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Determination of Genetic Integrity in Long-term Micropropagated Plantlets of *Allium ampeloprasum* L. Using ISSR Markers

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Abstract: Regeneration efficiency and genetic clonality are two major aspect of successful long-term *in vitro* culture to be studied extensively. The regeneration efficiency of 25 months sustained *in vitro* culture of *Allium ampeloprassum* L. was maintained through multiple shoot proliferation and root growth. No morphological variation was detected between the plantlets regenerated from initial and long-term shoot tip culture or with their mother, meaning a high likelihood of their clonal fidelity. Detection of genetic integrity for *in vitro* regenerated clones was carried out using 10 ISSR primers among which 4 primers produced a total number of 336 distinct and scorable bands with an average of 7 bands per primers where the other 6 primers were not reproducible. The ISSR primers base on AG motif and 3' anchoring produced more number of consistent bands. All monomorphic bands in the ISSR assay both for primary culture as well long term culture *in vitro* ascertained to a great extent their genetic integrity through their partial genetic coverage.

Key words: Clonality, elephant garlic, micropropagation, multiple shoot, sustained culture

INTRODUCTION

Allium ampeloprasum L., commonly known as elephant garlic is an important member of family *Alliaceae*. A single clove of this autotetraploid (2n = 4x = 32) (Silvertand *et al*., 1996) can be as large as a whole bulb of ordinary garlic. It is however, more perishable than ordinary garlic. It has multipurpose medicinal uses for lowering cholesterol, reducing high blood pressure and treating respiratory problems such as bronchitis and asthma. Cultivation of high value medicinal plants is now creating new dimension in the agricultural industry (Supe, 2007). Conventional propagation of elephant garlic by planting of cloves is rather slow and labour-intensive as other *Allium* sp. (Robledo *et al*., 2000). To overcome this problem *in vitro* mass multiplication in an accelerated pace within a short span of time was fruitfully attempted in several *Allium* sp. including elephant garlic using root tip (Haque *et al*., 1997; Martin-Urdiroz *et al*., 2004), shoot tip (Myers and Simon, 1998), seed (Wawrosch *et al*., 2001), mature clove (Robledo *et al*., 2000; Roksana *et al*., 2002) and stem dome (Kamstaityte and Stanys, 2004) explants, where most of the reports show that the mode of regeneration in elephant garlic was via callus (Buiteveld *et al*., 1993; Silvertand *et al*., 1996). But micropropagation itself has a major limitation regarding the clonality of the *in vitro* generated propagules with their mother, specially, long term *in vitro* culture poses a problem in recovering true to type regenerants due to chromosomal abnormality, gene amplification, point mutation (Saker *et al*., 2000). This clonal variation can be detected by DNA fingerprinting using different type of markers like RAPD, ISSR or RFLP. ISSR proves to be more effective and reproducible in detecting genetic uniformity (Martins *et al*., 2004). Although, there are some earlier reports on assessment of genetic integrity on other *Allium* sp. (Sudarmonowati *et al*., 2001) through phenotypical and cytological study (Novák, 1980, 1981; Mukhopadhyay *et al*., 2005) or cytological study with RAPD (Al-Zahim *et al*., 1999), the similar kind of work has not been extensively studied on *Allium ampeloprasum* L. particularly. The present study is concerned with the evaluation of regeneration efficiency of elephant garlic from shoot tip after a long period of sustained culture *in vitro* and assessment of the genetic clonality of the long term *in vitro* cultured clones through ISSR.

MATERIALS AND METHODS

Collection of explants and *in vitro* propagation: The whole experiment was conducted in the Tissue Culture

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Laboratory of Biotechnology department, Bidhan Chandra Krishi Viswavidyalay, W.B., India during 2006-09. Surface sterilized mature bulbs were grown under departmental greenhouse. Twenty days old shoot tips were collected, trimmed to 1 cm, surface sterilized and inoculated in artificial growth medium under laminar air flow. For establishment of explants MS (Murashige and Skoog, 1962) medium was supplemented with 0.25 mg L^{-1} of α-naphthalene acetic acid (NAA) and 2 mg L^{-1} of Kinetin or 6-furfurylaminopurine (KIN). Induced buds were excised and inoculated on MS, fortified with 2.5 mg L^{-1} of KIN plus 60 mg L^{-1} of adenine sulphate, for multiple shoot proliferation.

Physical condition of *in vitro* culture: In the present study MS basal medium (consisted of salts, vitamins and 3% sucrose) was used after solidifying with 0.7% (w/v) agar. Different growth regulators like NAA, KIN and additive like adenine sulphate were added in variable concentrations to MS before adjusting pH to 5.7 and autoclaved at 1.06 kg cm^{-2}, 121°C for 15 min. The MS basal salts, agar, vitamins and growth regulators were obtained from SRL, India. The *in vitro* cultures in all growth stages were accomplished at 25±2°C temperature, 60% RH and 16 h photoperiod (using white fluorescent tubes) with 3000 lux light intensity.

Rooting and acclimatization: Indole-3-acetic acid (IAA) at 1.5 mg L^{-1} was used in MS basal medium for root induction and elongation from individual *in vitro* generated multiple shoots as described by Gantait *et al.* (2009a). Rooted plantlets were acclimatized growing each of them on a pot mixture of autoclaved sand, soil and well decomposed farm yard manure (1:1:1) with intermittent supply of water, where pots were covered with transparent polyethylene to ensure high humidity. Acclimatized plantlets were then subject to ISSR assay.

Maintenance of sustained culture *in vitro*: *In vitro* generated shootlets were also subcultured for further multiple shoot proliferation in a sustainable manner on MS medium containing 2.5 mg L^{-1} of KIN and 60 mg L^{-1} of adenine sulphate. Five subcultures were performed at 5 months interval over a period of 25 months. Performance of the fifth subculture derived from secondary explants was assessed on the basis of response to multiple shoot formation, number of multiple shoots induced and their growth in terms of shoot length followed by rooting efficiency. They were then subjected to acclimatization. Randomly selected acclimatized plantlets were assessed for clonally fidelity through Inter Simple Sequence Repeats (ISSR) assay.

Extraction of genomic DNA and PCR amplification: Fresh young leaf samples were collected from three different sources such as mother plant, shoot tip derived acclimatized plantlets (considered as primary cycle of regeneration) and from the acclimatized plantlets regenerated through fifth subculture (referred to as secondary explants) after 25 months. Genomic DNA was extracted from 80 mg tender leaves from each sample plantlet under study, according to the procedure described by Chattopadhyay *et al.* (2008). Extracted DNA samples were subjected to PCR (Polymerase Chain Reaction) amplification using 10 selected ISSR primers (Gantait *et al.*, 2009b; Prakash *et al.*, 2009). The 25 µL PCR mixture contained 40 ng DNA, 2.5 µL of 10X Taq polymerase assay buffer, 3.5 µL of 2.5 mM dNTPs, 0.5 U Taq DNA polymerase (all from Chromous Biotech Pvt. Ltd., India) and 200 ng of primer (Bangalore Genei Pvt. Ltd., India). PCR consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 45 sec at 94°C, 45 sec at annealing temperature 90 sec 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 (Applied Biosystems, USA). The annealing temperature was adjusted according to the Tm of the primer being used in the reaction. The amplified PCR products, along with 50 bp DNA ladder were resolved by electrophoresis on 1.5% agarose (SRL) gel in 1X TBE buffer stained with ethidium bromide (10 µg L^{-1} TBE buffer). Bands were scored and photographed on Gel Logic 200 trans-illuminator system (Kodak).

Data analysis: The potentiality of producing multiple shoots and well developed roots were the criteria to assess the difference between plantlets regenerated from primary explants and plantlets regenerated from secondary explants (without any reference to explants of origin) after 25 months. Then plantlets of both phases were screened through ISSR assay which revealed the genetic clonality of all the propagules. Complete Randomized Design (CRD) was followed for the *in vitro* culture experiments. Each single explant was considered as an experimental unit. The experiments were carried out in six replications including 20 explants in each and standard deviation was calculated. For data scoring the percent response to multiple shoot proliferation, number of shoot and shoot growth including days to root induction, number and length of root were considered as morphological competence. Angular transformations of

the percent response values were done before the collected data were subject to Analysis of Variance (ANOVA). Significant difference among the treatments were tested by Duncan's multiple range test (Duncan, 1955) at 5% level using WINDOWSTAT 7.5 (Indostat services, Hyderabad, India) (Gantait *et al*., 2009a, b) software package from Uttar Banga Krishi Viswavidyalaya, India. For ISSR profiles, the well-resolved and consistently reproducible amplified DNA fragments were scored in terms of their presence or absence. To detect the genetic purity, the resulting banding patterns were compared between DNA samples for each ISSR primer.

RESULTS

Assessment of regeneration efficiency of long term culture: Morphological competence: The plantlets regenerated from primary explants and plantlets from secondary explants showed no significant difference in the percent response to shoot multiplication as it was around 92-93 in both cases (Table 1). It is also evident that there was uniformity in number of multiple shoots formed as the primary culture scored 5.83 and secondary culture scored 5.5 shoots per inoculum and the multiple shoot length was also similar in both the cases i.e., 4.97 and 5.08 cm, respectively. When the multiple shootlets were separated and transferred to the rooting media it also exhibited similar results between primary culture and sustained culture after 25 months. In this phase root induction occurred at 9.83 (in primary culture) days and 9.22 days (in secondary culture) after each inoculation where, the number and length of well developed roots recorded no significant difference according to Duncan multiple range test (Table 1). On an average around 4 (4.17 from primary and 3.67 from secondary culture) healthy roots under each shootlet were recorded and length of roots were around 4 cm (4.05 cm in primary and 4.25 cm in secondary culture) approximately in both of the cultures. All the plantlets regenerated from initial and long term sustained cultures equally responded to acclimatization (92% success), after which they screened for their genetic integrity through ISSR assay.

Confirmation of genetic stability: ISSR assay: In the present study 10 ISSR primers were used for checking the fidelity of *in vitro* generated acclimatized clones among which IS-9, IS-10 and IS-12 did not react with elephant garlic DNA. Between rest of the primers i.e., IS-7, IS-11 and IS-65 displayed a positive interaction but failed to reproduce any major scorable band whereas IS-6, IS-8, IS-61 and IS-65 showed positive reproducible bands (Table 2) (Fig. 1 A-D). Each of these four primers generated unique set of amplified products with the size range of 200 bp (in IS-61) (Fig. 1C) to 1.85 kb (in IS-8) (Fig. 1B). The number of bands from each of these successfully used primers varied from 4 (in IS-6) (Fig. 1A) to 11 (in IS-8) (Fig. 1B) per sample where IS-61 displayed 9 (Fig. 1C) and IS-65 displayed 8 bands (Fig. 1D) per primer. A total number of 336 reproducible monomorphic bands were scored from the clones including their mother with an average of 7 bands per primer.

Table 1: Performance of initial and long term culture on shoot multiplication and rooting of elephant garlic

Treatments	Response to shoot multiplication (%)	No. of shoot	Shoot length (cm)	Days to root induction	No. of root	Length of root (cm)
Primary culture	93.33±5.16a	5.83±1.47a	4.97±0.24a	9.83±2.04a	4.17±1.47a	4.05±0.38a
Secondary culture	92.50±5.24a	5.50±1.51a	5.08±0.35a	9.22±1.86a	3.67±1.36a	4.25±0.56a
Statistical analysis						
Overall mean	92.92	5.67	5.03	9.58	3.92	4.15
CD at 5%	6.695	1.922	0.386	2.513	1.827	0.621
SE	2.1246	0.6101	0.1226	0.7976	0.5798	0.1971

Mean±SD of 20 clones per treatment in six repeated experiments. Same letters indicate no significant difference at $p<0.05$

Table 2: List of primers with their sequences, anchoring, annealing temperature, mode of reaction, number and size of amplified fragments

Oligo-name	Tm (°C)	Sequences (5'-3')	Anchoring	Reaction to elephant garlic DNA	No. of scorable bands per primer	Total No. of scorable bands	Size range (bp)
IS-6	52	$(GA)_8C$	3' anchor	Positive, reproducible and monomorphic	4	36	500-1700
IS-7	50	$(GT)_8A$	3' anchor	Positive but not reproducible	-	-	-
IS-8	52	$(AG)_8C$	3' anchor	Positive, reproducible and monomorphic	11	121	350-1850
IS-9	46	$(TG)_7TA$	3' anchor	Negative	-	-	-
IS-10	52	$C(GA)_8$	5' anchor	Negative	-	-	-
IS-11	52	$(CA)_8G$	3' anchor	Positive but not reproducible	-	-	-
IS-12	52	$(GT)_8C$	3' anchor	Negative	-	-	-
IS-61	50	$(GA)_8T$	3' anchor	Positive, reproducible and monomorphic	9	99	200-1450
IS-63	52	$(AG)_8C$	3' anchor	Positive but not reproducible	-	-	-
IS-65	50	$(AG)_8T$	3' anchor	Positive, reproducible and monomorphic	8	80	300-1050
Total					28	336	200-1850

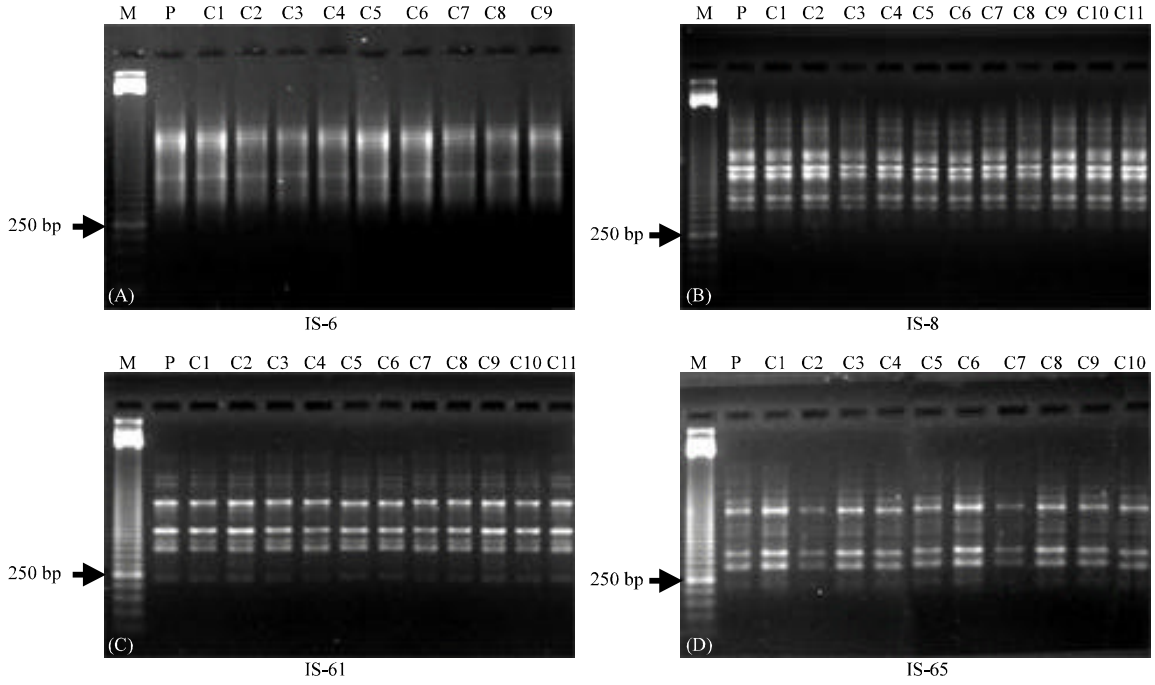

Fig. 1: Agarose gel electrophoresis of ISSR fragments of *in vitro* regenerated clones (C1-C5 from primary culture, C5-C9 and C11 from secondary culture) with their mother (P) showing monomorphic bands generated by (A) primer IS-6, (B) primer IS-8, (C) primer IS-61 and (D) primer IS-65. Lane M 50 bp ladder

DISCUSSION

The morphology of the *in vitro* generated clones was as per their mother plant which indicates no variation among them. Even, after long-term sustained culture of 25 months the morphogenetic competence was no way being handicapped both in plantlets regenerated from primary and secondary explants as revealed by their multiple shoot proliferation, root induction and growth maintained similar level. Regenerative ability and multiplication frequency has been previously reported as limiting factors for *in vitro* long term culture of *Allium cepa* (Hussey and Falavigna, 1980), but in this case such limitation was not observed. Interestingly, Hussey (1982) in narcissus and Rajneesh *et al*. (2009) in bamboo achieved an infinite micropropagation of by splitting and recycling shoots *in vitro*. In our study also multiple shoots were split into individual shoots and recycled for a period of 25 months with no depletion in regeneration frequency. Since, in this case the five cycles of multiple shoot formation in the same medium was performed, each cycle resulted in an exponential increase in the number of shoots.

In the present study di-nucleotide SSRs motifs AG, GA, GT, TG, CT and CA were used. Three positive and reproducible primers (two based on AG motif and two on GA) amplified distinct scorable number of bands (Table 2). Interestingly, IS-8 based on AG motif amplified more number of bands thus revealed more coverage of the genome. Similar results have been reported by Lakshmanan *et al*. (2007) in banana and Joshi and Dhawan (2007) in *Swertia chirayita*. Significantly, these three primers anchored at 3' end and are known to give clearer banding pattern as compared to those at 5' end (Blair *et al*., 1999). These primers produced 336 distinct and scorable bands in the size range of 200 bp-1.85 kb with an average of 7 bands per primer (Table 2). Elephant garlic being a tetraploid, it can be assumed that limited number of bands produced by these ISSR primers would partially cover the genome. However, none of the primer showed any difference in banding pattern. Considering observed similar morphological competence of the primary and long-term secondary culture *in vitro* and the displayed monomorphic banding pattern, it can be suggested that *in vitro* regenerated clones maintained their genetic integrity.

It was recognized that the presence or absence of variations during *in vitro* propagation depends upon the source of explants and the mode of regeneration (Goto *et al*., 1998) including levels of growth substances especially synthetics used (Martin *et al*., 2006). As shoot tip explants directly regenerated into clones (primary

culture) and clones from secondary explants cultured over a period of 25 months maintained clonal fidelity faithfully, it is suggested that shoot tip meristem based on micropropagation system is much more stable genetically. The protocol followed and the technique implemented (splitting and recycling of shoots *in vitro*) would further promote sustained long-term *in vitro* maintenance of shoot tip generated clones without any alteration or modification of DNA. This finding does not subscribe the view held by Bhatia *et al*. (2009), who suspected long-term multiplication of micropropagated plantlets might accelerate the rise of somaclonal or epigenetic variation.

CONCLUSION

The results revealed the significance of shoot tip explants in maintaining their regeneration frequency with morphogenetic competence even after prolonged *in vitro* culture. As per as could be ascertained through morphological similarity and ISSR marker fingerprinting the regenerant clones maintained their fidelity. The possibility of such prolonged sustained true to type cultures would help maintain steady supply of quality propagules for commercial application.

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