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In vitro Bulblet Formation from Shoot Apex in Garlic (*Allium sativum* L.)

R. Roksana, M. F. Alam, R. Islam and M. M. Hossain

Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh

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Abstract

Multiple bulblet formation was induced by repeated (fourth) subculture of regenerated plantlets on both liquid and semi-solid MS medium supplemented with 0.5 mg/l 2ip + 0.25 mg/l NAA. The regenerated bulblets were successfully established in soil.

Introduction

Cultivated garlic (*Allium sativum* L.) is a sexually sterile crop and exclusively propagated vegetatively (Novak et al. 1990). Conventionally the use of seed bulb is the only way for cultivation of garlic. For each plant one seed bulb is needed. The lack of availability of seed bulbs is the limitation for its large scale propagation. There are reports of using *in vitro* methods for propagation of garlic (Abo El-Nil 1977, Novak 1990, Nagakubo et al. 1994, Seabrook 1994, Zel et al. 1997). However, a few work reported using meristem for its micropropagation (Abo El-Nil 1977, Walkey et al. 1987, Bhojwani et al. 1982 and 1983, Matsubara and Chen 1989, Moriconi et al. 1990). The formation of multiple bulblets from single explant is the most desirable one. *In vitro* bulblet formation of garlic has also been reported (Abo El-Nil 1977, Bhojwani et al. 1982 and 1983, Matsubara and Chen 1989, Moriconi et al. 1990). Garlic is one of the major spice crops of Bangladesh. It is being cultivated on an area of 13077 hectare with a total production of 42805 tons, the average yield is 3.74 t/ha (BBS 1998). The yield of garlic in Bangladesh is very low in compare to other garlic growing countries, like China (7.9 t/ha), Thailand (7.8 t/ha) and Korea (5.0 t/ha). The local cultivars of Bangladesh are infected by viruses causing low yield. As garlic is propagated vegetatively, viruses are transmitted to the next generation. Therefore, the use of meristem as explant for micropropagation of multiple bulblet formation, is more suitable than other source of explants.

Considering the above facts present investigation was undertaken for : (i) standarization of a protocol for primary establishment of apical meristems and subsequently for rapid shoot proliferation, (ii) standarization of a protocol for bulblet formation following root induction and the elongation and (iii) acclimatization of *in vitro* bulblets/plantlets into soil under field condition.

Materials and Methods

The cloves were sterilized with 95% ethyl alcohol for 30 seconds for getting contamination free explants to isolate shoot apex. Isolated shoot apecies (0.2 - 0.3 mm) were inoculated aseptically on MS (Murashige and Skoog 1962) medium (with or without growth regulators) in both liquid and semi-solid conditions. Three kinds of cytokinins 2ip, BAP and Kn with different concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mg/l) were used singly in MS basal medium. In addition three cytokinins, two kinds of auxins NAA and IBA were also used. Three concentrations of 2ip (0.25, 0.5 and 1.5 mg/l) and five concentrations of NAA (0.05, 0.1, 0.2, 0.25 and 0.5 mg/l) were tested for initial shoot apex culture. Similar combinations were used for BAP + NAA, BAP + IBA and Kn + NAA. After 21 days of inoculation the shoot apecies were transferred from liquid to semi-solid medium was same as in liquid culture. Proliferated shoot apex with multiple clumps were repeatedly subcultured after every three weeks on fresh medium to initiate *in vitro* bulblets formation. The developed bulblets were separated as individual clove, and cultured on MS medium having different combinations of 2ip, BAP, Kn and 2ip + NAA, BAP + NAA, BAP + IBA, Kn + NAA to observe further bulblets formation. In all cases 3% sucrose and 1 g/l agar were used with pH 5.8. The medium was autoclaved at 15 Ib/sq. inch pressure and at 121_C for 20 mins. The culture tubes were placed in a growth chamber at 25 ± 1_C and light period 14 - 16 hrs (2000 - 3000 lux). The data were analyzed using mean $(\bar{X})$ and standard error (SE) for shoot multiplication and bulblet formation. Lastly the regenerated plantlets/ bulblets were transplanted on sterile soil (using formalin) with 1 : 1 soil and sand. A single plantlet was transplanted in a (9 ∇ 6 cm) poly bag in net house covered by large polythene sheet for maintaining shade and humidity. Necessary cultural practices were done for good growth of the plants. Field evaluation of the regenerated plants is under way.

Results and Discussion

From primary shoot apex shoot initiation was observed in all treatments on both liquid and semi-solid conditions. However, in all cases liquid medium was found suitable for early shoot initiation (Fig. 1A). Similar results were also

observed in others, viz. tomato (White 1943), carnation (Stone 1963), potato (Goodwin 1966), Mellor and Smith 1969) and strawberries (Vine 1968). Among the different liquid media earliest shoot initiation (three - four days) was observed in the combination of 0.5 mg/l 2ip + 0.25 mg/l NAA followed by in

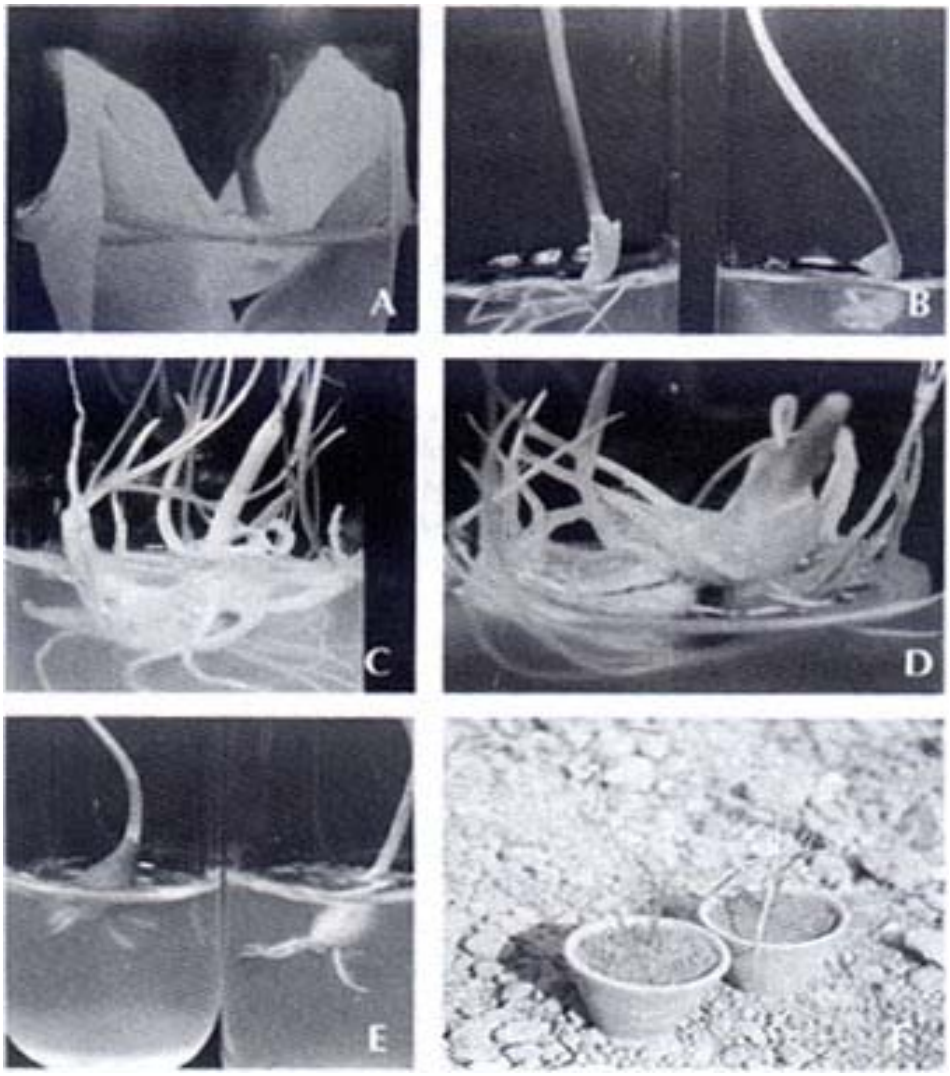

Fig. 1. A. Seven days old culture of meristem on paper bridge in MS liquid medium supplemented with 0.5 mg/l 2ip + 0.25 mg/l NAA. B. Single shoot with root from 21 days old culture of meristem in MS semi-solid medium. C. Forty two days old culture of meristem showing multiple shoot-root proliferation in MS medium containing 0.5 mg/l 2ip + 0.25 mg/l NAA. D. Eighty four days old culture of meristem showing multiple bulblets formation in MS medium containing 0.5 mg/l 2ip + 0.25 mg/l NAA. E. Meristem derived bulblet culture for observing further bulblet formation. F. *In vitro* regenerated plants established in soil (30 days after transplantation).

Table 1. Effects of different concentrations and combinations of phytohormones for shoot-root initiation and proliferation from shoot meristem in garlic.

Phyto-hormones (mg/l)	Shoot and root initiation								Shoot and root proliferation			
	Days taken to shoot-root initiation		Frequency of explants responsed (%)		Nature of morphogenic response from meristems				Number (after 21 days) ($\bar{X}$ ±SE)		Length (cm) (after 21 days) ($\bar{X}$ ±SE)	
					L		S					
	L	S	L	S	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
MS0	4 - 8	5 - 8	60	70	+	-	+	+	1.0 ±0.0	0.9 ±0.34	9.0 ±0.0	0.5 ±0.1
2ip (0.5)	3 - 6	4 - 8	90	90	+	-	+	+	8.1 ±1.4	1.2 ±0.52	8.4 ±0.42	0.75 ±0.5
2ip + NAA (0.5 + 0.25)	3 - 5	4 - 6	100	90	+	-	+	+	9.8 ±1.2	2.4 ±0.73	9.5 ±0.54	0.9 ±0.55
BAP (0.5)	4 - 6	6 - 8	80	80	+	-	+	+	5.4 ±0.92	1.0 ±0.10	7.7 ±0.43	0.3 ±0.10
BAP + NAA (1.5 + 0.5)	4 - 6	5 - 7	90	90	+	-	+	+	7.9 ±0.87	2.7 ±0.98	7.2 ±0.22	0.97 ±0.71
BAP + IBA (1.5 + 0.5)	4 - 6	5 - 7	85	85	+	-	+	+	5.0 ±0.79	1.8 ±0.84	6.9 ±0.33	0.88 ±0.40
Kn (2.0)	4 - 7	5 - 8	66.6	66.6	+	-	+	-	5.6 ±0.88	-	7.2 ±0.41	-
Kn + NAA (2.0 + 0.5)	5 - 8	6 - 9	70	60	+	-	+	+	6.0 ±0.91	1.1 ±0.18	5.5 ±0.37	0.2 ±0.52

Selected results are presented. L = liquid medium, S = semi-solid medium, + = positive response, - = no response.

treatment 1.5 mg/l BAP + 0.5 mg/l NAA and 0.5 mg/l 2ip alone. On the other hand late (eight - ten days) initiation was noticed in 5.0 mg/l of Kn (Table 1). In case of semi-solid medium the earliest (four - six days) and longest (10 - 12 days) period for shoot induction was observed in treatments : 0.5 mg/l 2ip + 0.25 mg/l NAA and 2.0 mg/l Kn + 2.0 mg/l NAA, respectively. However, the advantage of using semi-solid medium is that it can produce both shoot and root from shoot apex (Fig. 1B).

From this result it is revealed that combination of cytokinins and auxins is better for early establishment of shoot apex than using cytokinins alone. Data for elongation and multiple proliferation of shoot were taken after 21 and 42 days from first subculture of primary established shoot apex. At 21 days interval highest mean (9.8 ± 1.2) shoot proliferation was observed in treatment of 0.5 mg/l 2ip + 0.25 mg/l NAA (Fig. 1C), followed by 7.9 ± 0.87 in 1.5 mg/l BAP + 0.5 mg/l NAA. However, longest (9.8 cm ± 0.63) shoot was measured in treatment of 1.0 mg/l 2ip + 0.5 mg/l NAA (Table 1). Similar observation was recorded after 42 days of interval. These results showed that the use of low concentration of 2ip + NAA was found suitable for high percentage of shoot-root proliferation rather than use of high concentration and combinations of phytohormones. Beside 2ip + NAA combination, BAP + NAA was also suitable (Havel and Novak 1985, Nagaswa and Finer 1988). This indicates that the response of shoot-root prolfieration was varying from genotype to genotype. Here it is also noticed that no shoot proliferation was observed in medium without any growth regulators. Therefore, it is proved that the use of phytohormone is essential for shoot-root multiplication and prolfieration. The shoot clumps which had well developed roots and shoots were treated with different combinations of cytokinins (2ip, BAP and Kn) and auxins (IBA, NAA) for bulblets formation (Fig. 1D). The shoot clumps were subcultured after every 21 days interval. The first bulblets were observed after fourth subculture in medium containing 0.5 mg/l of 2ip with 0.25 mg/l of NAA. In case of other media more subcultures (five - seven) were needed for bulblet formation. The highest frequency (6.1 ± 0.53) of bulblet formation was also observed in medium containing 0.5 mg/l of 2ip + 0.25 mg/l of NAA. The lowest frequency (2.5 ± 0.62) of bulblet formation was observed in treatment 1.0 mg/l of Kn at seventh subculture (Table 2). These results suggested that use of repeated subculture can initiate bulblets formation without giving any other stress like high concentration of sucrose (Abo El-Nill 1977, Bhojwani 1980, Nagakubo et al. 1993, Seabrook 1994, Mohamed Yasseene et al. 1994, Zel et al. 1997) or manitol + glucose combination (Nagakubo et al. 1993) and low temperature treatment

(Seabrook 1994). These types of stress can result abnormal genetic change among the regenerated plants.

The shoot apex derived bulblets were separated and cultured on medium containing different combinations of phytohormones to observe further proliferation of bulblets and no proliferation was observed (Fig. 1E). Additional experiment can be undertaken for further confirmation.

Table 2. Effects of repeated subculture on bulblets formation from *in vitro* established mericlones of garlic in MS semi-solid media with different concentrations and combinations of phytohormones.

Phytohormones (mg/l)	No. of subculure	Days taken to bulb initiation	Frequency of bulb formed (%)	Mean No. of bulbs/culture ($\bar{X}$ ± SE)
2ip (0.5)	Fourth	84	80	4.3 ± 0.52
2ip + NAA (0.5 + 0.25)	Fourth	84	90	6.1 ± 0.53
BAP (1.5)	Sixth	126	80	3.8 ± 0.88
BAP + NAA (1.5 + 0.5)	Fifth	105	80	4.8 ± 0.88
BAP + IBA (1.0 + 0.5)	Sixth	126	70	4.3 ± 0.88
Kn (1.5)	Sixth	126	60	3.2 ± 0.7
Kn + NAA (1.5 + 0.5)	Fourth	84	60	4.8 ± 0.64

Selected results are presented.

The acclimatized plantlets were transplanted in formaline sterile soil to observe their survival rate. About 98% transplanted plants survived at the field condition and initially no abnormalities were observed among the regenerated plants (Fig. 1F).

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