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Effects of Sucrose, Mannitol and KH_2PO_4 on Proliferation of Root Tip Derived Shoots and Subsequent Bulblet Formation in Garlic

[1]Muhammad Shahidul Haque, Tomikichi Wada and Kazumi Hattori
Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan
[1]Department of Crop Botany, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

Abstract: Root tips (2-3 mm) of garlic (*Allium sativum* L.) were cultured on MS medium supplemented with 1 FM NAA and 10 FM BA for shoot initiation. Initiated shoots were transferred to growth regulator free MS medium supplemented with 3, 6, 9 and 12% sucrose, 3% sucrose + 0.8% mannitol and 3% sucrose + 0.75 g l^{-1} KH_2PO_4 for proliferation and bulblet formation. The additives effectively proliferated the shoots. The highest number of shoots was produced by mannitol. Fresh weight (root and shoot) was highest in 6% sucrose and the longest shoots were found in the control (3% sucrose). Best root development in terms of root number and length was found in 6% sucrose. On the medium containing 9% sucrose, 90% of the shoots produced bulblets yielding the highest amount of bulblets/explant. However, maximum number of bullets/explant (smaller in size) was found on 6% and the heavier bulblets were found on 12% sucrose. The shoots on medium containing mannitol failed to produce bulblet and some of the shoots on 12% sucrose containing medium became abnormal and did not produce any bulblets.

Key words: Carbon sources, bulblet formation, mannitol, shoot multiplication, sucrose

Introduction

Garlic (*Allium sativum* L.) is one of the important crops with significant medicinal value within the Alliaceae family. It is propagated vegetatively due to lack of fertile flowers. It has a low efficiency of propagation (six to ten times per year) in the field (Ebi *et al*., 2000). An efficient method for mass propagation of garlic cloves is highly desirable (Ayabe and Sumi, 1998). One of the advantages of *in vitro* micro propagation is the higher rate of multiplication and growth, especially of the vegetatively propagated crops (Dirk and Rhodomiro, 1996).

Tissue culture techniques were used for the propagation of garlic, using explants derived from basal parts of the bulblets, scape tips, shoot tips and leaves within cloves (Masuda *et al*., 1994; Ma *et al*., 1994; Nagakubo *et al*., 1993; Verbeek *et al*., 1995; Abo El-Nil, 1977), but all have inherent defects as practical methods- the need for long-term cultivation, relatively low propagation rates and the necessity of mastering skillful techniques (Ayabe and Sumi, 1998). These techniques used explants limited to only one or few per clove of garlic that seems the major cause of low multiplication rate *in vitro*. We proved that root can be efficiently used as explant source in garlic (Haque *et al*., 1997).

The growth and multiplication of shoots *in vitro* were affected by many factors (Israeli *et al*., 1996), one of which was the concentration and type of exogenous carbon source added to the medium to serve as energy and also as an osmoticum. There have been various opinions on the beneficial effects of the various carbon sources on the growth of plants *in vitro*. In general, sucrose is the carbohydrate of choice as carbon source for *in vitro* plant culture, probably because it is the major transport sugar of many plants (Murashige and Skoog, 1962; Thorpe, 1982). However, carbohydrate other than sucrose can be used for *in vitro* growth and storage organ formation in many plants including garlic (Garcia *et al*., 2002; Seabrook, 1994).

In vitro formed bulblets have many properties that make them ideal propagules for producing high quality seed bulbs. Due to *in vitro* formation, bulblets are free from pathogens. They are easy to handle, more amenable to automatic planting without acclimatization, can be stored for extended periods and also provide more flexible planting options. There many reports of *in vitro* shoot multiplication and bulblet formation from various explants using various methods (Nagakubo *et al*., 1993; Seabrook, 1994; Ayabe and Sumi, 1998; Haque *et al*., 1998). However, a simple and efficient method is yet to be established. The aim of this study to compare the influence of sucrose, mannitol and other additives like KH_2PO_4 on *in vitro* multiplication and bulblet formation from shoots regenerated from root tip explants in order to improve the *in vitro* propagation potential of garlic.

Materials and Methods

Cloves of garlic cv. 'white roppen' were surface sterilized with 70% ethanol for 30 s, 0.1% sodium hypochlorite

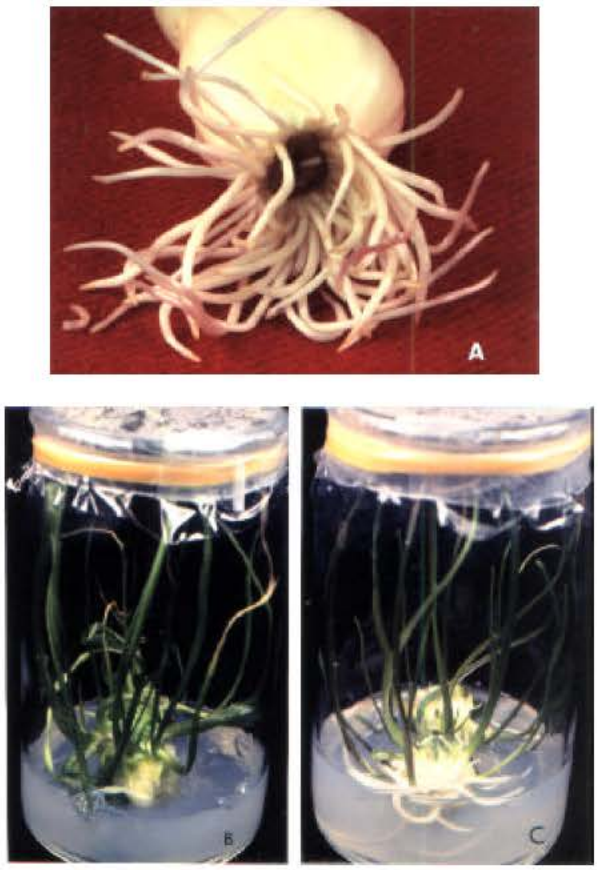

Fig. 1:
A) A sprouted garlic clove showing more than 40 roots, Root tips were excised from this plantlet.
B) Shoot multiplication on MS medium supplemented with 3% sucrose.
C) Shoot multiplication on MS medium supplemented with 6% sucrose.

solution with two drops of Tween 20 per 100 ml for 20 min and then washed three times with sterile distilled water. The cloves were sprouted on sterile solidified agar (0.7% agar in distilled water) under continuous light (13.5 μmol m^{-2} s^{-1}). They developed into plantlets with a well-developed root system. The root system of sprouted clove (garlic plantlet) comprised of more than 40 roots those had no laterals (Fig. 1A). Root tips measuring 2 to 3 mm from plantlets 15 to 20 days after sprouting (DAS) were excised and placed horizontally on the shoot initiation media that comprised of MS (Murashige and Skoog, 1962) salts and vitamins supplemented with 1 μM NAA and 10 μM BA (Haque *et al*., 1997). Direct shoot regeneration was achieved from the root tips within 20 days of culture. After 30 days on shoot initiation medium, the root tip explants that had regenerated shoots were used in the study.

For shoot proliferation and bulblet formation, various media compositions were examined. Growth regulator-free MS media supplemented with various concentrations of sucrose (3, 6, 9 or 12%) were compared. Medium containing 3% sucrose was used as control. MS media supplemented with (a) 0.8% mannitol, (b) 750 mg l^{-1} KH_2PO_4 and © 1% activated charcoal + 16 g l^{-1} glucose + 0.8% mannitol + 750 mg l^{-1} KH_2PO_4 + 250 mg l^{-1} $KHCO_3$ in addition to the usual 3% sucrose (Seabrook, 1994) were also compared.

Ten bottles with single root tip explant that regenerated shoot on the shoot initiation medium in each were used per treatment. Each experiment was run twice. Cultures were incubated at 28±1°C with constant cool white fluorescent light (13.5 μmol m^{-2} s^{-1}). Data on fresh weight, shoot and root number per explant and length of the longest shoot and root within the same explant were recorded 30 days after subculture. Fresh weight of bulblets, number of bulblets per explant, bulblet weight per explant and percentage of shoots forming bulblet were recorded after 45 days. The data were analyzed using analysis of variance (ANOVA) and t-test or Duncan's multiple range test (DMRT).

Results

Sterile garlic cloves sprouted on water agar within 5 to 7 days and developed into plantlets with a well-developed root system. In this Japanese cultivar of garlic (White roppen), more than 40 roots were produced in each clove after sprouting (Fig. 1A). The roots were very healthy and robust. All the root tips could be used in the study. Previously we reported 95% regeneration from root tip explants (Haque *et al*., 1997). Similar rate of regeneration was found during the study.

Shoot proliferation was found to occur well on all the media compositions of the present study. Fresh weight of the regenerated shoots was taken after 30 days on the proliferation medium. Among the sucrose concentrations, the highest fresh weight (3.80 g) of the shoots was achieved by 3% sucrose followed closely by 6% sucrose (3.43 g). Medium supplemented by 12% sucrose had the lowest fresh weight (2.37 g) (Table 1).

Number of shoots per explant varied significantly ($p \leq 0.05$) among the additives used (Table 1). The highest number of shoot (7.4) per explant was found with Mannitol Followed by 6% sucrose (6.2) and KH_2PO_4 (6.0). However, the shoots on medium containing mannitol were shorter and thinner (Fig. 2A) while those on medium supplemented with 3 and 6% sucrose and KH_2PO_4 were larger (Figs. 1B, C and 2B). The medium supplemented with 1% activated charcoal + 16 g l^{-1} glucose + 0.8% mannitol + 750 mg l^{-1} KH_2PO_4 + 250 mg l^{-1} $KHCO_3$ (Seabrook, 1994) had the least number of only

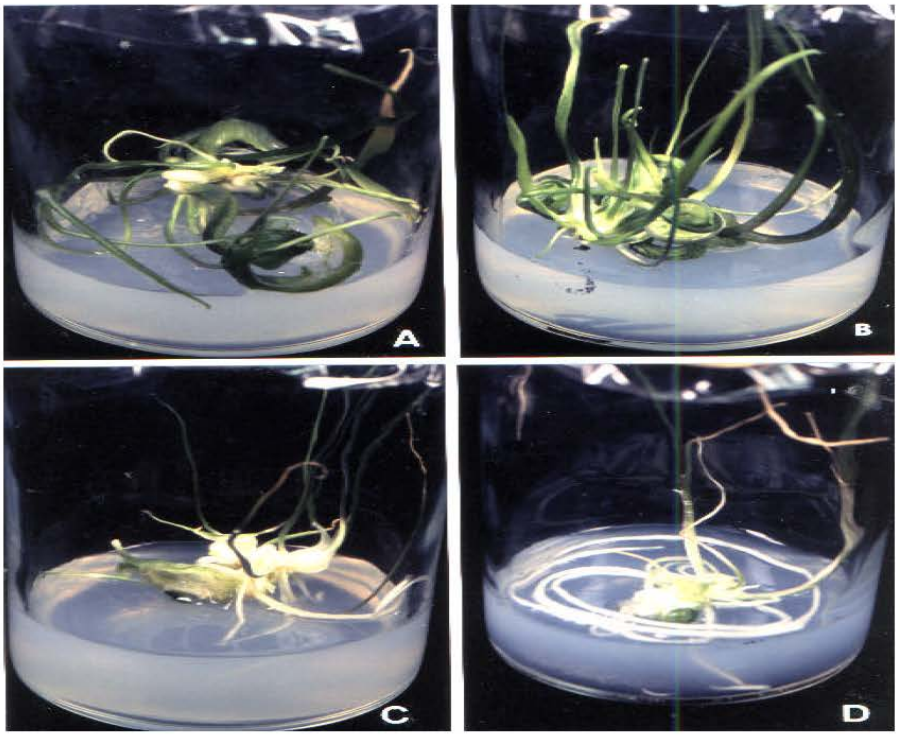

Fig. 2: A) Shoot multiplied on 0.8% mannitol. B) Shoot multiplied on 750 mg l^{-1} KH_2PO_4. C) Bulblet formation on 6% sucrose. D) Failure of bulblet formation on 1% activated charcoal+16 g l^{-1} glucose+0.8% mannitol+ 750 mg l^{-1} KH_2PO_4 + 250 mg l^{-1} $KHCO_3$

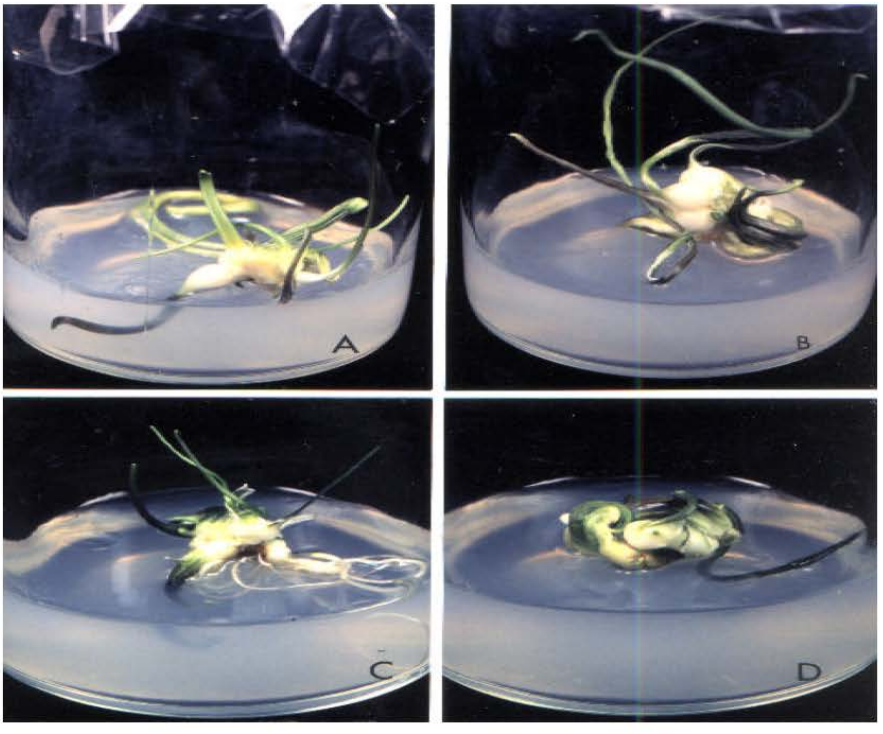

Fig. 3: A) Bulblet formation on medium containing 9% sucrose. B) Bulblet formation on medium containing 12% sucrose. C) Shoot on medium containing 9% sucrose. It had no bulblet formation only. D) Shoot on medium containing 12% sucrose . It had no bulblet formation, in stead became a bulbous mass of abnormal shoot

Table 1: Effects of sucrose, mannitol and KH_2PO_4 on shoot proliferation in garlic

Additives used	Fresh weight (g)	Shoot explant^{-1}	Shoot clove^{-1}	Root explant^{-1}	Shoot length (cm)	Root length (cm)
3% Sucrose	3.80±0.51a	5.0±0.7b	200	6.8±1.6b	16.4±2.4a	8.0±1.2b
6% Sucrose	3.43±0.84ab	6.2±1.9a	248	11.0±1.4a	11.8±2.5b	12.8±1.5a
9% Sucrose	2.73±0.75bc	5.6±1.1b	224	4.0±1.7c	7.2±2.3c	12.4±2.1a
12% Sucrose	2.37±0.56c	5.4±1.1b	216	1.2±0.4d	4.6±1.3c	3.6±0.9c
0.8% Mannitol[a]	2.80±0.81bc	7.4±1.1a	296	8.6±1.8b	13.0±2.5b	8.2±1.3b
750 mg l^{-1} KH_2PO_4[a]	2.81±0.57bc	6.0±1.2b	240	11.8±1.5a	11.8±2.9b	11.4±1.9a
Level of significance	**	*		**	**	**

* Significant at 5% level, ** Significant at 1% level, Figures (mean ± standard deviation) followed by different letter differ significantly.
[a] In addition to the normal 3% sucrose.

Table 2: Effects of sucrose, mannitol and KH_2PO_4 on bulblet formation in garlic

Additives used	% Shoot with bulblet	Bulblets explant^{-1}	Bulblets Clove^{-1}	Average bulblet wt. (g)	Total bulblet wt. explant^{-1} (g)
3% Sucrose	50	1.8±0.8	72	0.08±0.03b	0.15±0.1c
6% Sucrose	60	3.2±0.8	128	0.22±0.03ab	0.68±0.2ab
9% Sucrose	90	3.0±1.4	120	0.26±0.04ab	0.78±0.4a
12% Sucrose	50	2.2±0.4	88	0.34±0.17a	0.70±0.3ab
0.8% Mannitol[a]	00	0.0±0.0	00	0.00±0.00	0.00±0.0
750 mg l^{-1} KH_2PO_4[a]	50	3.0±1.0	120	0.17±0.03b	0.40±0.1b
Significance level		ns		**	**

ns = Not significant , ** Significant at 1% level, Figures (mean ± standard deviation) followed by different letter differ significantly.
[a] In addition to the normal 3% sucrose.

one shoot per explant (Fig. 2D). The number of shoots per clove was calculated on the basis of 40 roots per clove and it was found that the number ranged from 200 to 296 per clove (Table 1). This (200 to 296 times) is the rate of *in vitro* multiplication using root tip explant in cv. White roppen garlic. The time needed for this multiplication is only 2 months.

Number of roots per explant was different among the treatments. The highest number (11.8) of roots was found with 750 mg l^{-1} KH_2PO_4 (Table 1). However, the medium containing 6% sucrose had statistically same number (11.0) of roots per explant. Number of roots produced per explant decreased with sucrose concentration higher that 6%. The lowest number of roots per explant was found in 12% sucrose. This concentration of sucrose had the least number of shoots also.

Length of shoots varied significantly ($p \leq 0.05$) among the treatments. The longest shoots (16.4 cm) were recorded in control, the lowest concentration of sucrose (Table 1). The length of shoots decreased with the increase of the sucrose concentration and the shortest shoots were found with 12% sucrose. The addition of KH_2PO_4 to the medium promoted better vegetative growth (Fig. 2B). Medium containing mannitol had a poor vegetative growth (Fig. 2A). Root length was highest in 6% sucrose (12.8 cm) followed closely by 9% sucrose (12.4 cm) (Table 1). The shortest root length was found on 12% sucrose.

With the further culture of the shoots on the same medium there was bulblet formation at the base of the shoots. However, some of the shoots did not have bulblets formation and the percentage of shoot forming bulblets varied greatly with the media composition (Table 2). The percentage of bulblet formation increased with the increase of the sucrose concentration. With 9% sucrose, the maximum 90% shoots had bulblet formation. The media supplemented with mannitol and 1% activated charcoal + 16 g l^{-1} glucose + 0.8% mannitol + 750 mg l^{-1} KH_2PO_4 + 250 mg l^{-1} $KHCO_3$ had no bulblet formation (Fig. 2A&D). However, the medium containing 750 mg l^{-1} KH_2PO_4 had 50% bulblet formation. (Table 2). Bulblet formation was better observed on media containing 6 to 12% sucrose (Fig. 2C, 3A, 3B). In addition to bulblet formation on media containing 9% and 12% sucrose, in some cases shoots developed into masses of bulbous structures (Fig. 3C, D).

Number of bulblet was highest (not significant) on 6% sucrose and lowest on 3% sucrose (Table 2). The medium containing mannitol had no bulblet formation. We calculated the number of bulblets produced per explant. The number varied from 72 to 128. It implies that 128 bulblets were produced from a single clove within 2.5 months.

Average bulblet weight was highest on medium supplemented with 12% sucrose (0.34 g) (Table 2). The smallest bulblets were produced by 3% sucrose. Total highest bulblet yield per explant was recorded on medium supplemented with 9% sucrose (Table 2). The lowest bulblet yield was recorded in 3% sucrose.

Discussion

The results of this study indicated that *in vitro* shoot proliferation and bulblet formation of garlic were affected by both type and concentration of sugar in the shoot multiplication medium. In general, sucrose was better for inducing shoot proliferation and bulblet formation than

mannitol or mannitol and glucose. Several studies have indicated that sucrose is the carbohydrate of choice as carbon source for *in vitro* culture, of many plant species probably because it is the major transport sugar of many plants (Murashige and Skoog, 1962; Thorpe, 1982). A high concentration of sucrose has increased shoot regeneration capacity in root explants of cactus (Bhau, 1999). A high sucrose concentration may prove beneficial. In the absence of sucrose, organogenesis does not occur in callus cultures which highlights the vital role of sucrose in shoot differentiation (Bhau, 1999).

Sucrose concentration had an inhibitory effect on the fresh weight and shoot length and a promotive effect on the shoot and root number and the elongation of root. Addition of 750 mg l^{-1} KH_2PO_4 in the medium also decreased fresh weight and suppressed elongation of shoot but increased shoot and root number slightly and elongated root better. The decrease in fresh weight might be due the supra-optimal level of KH_2PO_4. Dunstan and Short (1977) recommended a phosphate level about two times that of in B5 medium for optimum growth in *Allium* and further increase in phosphate level decreased in the fresh weight.

Mannitol, the most widely distributed sugar alcohol in the plant kingdom can substitute for sucrose in suspension and embryo cultures (Stoop and Pharr, 1993; Garcia *et al*., 2002). In root tip culture of garlic addition of 0.8% mannitol in addition to the 3% sucrose that is normally used in MS medium, shoot multiplication was better than all the sucrose concentrations. However, *in vitro* bulblet formation was inhibited by mannitol. It indicates that sucrose acts as a source of carbon and energy more than as an osmotic agent in the bulbing medium. If sucrose would play its role solely as osmoticum in the bulbing medium, mannitol could substitute it. However, a detailed study is needed to clarify it.

In this study more than five shoots per root tip explant was produced within 2 months culture period. Considering 40 root tip explants per clove, the multiplication rate of 200 to 296 by the production of multiple shoots from root tips was achieved within a short period. Previous reports show a multiplication rate of only 8.2 using shoot tip and cut stem base (Mohamed-Yasseen *et al*., 1994, 1995) and 8.0 using bulb scale explant (Seabrook, 1994). By shoot tip culture of the same cultivar (white roppen), Nagakubo *et al.* (1993) reported 137.7 shoots/explant in 27 weeks. It is clear from the above reports that, the present report is a significant improvement in the *in vitro* propagation of garlic. We reduced the requirement of source material and length of culture period and increased the number of regenerated shoots per explant. However, by the production of bulblets, the multiplication rate was reduced to 88 to 128 by using 6 to 12% sucrose. This multiplication rate is higher and the process is easier than that of Ayabe and Sumi (1998) who reported that by the stem disc culture method combined with the pre-treatment of garlic at 4°C for approximately 8 weeks consistently produced more than 100 bulblets from each bulb.

Production of bulblets *in vitro* is preferred to plantlet formation because it avoided acclimatization step in the micro propagation process. Moreover, the bulblets are easy to handle, can be stored for longer periods using small place in the refrigerator and also provide more flexible planting options. In vitro bulblet production from root tips provides the unique opportunity of a cyclic and continuous micro propagation (Haque *et al*., 1998).

In conclusion, a higher sucrose in the medium has the potentiality to multiply shoot and produce *in vitro* bulblets. Mannitol cannot substitute sucrose for bulblet formation in garlic. Further study can clarify the role of sucrose in *in vitro* bulblet formation of garlic.

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