In vitro mass multiplication with genetic clonality in elephant garlic (*Allium ampeloprasum* L.)

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ABSTRACT

A novel protocol was developed for Allium ampeloprasum L. to enhance in vitro cloning through multiple shoot induction. First bud induction was recorded from shoot tip within 6 days in MS with 0.25 mgl^{-1} NAA and 2 mgl^{-1} Kn. A maximum of 3 buds from a single explant appeared within the 15 days after the first bud induction. MS with 2.5 mgl^{-1} Kn plus 60 mgl^{-1} of adenine sulphate proved best for multiple shoot proliferation resulting in 6 shoots per inoculated shoot bud within next 30 days. Maximum 5 roots per shoot were recorded when cultured on MS with 0.5 mgl^{-1} IAA for 20 days. The combination of soil, sand and vermicompost with intermittent water spraying proved to be the best for hardening of micropropagated plantlets ensuring 90% success in next 25 days. Selected ISSR primers were used to ensure genetic clonality for the in vitro generated propagules.

Key words: Acclimatization, *Allium*, in vitro cloning, ISSR, multiple shoot

Allium ampeloprasum L, commonly known as elephant garlic, is an important family member of *Alliaceae*. It is more closely related to the leek than to ordinary garlic. A single clove of elephant garlic can be as large as a whole bulb of ordinary garlic. It is more perishable than ordinary garlic so it is difficult to keep as long. Elephant garlic is considered to be nature's very own antibiotic. It has also been used for lowering cholesterol, reducing high blood pressure, and treating respiratory problems such as bronchitis and asthma. Conventional propagation of elephant garlic by planting of cloves, used for both culinary purposes and propagation is slow, labour-intensive, time-consuming and non-economical like anyother *Allium sp.* (Robledo-Paz *et al.,* 2000). To overcome this drawback *in vitro* mass multiplication was fruitfully attempted in several *Allium spp*. using root tip (Haque *et al.,* 1997), shoot tip (Myers and Simon, 1998), seed (Wawrosch *et al.,* 2001), mature clove (Roksana *et al.,* 2002) and stem dome (Kamstalityte and Stanys, 2004) explants. The present study aims at to develop a novel protocol of *in vitro* cloning and establishment of an effective *ex vitro* acclimatization process; to enhance the multiplication rate through induction of multiple shoots; to raise and maintain a sustainable pool of propagules for further multiplication and constant supply and to ensure the genetic clonality of the regenerated propagules through DNA fingerprinting.

MATERIALS AND METHODS

Thirty days-old shoot tips of elephant garlic were collected from healthy disease free plants. Collected explants were surface sterilized with10% (v/v) NaOCl for 5 min, washed with sterile water and treated again with 0.1% (w/v) $HgCl_2$ for 3 min. The explants were then thoroughly washed 4-5 times in sterile double distilled sterile water, and finally trimmed to 2 cm. The whole process was performed under strict aseptic conditions. To induce bud break from shoot tips the explants were inoculated in MS basal medium (Murashige and Skoog, 1962) plus 3% (w/v) sucrose supplemented with different levels of α-naphthalene acetic acid (NAA) and Kinetin (Kn). The MS basal media (consisted of salts, vitamins and 3% sucrose) was solidified with 0.7% (w/v) agar. The growth regulators NAA, Kn, IAA and IBA were added in the medium before the pH adjustment to 5.7 (with 0.5 N NaOH) and autoclaving at 1.06 kg. cm^{-2}; 121°C for 15 min. MS basal salts, sucrose, agar, vitamins and growth regulators were obtained from SRL, India. Incubations of *in vitro* cultures were done at 25± 2°C temperature with 60% RH under a 16 hr photoperiod (using white fluorescent tubes) and 3000 lux light intensity.

Excised explants were inserted in the media dipping approximately up to 0.5 cm for good contact and anchor. The induced buds were separated and cultured on MS with different concentrations of Kn plus adenine sulphate for multiple shoot proliferation where MS devoid of growth regulator act as a control. During shoot multiplication and proliferation the cultures were incubated for 30 days. The best resulting media formulation was identified in respect of response to multiple shoot formation, number and length of shoots. For root induction and elongation MS basal medium was supplemented with different concentrations of indole acetic acid (IAA) or indole butyric acid (IBA). Multiple shoots were separated and then transferred individually to the rooting media. The efficiency of IAA and IBA were tested in respect of response of shoots to root induction, days to root induction, number and length of roots which ultimately determined the most successful media

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composition in this purpose. Two steps of acclimatization process were used to select the plantlets were transferred in to small plastic pots containing autoclaved sand with sprayed water and covered with transparent polythene sheet ensured high humidity. These plantlets were allowed to grow for 10 days. Then, the partially-acclimatized plantlets with 3-4 primary leaves were transferred to large pots containing sand, soil and vermicompost mixture (1:1:1) Water was sprayed intermittently to ensure high level of humidity for plant survival. The entire procedure was done under the shade and well acclimatized plants were then recovered for further clonal fidelity study.

Genetic purity of *in vitro* generated and acclimatized plantlets was estimated through DNA fingerprinting using 10 selected ISSR primers (Prakash *et al.* 2009). DNA extraction from fresh leaves was done according to the procedure described by Bhattacharyya and Mandal (1999). The 25 µl optimized PCR mixture contained 40 ng DNA, 2.5 µl 10X Taq polymerase assay buffer, 3.5 µl 2.5 mM dNTPs, 0.5 U Taq DNA polymerase and 200 ng of primer. PCR performance consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of 45 s at 94 °C, 45 s at annealing temperature 90 s at 72 °C and final extension at 72 °C for 7 min, 4 °C for 5 min was done using Gene Amp PCR system 2400. The annealing temperature was adjusted according to the Tm of the primer being used in the reaction. Banding patterns were visualized in 1.5% agarose and documentation was done on Gel Logic 200 trans-illuminator system. Similarly, shootlets from each remaining subcultures were also examined for their clonal fidelity. All the experiments were repeated thrice using 20 explants in each replication and Completely Randomized Design were used for these experiments. Each single explant was considered as an experimental unit. Data were subjected to ANOVA and significant differences among the treatments were tested by Duncan's multiple range test (DMRT) (Duncan 1955) at 5% level.

RESULTS AND DISCUSSION

Direct organogenesis

Surface-sterilized shoot tips were cultured on MS supplemented with different concentrations of NAA and Kn. Bud induction was observed on the 6th day after inoculation. It was observed that around 90% inoculants showed bud proliferation in a MS media with 0.25 mgl^{-1} NAA and 2 mgl^{-1} Kn (Table 1). Only 3 greenish shoot buds were appeared after 15 days of prolonged culture (Fig. A). So, the identified low concentrations of Kn and NAA combination proved effective for induction and establishment of shoot buds. This supports the previous report of George and Ravishankar (1997) who observed the positive role of low concentration of cytokinin and auxin in shoot bud initiation.

In case of multiple shoot culture MS with 2.5 mgl^{-1} Kn plus 60 mgl^{-1} of adenine sulphate proved most effective formulation for multiple shoot formation and proliferation than any other tried combinations confirmed by Duncan's multiple range test. More than 18 buds (90.6%) out of 20 (from each replication) responded to shoot multiplication and proliferation (Table 2). On an average 5.66 multiple shoots per inoculant were scored at 30th day after shoot bud inoculation. At that time shoot length was 5 cm and the multiple shoots were healthy with light green in colour (Fig. B).The results of multiple shoot poliferation demonstrated that cytokinin without auxin successfully induced maximum multiple shoots (Sugla *et al.* 2007). The significance of the addition of adenine sulphate was to enhance shoot proliferation in positive sense. The similar effect of addition of the adenine sulphate for increased shoot multiplication has also been reported in *Anthurium* (Gantait *et al*. 2008).

Selected 20 individual dissected shootlets were transferred to MS with varying concentrations of IAA or IBA to induce roots. MS supplemented with 1.5 mgl^{-1} IAA induced roots for more than 93% shootlets within 7 days after inoculation (Fig. C). However, average number of roots per shootlet was around 5 and average root length was 4.5 cm on 20th days after culturing (Table 3). During this time average shoot length was 8.1 cm. Profuse *in vitro* rooting was recorded in very low concentration of IAA proved to be very efficient in inducing as well as promoting root growth for *Allium*.

Acclimatization

Development of good number healthy roots on each shootlet is necessary for better acclimatization. Henceforth, after profuse robust rooting of 20 plantlets were first washed thoroughly in running tap water to remove traces of culture medium to prevent any type of bacterial or fungal contamination as suggested by Sharry and Teixeira da Silva (2006). In the first stage of acclimatization the mixture of autoclaved sand provided optimum anchorage to the plantlets and helped in primary regeneration of roots (Fig. D) for 10 days.Pruning of elongated roots before acclimatization was done for easy and *ex vitro* regeneration of fresh roots. Moreover, root pruning helped in reducing the variability arising due to the difference in the number and length of *in vitro* generated roots and gave more uniform plants (Thomas and Ravindra, 1997). In the next stage of 15 days the sand, soil and vermicompost mixture (1:1:1) resulted to the 18 well acclimatized plantlets **(**Fig. E**)** out of 20. The maximum credit of this successful acclimatization was gained by the

retention of high humidity. Except the intermittent water spraying and covering with transparent polyethylene sheet (Thomas and Ravindra, 1997) we supposed that use of vermicompost had major contribution in retaining the moisture. Overall 90% success was achieved following the above mentioned protocol of acclimatization process.

Test of clonal fidelity

In the present study 10 selected ISSR primers were used for the clonality assessment of *in vitro* generated propagules **(**Table 4) among which three primers (IS-8, IS-61 and IS-65) showed to be reproducible. A total number of 105 reproducible monomorphic bands were scored from the clones including their mother. None of the primers showed any difference in banding pattern proving that the purity of the *in vitro* regenerated clones was maintained (Fig. F). The test of clonal fidelity using ISSR primers was successfully attempted in different crops (Joshi and Dhawan 2007; Lakshmanan *et al.* 2007; Bhatia *et al.* 2009). It was proven from this experiment that clones developed form *in vitro* direct organogenesis are true to its genetic identity and safest mode of micropropagation to have true to type progeny. The reports of Shu *et al*. (2003), Carvalho *et al*. (2004) and Martins *et al.* (2004) also cite similarity with this result.

Thus the present study revealed that the source of explant and proper formulation of auxin-cytokinin have significant effect on large scale micropropagation of elephant garlic maintaining its genetic purity. So the present exercise by and large offers a promising approach to engage in commercial activity through micropropagation.

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Table 1: Bud induction of elephant garlic from shoot tip explants

Sl. No.	Growth regulators (mgl^{-1})		Responding explants	Days to bud induction	No. of buds per explants
	NAA	Kn			
1	0	0	0.67f	35.67a	0.67c
2	0.1	1	7.33e	16.33b	2.33ab
3	0.25	1	14.33b	13.00c	1.67bc
4	0.5	1	10.67cd	17.33b	2.00b
5	1.0	1	8.67de	17.67b	2.00b
6	0.1	2	11.33c	10.00d	1.33bc
7	0.25	2	17.67a	5.33e	3.33a
8	0.5	2	15.67ab	9.67d	2.33ab
9	1.0	2	14.33b	12.00cd	2.00b
Overall mean			**11.19**	**15.22**	**1.96**
SE			**0.6894**	**0.8471**	**0.3379**

Different letters indicate significant difference at P<0.05

Table 2: Multiple shoot proliferation in elephant garlic

Sl. No.	Growth regulator (mgl^{-1})		Additive (mgl^{-1})	Responding shoot buds out of 20	No. of shoots	Shoot length (cm)
	BAP	Kn	ADS			
1	0		0	1.33h	1.33e	1.30e
2	2.0		60	7.67g	2.00de	2.07d
3	2.5		60	9.67f	2.33cde	2.37d
4	3.0		60	16.33b	3.67b	3.83b
5	3.5		60	13.67de	2.67bcd	2.97c
6		2.0	60	14.33cd	2.33cde	3.10c
7		2.5	60	18.33a	5.67a	5.00a
8		3.0	60	15.33bc	3.33bc	4.10b
9		3.5	60	12.67e	2.67bcd	3.83b
Overall mean				**12.15**	**2.89**	**3.17**
SE				**0.556**	**0.3727**	**0.1544**

Different letters indicate significant difference at P<0.05

Table 3: Root induction and elongation in elephant garlic

Sl. No.	Growth regulators (mgl^{-1})		Responding shootlets out of 20	Days to root induction	No. of roots	Root length (cm)	Shoot length (cm)
	IAA	IBA					
1	0	0	7.33e	27.67a	1.67d	1.87f	2.73f
2	0.5	0	13.67d	14.67cd	2.00cd	2.57de	4.73de
3	1.0	0	16.33b	13.67de	2.67bcd	2.83cd	6.73c
4	1.5	0	18.33a	9.67f	5.33a	4.50a	8.10a
5	2.0	0	17.67ab	12.33e	3.33bc	3.20bc	7.47b
6	0	0.5	14.33d	16.33bc	2.33cd	2.13ef	4.27e
7	0	1.0	16.33b	16.67b	4.00ab	3.33bc	5.13d
8	0	1.5	16.67ab	12.67e	3.00bcd	3.50b	5.03d
9	0	2.0	17.67ab	14.67cd	2.67bcd	4.17a	5.13d
Overall mean			**15.37**	**15.37**	**3.00**	**3.12**	**5.48**
SE			**0.6407**	**0.5720**	**0.4953**	**0.1926**	**0.2077**

Different letters indicate significant difference at P<0.05

Table 4: ISSR primers used for the test of clonal fidelity

Sl No.	Oligo-name	Sequences	Tm (°C)
1	IS-6	5' GAG AGA GAG AGA GAG AC 3'	52
2	IS-7	5' GTG TGT GTG TGT GTG TA 3'	50
3	IS-8	5' AGA GAG AGA GAG AGA GC 3'	52
4	IS-9	5' TGT GTG TGT GTG TGT A 3'	46
5	IS-10	5' CGA GAG AGA GAG AGA GA 3'	52
6	IS-11	5' CAC ACA CAC ACA CAC AG 3'	52
7	IS-12	5' GTG TGT GTG TGT GTG TC 3'	52
8	IS-61	5' GA GA GA GA GA GA GA GA T 3'	50
9	IS-63	5' AG AG AG AG AG AG AG AG C 3'	52
10	IS-65	5' AG AG AG AG AG AG AG AG T 3'	50

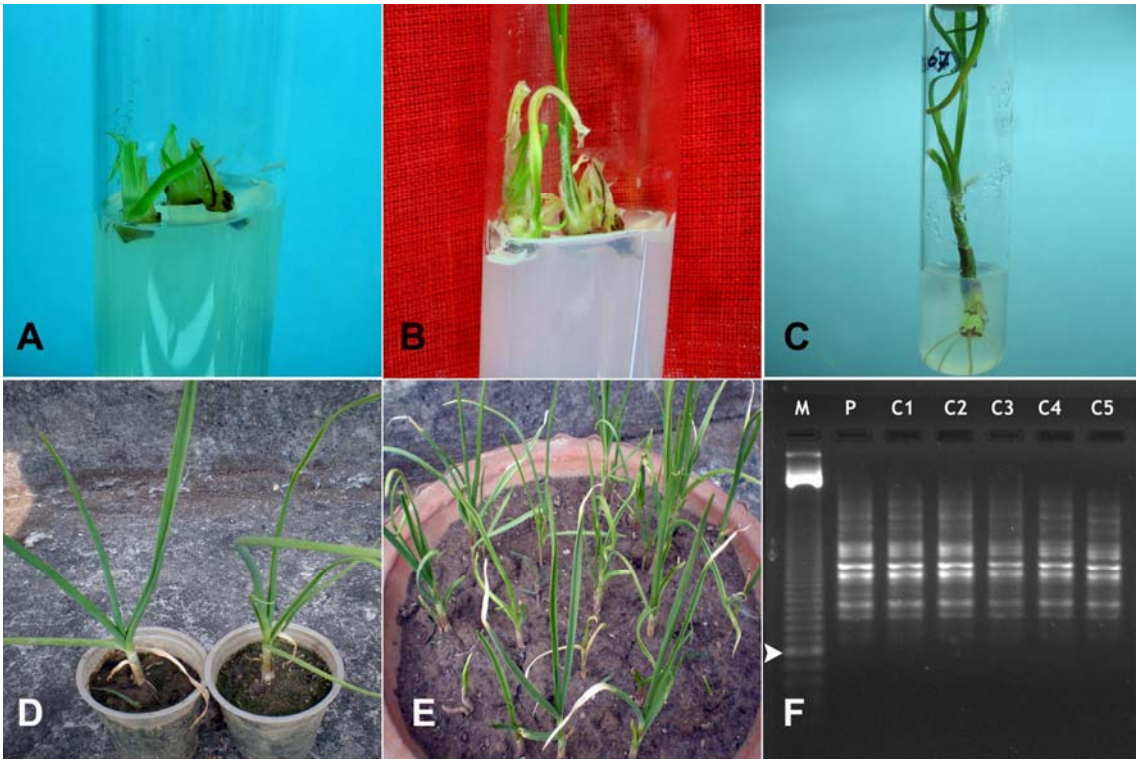

Fig: A- Bud induction, B- Shoot multiplication, C- Root induction and elongation, D- Primary acclimatization, E- Secondary acclimatization, F- Gel electrophoresis of amplified ISSR products of *in vitro* regenerated propagules (C1 – C5) and their mother (P) using IS-8 primer. Lane M- 50bp ladder.