSB, (ii) semi-solid and (iii) liquid culture systems and (b) regenerated plantlets obtained from three treatments.

regulators in bioreactor cultures more effective in controlling proliferation and regeneration potential (Zic, 2005).

Sarkar et al. (1997) reported that for an optimum explants density required for an efficient growth of potato shoot growth and microtuberization. Our study showed that increasing explants density improves shoot growth and microtuberization for potato. For instance, number of internodes per plantlet, shoot length, total chlorophyll, as well as number and fresh weight of microtubers increased linearly as density increased up to maximum of 60 explants per bioreactor, beyond which it decreased once again. Similar patterns have been reported by other studies, which showed that low inoculum densities could result in the sub-utilization of the bioreactor, whereas high densities could cause phenotypic malformations, thereby resulting in lower quality plants and microtubers (Piao et al., 2003; Hahn and Paek, 2005; Pérez-Alonso et al., 2007).

Our system also induced better growth of potato shoots and microtuber in cultured explants when compared to the other systems tested. Similar results were reported from temporary immersion bioreactor system reported by Wawrosch et al. (2005), Zhu et al. (2005) and Adelberg (2005). According to Etienne and Berthouly (2002), bioreactor systems combine the advantages of solid cultures (maximum gas exchanges) and liquid media (increased nutrient uptake). The efficiency of the NSB system is probably driven by its ability to provide ventilation and intermittent contact between entire explants and the liquid medium, a feature which is absent in classical liquid and semi-solid culture procedures.

In summary, this study developed a system that produces better quality plantlets, with increased number of shoots and suitable size of microtubers, than conventional liquid and semi-solid culture methods. The nutrients spray interval, BAP concentration and inoculum density for this system was optimized for the mass propagation of potato microtubers. More efficient than other currently used systems, our NSB system may open a new dimension of opportunity for commercial laboratories, in the field of potato micropropagation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2015.06.014

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