In Vitro Culture and Microtuberization of 'Spunta' Potato (*Solanum tuberosum* L.)

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ABSTRACT

Effect of different levels of 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) and BAP and sucrose on *in vitro* shoot (SH) proliferation and microtuberization of 'Spunta' potato was studied using virus-free plantlets on modified Murashige and Skoog (MS) media.

NAA at 2.0 mg l^{-1} and BAP at 0.5 mg l^{-1} resulted in the longest main SH (22 cm) with highest node numbers (23 nodes). A maximum of 15 axillary SHs per main SH was produced after 10 weeks of incubation on the modified MS proliferation media in presence of 2.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA.

Sucrose at 40 g l^{-1} when associated with 0.1 mg l^{-1} BAP was optimal for obtaining the maximum number of microtubers (MTs) *in vitro*. Severn MTs per SH and 1.35 MTs per node were obtained after 20 and 8 weeks, respectively. Largest MT weight and size resulted from media with 80 g l^{-1} sucrose supplemented with 0.1 mg l^{-1} BAP.

On basis of space required and media used, single nodal cuttings with leaf (SNCs) seem to be more applicable for induction of MTs *in vitro*; contamination hazards are also reduced.

INTRODUCTION

Propagation by tissue culture provides the opportunity for having virus free *in vitro* cultures used for micropropagation of large quantities of plantlets (Estrada et al., 1986) to produce MTs *in vitro* or minitubers *in vivo*. MTs can be used as an additional component to the standard methods of rapid propagation used in seed tuber production (Dodds, 1988) and utilized for the distribution of germplasm (Dodds, 1988; Epinoza et al., 1989) quite conveniently.

In vitro potato microtuberization has been reported (Harmey et al., 1966; Hussey and Stacey, 1984; Lillo, 1989) using different types of explants including nodal (Harmey et al., 1966; Hussey and Stacey, 1984) and *in vitro* SH (Lillo, 1989) cuttings.

Several media for propagation under aseptic conditions were also tested (Miller et al., 1985; Estrada et al., 1986; Dodds, 1988). Most multiplication programmes use modified MS media (Murashige and Skoog, 1962) with variable supplements of vitamins and plant bioregulators (PBRs) (Estrada et al., 1986). In general, increasing the sucrose concentration from 10 to 80 g l^{-1} increased the percentage and earliness of microtuberization (Wang and Hu, 1985). Wang and Hu, (1982) found that sucrose at the concentration 80 g l^{-1} was optimum for the *in vitro* microtuberization from *in vitro* rooted SHs.

In vitro microtuberization always resulted when exogenous cytokinins were used (Wang and Hu, 1985). 10 mg l^{-1} BAP induced the highest number of MTs from *in vitro* rooted SHs within a concentration range of 0.01 to 30.0 mg l^{-1} (Wang and Hu, 1982).

Hence, this investigation was initiated to study the possible effect of different levels of cytokinin, auxin and sucrose on *in vitro* SH proliferation and microtuberization of the locally used 'Spunta' potato.

MATERIALS AND METHODS

In vitro virus-free potato plantlets of 'Spunta' (accession number 800923) were provided from the International Potato Center (CIP), Lima, Peru. The plantlets were certified to be free from: potato

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virus A (PVA), potato virus S (PVS), potato virus X (PVX), potato virus Y (PVY), potato leaf roll virus (PLRV) and other viruses.

Upon arrival, the imported plantlets were propagated using SNCs and shaken liquid culture.

Solidified agar and liquid media were prepared as described by Estrada et al. (1986). Forty and 10 ml of the agar medium were poured into each of autoclavable polypropylene plant cell culture vessels (110 x 86 x 70 mm) and pyrex test tubes (25 x 150 mm), respectively. In addition, aliquotes of 20 ml of the liquid medium were dispensed in 250 ml erlenmeyer flasks. Vessels, tubes and erlenmeyer flasks were autoclaved for 20 minutes at 121°C and 15 psi and cooled to room temperature inside a laminar air flow hood.

Plant cell culture vessels and test tubes with solidified agar medium were inoculated each with a SNC, while the flasks with liquid media were layered each with two 4–5 cm-long stem pieces with 4 nodes each, under a laminar air flow hood. Liquid cultures were shaken continuousely (80 rpm) using an orbit shaker. All cultures were then incubated in a growth chamber at 23–25°C with supplemental light of 70 µmol $m^{-2} s^{-1}$ for 16 hour photoperiod. Every 3 to 4 weeks, 7 to 10 node plantlets/ node and 30–40 nodes/ flask become available for subculturing.

Effects of 16 treatment combinations of 4 levels (0.1, 0.5, 1.0, 2.0 mg l^{-1}) of each of BAP and NAA on *in vitro* propagation of potato as well as effects of 16 treatment combinations of 4 levels of each of BAP (0.0, 0.1, 5.0, 10.0 mg l^{-1}) and sucrose (20, 40, 80, 100 g l^{-1}) on *in vitro* microtuberization of potato, were studied using solidified agar (0.4 mg l^{-1} thiamine HCl, 2.0 mgl^{-1} Ca-pantothenic acid, MS basal salts (Murashige and Skoog, 1962), 30 gl^{-1} sucrose, 8.0 g l^{-1} agar and 0.1 g l^{-1} inositol) and liquid (0.4 mgl^{-1} thiamine HCl, MS basal salts and 0.1 gl^{-1} inositol) media, respectively.

For the *in vitro* propagation experiment, each treatment consisted of one vessel with 40 ml of the solidified agar medium inoculated with a SNC; each treatment was replicated 4X.

For the *in vitro* microtuberization, two experiments were conducted. In one experiment, each treatment consisted of one vessel with 60 ml of liquid medium layered with six week old *in vitro* plantlets (8–10 nodes each) and replicated 3X. In the second experiment, each treatment consisted of one test tube with 10 ml of liquid medium inoculated with a SNC excised from a 3 week old *in vitro* potato plantlets; each treatment was replicated 20X.

All treatments in all experiments were arranged in a completely randomized design. Cultures of the *in vitro* propagation experiment were kept in a growth room under conditions similar to those of the initial propagation, while conditions of the *in vitro* microtuberization experiments were 20 – 22°C and complete darkness. After 10 weeks, plantlets of the *in vitro* propagation experiment were removed from the medium and length of the main SH, number of nodes per main SH and number of axillary SHs were measured. On the other hand, for the *in vitro* microtuberization experiments MT number was measured in the first experiment and MT number, weight and size were measured in the second experiment.

Data for all experiments was analyzed as for the factorial completely randomized design (Little and Hills, 1978).

RESULTS AND DISCUSSION

A. *In vitro* Propagation

Similar to earlier findings (Estrada et al., 1986; Epinoza et al., 1989), 3 to 4 weeks were required for SNCs to develop into potato plantlets on PBR-free solidified media. In the present experiments, however, when virus-free nodes were cultured for more than one month in an attempt to get more nodes for future propagation, growth of plantlets was poor, and signs of senescence developed. Therefore, the idea was abandoned and cytokinin was introduced following reports that cytokinins, natural or synthetic, are of prime importance in deferral of leaf senescence (Moore, 1979).

Results of preliminary studies with BAP alone (0.5, 1.0 and 2.0 mg l^{-1}) or in combination with NAA (0.1 mg l^{-1} revealed callus formation at 2.0

mg l^{-1} of BAP only. This agrees with the findings of Novak et al. (1980) where higher BAP concentrations resulted in callus formation on bases of meristem tips of potato explants. When all BAP concentrations were combined with 0.1 mg l^{-1} NAA no callus was formed. 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 SH development.

Ten weeks after culturing single nodes, number of nodes per SH increased with SH length (R = 0.87**). SH length and node number (Fig. 1), however, varied with the levels of BAP and NAA incorporated with the MS solidified media, but gave almost similar patterns except for some minor discrepancies at 0.5 mg l^{-1} NAA in presence of either 0.5 or 1.0 mg l^{-1} BAP. In general, low NAA (0.1 mg l^{-1}) combined with high BAP (2.0 mg l^{-1}) or high NAA (2.0 mg l^{-1}) combind with moderate amount of BAP (0.5 mg l^{-1}) resulted in highest node number (21 – 23 node/ main SH). In contrast, main SHs were taller when high NAA levels (1.0 to 2.0 mg l^{-1}) were used with moderate (0.5 mg l^{-1}) to high (2.0 mg l^{-1}) BAP levels (Fig. 1).

Epinoza et al., (1989) reported 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^{-1} BAP, 0.01 mg l^{-1} NAA and 0.4 mg l^{-1} GA_3; their 1:50 ratio, NAA to BAP, seems to fall within the range 4:1 and 1:20 where best response occurred under the conditions of the present investigation (Fig. 1). According to Miller et al., (1985) the combination of 1.0 mg l^{-1} GA_3 and 0.1 mg l^{-1} NAA was more effective in increasing the number of nodes which could subsequently be cultured; contribution of potato cultivars to observed discrepancies could not be ignored as 'Spunta' was the only cultivar used in this study.

Growth and morphogenesis *in vitro* are not only regulated by the interaction and balance between the PBRs supplied in the medium, but also endogenous PBRs produced by cultured cells have a role as well (George and Sherrington, 1984). That auxin application induce rooting of *in vitro* grown SHs and roots act as main center for cytokinin biosynthesis (George and Sherrington, 1984), endogenously produced cytokinin could have affected the optimal ratios reported herein (Fig. 1). Miller et al., (1985) reported that 1.0 mg l^{-1} GA_3 substantially increased the plantlet height of most potato cultivars. Amirouche (1982), as cited by Miller et al., (1985) found that when applied to 'Record' potato, NAA alone had no appreciable effect on the overall height but stimulated root production. A combination of kinetin, IAA and GA_3 induced cultured meristems to form multiple SHs in several potato cultivars (Novak et al., 1980). However, PBR-free media was almost higher than all BAP and NAA combinations (Fig. 1) in terms of SH length and node number (17.1 cm and 14.7 nodes). This implies that, PBR-free media is an effective substitute for PBRs for *in vitro* propagation. The high multiplication rates for several potato cultivars on modified MS media without any PBR (Hagman, 1990) confirms the above implication.

In the SNC method, PBRs, auxins and cytokinins, are generally added to the media at concentrations sufficient to support active SH growth, not tissue proliferation or lateral bud growth, as lateral bud break is promoted by cutting the main SH (George and Sherrington, 1984). Therefore, PBR combinations were used herein to affect proliferation, lateral bud growth and active SH growth to develop more axillary SHs rendering more nodes available for subsequent subculturing. Results revealed significant relationships between "SH length and axillary SH number" (R = 0.62**) and between "node number and axillary SH number" (R = 0.74**). Depending on the number of nodes on developing axillary SHs over an extended period (10 weeks) without deterioration of plantlet health is possibly better than the 3–4 weeks continuous culturing. PBR treatment combinations were more effective (Fig. 1) in inducing axillary SHs than the control (2.75 axillary SHs). Higher BAP levels (2.0 mg l^{-1}) combined with moderate NAA levels (0.5 mg l^{-1}) gave the highest ratio of axillary buds developing into SH (5 X the control) along the

main stem. These results could be attributed to the fact that relatively high levels of cytokinin, would encourage growth of axillary buds by reducing apical dominance (George and Sherrington, 1984).

B. *In vitro* Microtuberization

In the microtuberization experiments, when whole SHs and single nodes were cultured in MT induction liquid media, axillary buds developed into stolons. Hooked stolon tips were first observed followed by swelling in the node next to the stolon tip, until MT formation was completed either at the end of or along the stolon (sessile).

B.1. MT Number

On MT induction liquid media, microtuberization from whole potato SH cultures started 12 days earlier than the SNC cultures. This earliness could be related to explant type or age, as whole SHs were grown for 6 weeks and single nodes were derived from 3 week old SHs. Furthermore, Forsline and Langille, (1976) reported tuber formation to be under the control of environmental factors, mainly temperature and photoperiod, which regulate levels of endogenous PBRs within the plant. But this is unlikely possible under the conditions of the present investigation, as both explants were cultured on similar media under 16-hour photoperiod. In general agreement with the findings of Harmey et al., (1966), earliness of microtuberization could be further explained on basis of higher IAA levels in the apical tips of whole SHs compared with nodes.

Importance of sucrose for microtuberization in culture has been described earlier (Wang and Hu, 1982; Meulemans et al., 1986; Garner and Blake, 1989; Lillo, 1989). According to Wang and Hu, (1985) carbohydrates needed for tuberization under field conditions is favoured by high light intensity, while *in vitro*, sucrose is added to the media.

Several workers (Wang and Hu, 1982; Hussey and Stacey, 1984; Meulemans et al., 1986; Lillo, 1989) reported on the role of cytokinins in stilulation of the microtuberization process *in vitro*. Present results showed that 40 g l^{-1} sucrose in presence of 0.1 mg l^{-1} BAP gave optimal MT number per SH in liquid media cultures. At the end of the season, the number increased to a climax of seven per *in vitro* rooted SH after 20 weeks of incubation (Fig. 2). This finding disagrees with the results of Wang and Hu, (1982) who reported that 10.0 mg l^{-1} BAP were optimal for inducing highest number of MTs (0.49/ *in vitro* rooted SH) when 80 g l^{-1} sucrose were used under short day conditions. When results on MT numbers were compared on similar levels of BAP and sucrose, 3.6 X to 4.5 X the number of MTs were obtained after 7 and 20 weeks (Fig. 2), compared to the numbers obtained elsewhere (Wang and Hu, 1982) after 16 weeks. These differences could be due to differences among cultivars or/and incubation conditions. After eight weeks of incubation of SNCs, maximum number of MTs (1.35/node) were obtained when media was supplemented with 40 g l^{-1} sucrose and 0.1 mg l^{-1} BAP (Fig. 3). In contrast, lower MT numbers (9.3/ 10 nodes) were obtained by Hussey and Stacey (1984) within 6–8 weeks incubation under short day conditions when higher BAP (2.0 mg l^{-1}) and sucrose (60 g l^{-1}) levels were used.

Comparing the SH and node culture methods 7 weeks after incubation, the highest MT numbers per SH and node were 3.67 and 0.85, respectively. On basis of similar media and space, microtuberization in node culture turns to be superior by 38%. Use of SNC culture also reduces losses by contamination. It is therefore more convenient to discard a contaminated test tube (10 ml media and 0.85 MT/node) rather than discard a vessel (60 ml media and 3.67 MTs / SH).

B.2. MT Weight, Size and Shape

Upon termination of the experiment, both MT weight and size (Fig. 4) were significantly the highest on media with high sucrose (80 g l^{-1}) combined with low BAP (0.1 mg l^{-1}). Similarly, Garner and Blake, (1989) reported that increasing the level of sucrose from 40 to 80 g l^{-1} increased weight of MT, particularly in 'Pen' and 'Javelin' potato. The largest MT weight (250 mg) (Fig. 4) was considerably higher than that (150 mg) obtained by Meulemans et al., (1986) after 3 weeks of incubation in media containing 0.1 mg l^{-1} BAP, 70 g l^{-1} sucrose and 10.0 mg l^{-1}

coumarin. After 10 weeks of incubation in 100 g l^{-1} sucrose, 10.0 mg l^{-1} BAP and 1.2 ml l^{-1} cycocel, Lillo, (1989) reported MT weight to fall within the range 200–400 mg. Length and width measurements showed an oblong MT shape in treatments where microutuberization response was observed.

SUMMARY AND CONCLUSION

Responses of 'Spunta' potao plantlets to "micropropagation" and "microtuberization" were investigated using different levels of "cytokinin and auxin" and "cytokinin with sucrose", respectively. Solidified media containing 2.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA tends to be the best in terms of axillary SH proliferation, SH length and node number per main SH making higher multiplication rates possible when 'Spunta'potato plantlets are cloned *in vitro*. Induction of microtuberization was best on liquid media with 40 g l^{-1} sucrose and 0.1 mg l^{-1} BAP for both SH and node cultures. However, MT weight and size were largest at 80 g l^{-1} sucrose supplemented with 0.1 mg l^{-1} BAP.

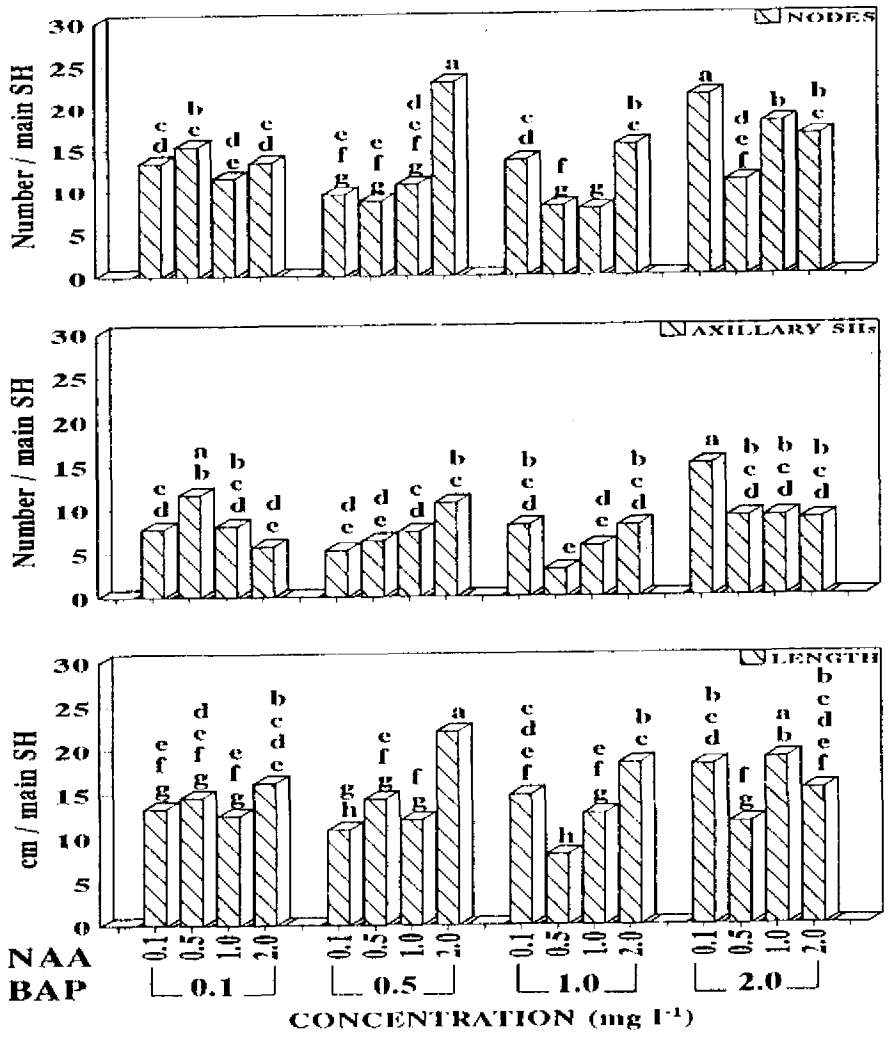

Fig. 1: Effect of BAP and NAA concentrations on axillary SH number per main SH, node number per main SH and main SH length, 10 weeks after single node culturing on modified MS proliferation media (bars having different letters are significantly different according to DMRT at P≤ 0.05).

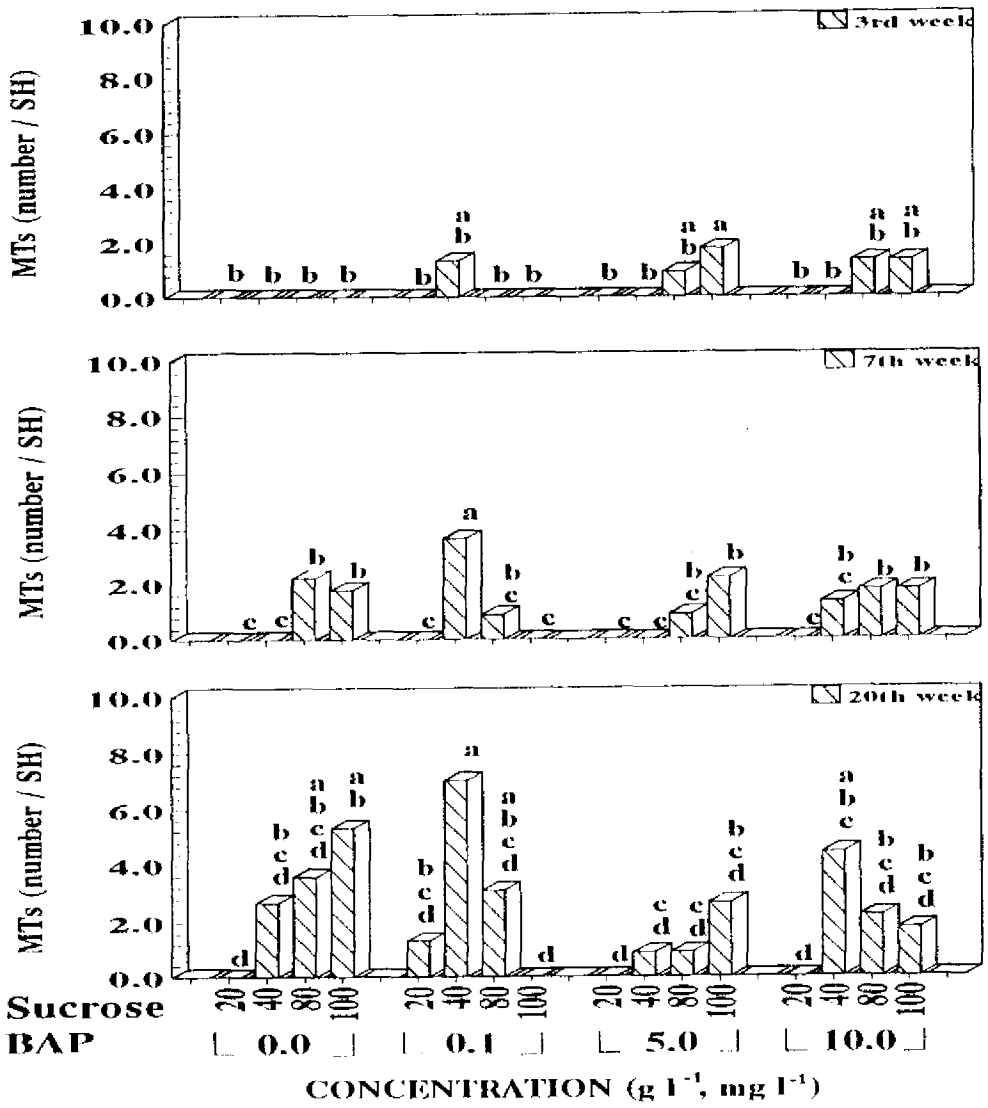

Fig. 2: Effect of BAP and sucrose concentrations on MT number per SH 3, 7 and 20 weeks after whole SH culturing in tuber induction liquid media (bars having different letters are significantly different according to DMRT at $P \leq 0.05$).

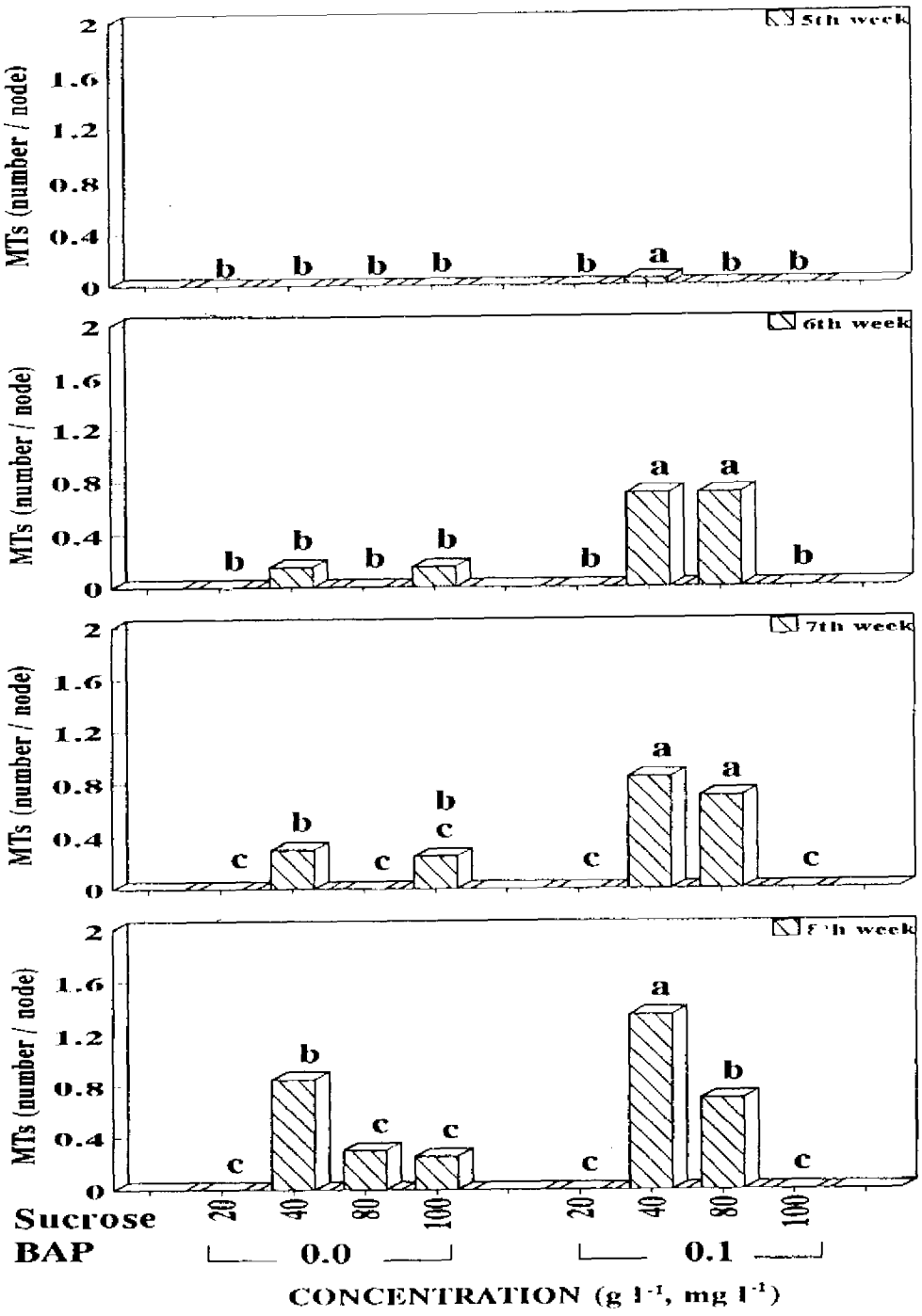

Fig. 3: Effect of BAP and sucrose concentrations on MT number per node 5, 6, 7 and 8 weeks after single node culturing in tuber induction media (bars having different letters are significantly different according to DMRT at P≤ 0.05).

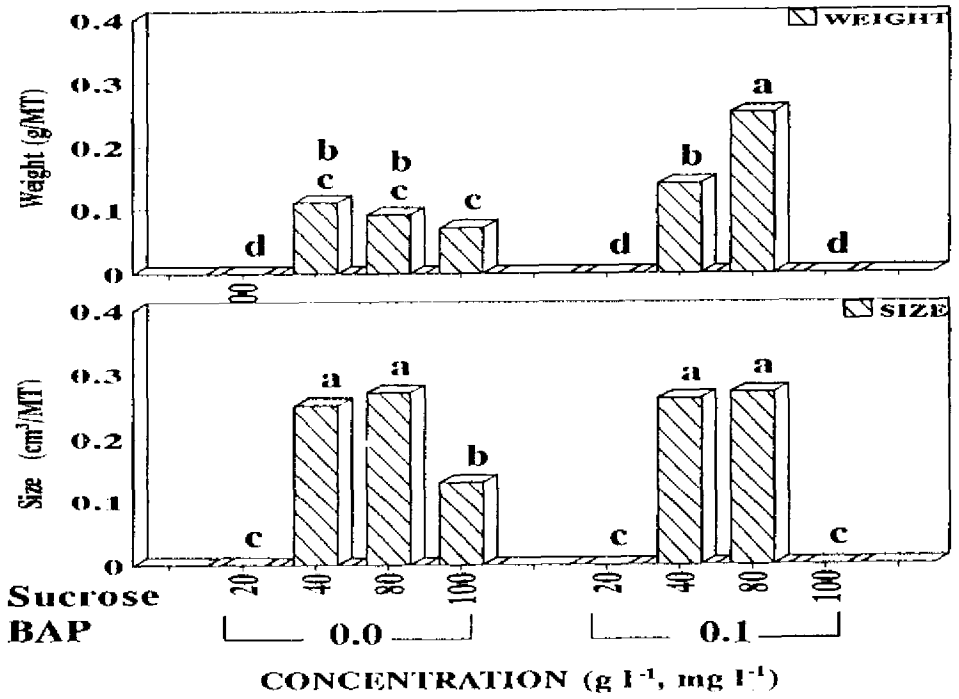

Fig. 4: Effect of BAP and sucrose concentrations on weight per MT and size per MT, 8 weeks after single node culturing in tuber induction liquid media (bars having different letters are significantly different according to DMRT at $P \leq 0.05$).

الإكثار وتكوين الدرنات الدقيقة داخل الأنابيب في صنف البطاطا (سبونتا)

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ملخص

تمت دراسة تأثير مستويات مختلفة من ٦ - بنزل امينوبيورين (BAP) ونفثالين استيك اسد (NAA) وكذلك BAP والسكروز على تكوين الافرع والدرنات داخل الأنابيب وذلك بزراعة اشتال من صنف 'Spunta' خالية من الفيروسات على بيئة Murashige and Skoog (MS) المعدلة.

أدى استعمال ٢ ملغم/ لتر من NAA و ٠.٥ ملغم/ لتر من BAP الى الحصول على افرع اطول (٢٢ سم) وعدد اكبر من العقد لكل فرع (٢٣ عقدة). كما تم الحصول على اعلى عدد من الافرع الابطية (١٥/ فرع رئيسي) بعد عشرة اسابيع من الحضانة في بيئة MS المعدلة وفي وجود ٢ ملغم/ لتر من BAP و ٠.١ ملغم/ لتر من NAA.

وتبين ان استخدام السكروز بتركيز ٤٠ غم/ لتر في وجود ٠.١ ملغم/ لتر BAP كان مثالياً لانتاج اكبر عدد ممكن من الدرنات داخل الانابيب. فقد تم انتاج ٧ درنات/ فرع و ١.٣٥ درنة/ عقدة بعد ٢٠ اسبوعاً و ٨ اسابيع، على التوالي. ونتج اكبر وزن وحجم للدرنة عند استخدام السكروز بتركيز ٨٠ غم/ لتر في وجود ٠.١ ملغم/ لتر من BAP.

وبناء على كميات البيئة المستعملة والمكان المطلوب للاكثار يبدو ان استخدام «العقل ذات العقدة الواحدة» (SNCs) اكثر قابلية للتطبيق لتحفيز تكوين الدرنات داخل الانابيب. هذا بالاضافة الى ان اخطار التلوث في هذه الحالة تصبح اقل.

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