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Field evaluation of micropropagated vs. conventionally propagated elephant garlic

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Micropropagated and conventionally propagated plantlets of same genotype of elephant garlic were transferred to the similar field condition and growth stage. A comparative study was conducted using morphological parameters as well as genetic assessments using ISSR markers. The *in vitro* generated elephant garlic plants exhibited significantly vigourous morphological growth when compared to plants propagated through planting of cloves. Genetic appraisal through ISSR showed no polymorphism in banding pattern and thus it was revealed that, there was no significant variation between micropropagated and conventional propagated plants at molecular level.

Key words: *ex vitro* performance, ISSR, *Allium ampeloprasum* L.

Introduction

Elephant garlic (*Allium ampeloprasum* L.), belongs to family Alliaceae, and is an important crop that has been used for centuries in table purpose as a spice and more importantly as medicine. Elephant garlic was named so, as single clove of it can be as large as a whole bulb of ordinary garlic. It has more closely related with the leek than to ordinary garlic. Elephant garlic is considered to be nature's antibiotic. It has also been used for lowering cholesterol, reducing high blood pressure, and treating respiratory problems such as bronchitis and asthma. Because all known cultivated elephant garlic cultivars are sterile, they can only be propagated through vegetative means. The 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 any other *Allium* sp. (Robledo-Paz *et al.,* 2000).

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Hence, tissue culture method appeared to be successful alternative to produce large number of propagules in a short time span and space in several *Allium* spp. using root tip (Haque *et al.,* 1997), shoot tip (Gantait *et al.*, 2009), seed (Wawrosch *et al.,* 2001), mature clove (Roksana *et al.,* 2002) and stem dome (Kamstalityte and Stanys, 2004) explants. There is still no well described work regarding the *in vivo* performance of *in vitro* derived elephant garlic plants after field transfer. Hence, the present investigation was carried out to assess the relative performance of *in vitro* generated and *ex vitro* propagated elephant garlic plants both in terms of morphological and molecular levels.

Materials and methods

In vitro elephant garlic plantlets were mass propagated by *in vitro* culturing of shoot tips on MS media and subsequent acclimatization following the methods developed earlier (Gantait *et al*., 2009). Well acclimatized *in vitro* generated 15 days old plantlets with 8-10 cm height were shifted to earthen pots of 5 cm diameter in the month of August at field condition. Conventionally propagated plants were also transferred *ex vitro* following the similar methods and at same growth stage. The pots were filled with a mixture of soil and farm yard manure (1:1 v/v). All plants were kept under detailed observation at the experimental garden of Bidhan Chandra Krishi Viswavidyalay, W.B., India and agronomic practices were applied in accordance with the plantation protocols during this period. After 3 months of growth period (during the month of November) different important morphological attributes like plant height (cm), leaf length and width (cm), number of leaves, and weight of single bulb (g) were recorded. Along with the morphological response study, the genetic status of micropropagated and conventional propagated plantlets was assessed through ISSR fingerprinting to confirm if there is any alteration in genetic integrity. DNA extraction followed by PCR amplification and ISSR analysis was done using 10 ISSR primers (Gantait *et al*. 2009) as seen in Table 2. Genomic DNA was extracted from 80 mg tender leaves according to the procedure described by Chattopadhyay *et al*. (2008). Extracted DNA samples were subjected to PCR (Polymerase Chain Reaction) amplification using 10 ISSR primers mentioned above. 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 (all from Chromous Biotech Pvt. Ltd., India) and 200 ng of primer (Bangalore Genei Pvt. Ltd., India). PCR performance consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 45 second at 94°C, 45 second at annealing temperature and 90 second 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). The well-resolved and consistently reproducible amplified DNA fragments as bands were scored in terms of their presence or absence and photographed on Gel Logic 200 trans-illuminator system (Kodak).

Treatments were distributed to a Complete Randomized Design (CRD) including three replicants in fifteen repetitive experiments for *ex vitro* study of morphological competence. Each plant was considered as an experimental unit. Data on *ex vitro* evaluation studies were collected and subjected analysis of variance (ANOVA) where 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) 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 and discussion

Micropropagation results in uniform batches of plants which normally grow, flower and fruit. Although, it can be expected that eventually plants multiplied *in vitro* would equivalent or superior to those propagated by traditional techniques, it can not be assumed that their *ex vitro* growth behaviour in long term would necessarily be the same (George, 1996). The utmost importance was assessed the field performance of micropropagated plantlets to ensure their fidelity or superiority to conventionally propagated plantlets.

Morphological performance ex vitro

The *ex vitro* assessment of morphological competence proved to be the potent factor in discriminating the micropropagated and traditional propagated elephant garlic plants. A comparison was made between these two types of regenerated plants, where both of these were not significantly different performers in terms of plant height, leaf length and width. The morphological competences, assessed at the 3 months growth stage, are presented in Table 1 where there was no significant difference in plant height between micropropagated and conventional propagated. The *in vitro* generated plants at

the 3 month stage was averaged height of 35.27 cm which 26.9 cm and 0.47 cm of leaf length and width respectively (Fig. 1a). On the other hand, control plants attained 34.47 cm height with 26.53 cm leaf length and 0.43 cm leaf width (Table 1). But regarding the number of leaves and weight of single bulb, the micropropagated plantlets revealed their superiority over control elephant garlic plants where previous ones produced 7.34 leaves per plant, and the later were 6.77 leaves per plant. The weight of single bulb, micropropagated plants attained 10.13 g and conventionally propagated plants were 8.53 g of single bulb. The better performance *in vitro* generated elephant garlic plantlets over conventional ones opposed the earlier observation of Biswas *et al*. (1999) in *Eucalyptus tereticornis*. It is noted that earlier study was on a tree species but the present experiment was carried out on a herb. The *in vitro* generated plants developed higher number of leaves to be able to intercept incoming radiation for photosynthesis earlier than conventional propagated plants. This may explain the differences between the micropropagated and clove-derived plants (Buah *et al*. 2000). Moreover, the better vegetative growth in micropropagated plants means the support in better establishment of plants against any biotic stress. The protected *in vitro* environment during micropropagation favoured the plantlets to express their complete potentiality towards morphological attributes *ex vitro* too. This study supported the earlier report of Gustavsson and Stanys, (2000) who observed the better performance of *in vitro* derived lingonberry plants than the conventional propagated plants.

Table 1. *Ex vitro* field performance* of micropropagated in comparison to conventionally propagated elephant garlic.

Treatments	Plant height (cm)	No. of leaves/plant	Leaf length (cm)	Leaf width (cm)	Wt. of single bulb (g)
Conventional	34.47[a]	6.77[b]	26.53 [a]	0.43 [a]	8.53[a]
Micropropagated	35.27 [a]	7.43 [a]	26.90[a]	0.47 [a]	10.13[b]
Mean	34.87	7.10	26.71	0.45	9.33
SE (±)	0.3568	0.1764	0.1795	0.0126	0.2308
CD at 5%	1.034	0.511	0.520	0.036	0.669

Data represent mean of 3 replicants per treatment in fifteen repeated experiments; Means within columns separated by DMRT (P=0.05); *Data were recorded at 3 months growth stage after field transfer

Genetic assessment using ISSR

In the assessment of the fidelity of *in vitro* generated acclimatized clones IS-9, IS-10 and IS-12 did not react with elephant garlic DNA among the 10 selected ISSR primers. Between the rest of the primers *i.e.* IS-6, IS-7, IS-11 and IS-65 displayed a positive interaction but failed to reproduce any major

scorable band whereas IS-8 (Fig. 1b), IS-61 (Fig. 1c) and IS-65 (Fig. 1d) showed positive reproducible bands (Table 2). Each of these three primers that generated a unique set of amplified products with the size range of 200 bp in IS-61 to 1850 bp in IS-8. The number of bands from each of these successfully used primers varied from 8 (in IS-65) to 11 (in IS-8) per sample. A total number of 168 (number of plants used as sample × average number of bands per sample for all primers) reproducible monomorphic bands were scored from the clones including their mother with an average of 9.33 bands per primer per sample. With 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. 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 one on GA) amplified a distinct scorable number of bands. Interestingly, IS-8 based on AG motif amplified a higher number of bands revealed more coverage of the genome. Similar results have been reported by Laxmanan *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). However, none of the primers showed any difference in banding pattern. It is displayed monomorphic banding pattern, it can be suggested that both micropropagated and conventional propagated plants that maintained similar genetic clonal integrity though micropropagated plants would better performer in *ex vitro* condition.

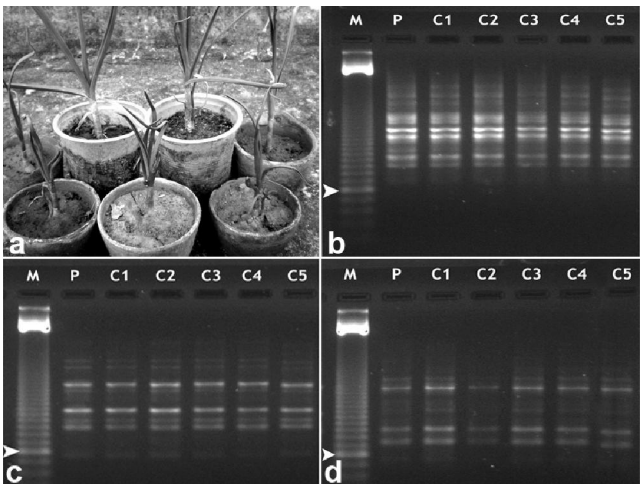

Fig. 1. (a) Micropropagated elephant garlic plantlet with vigorous growth after *ex vitro* transfer. Agarose gel electrophoresis of ISSR fragments of micropropagated (C1-C3) and conventional propagated (C4-C5) vanilla plants with their mother (P) showing monomorphic bands generated by primer IS-8 (b), IS-61 (c) and IS-65 (d). Lane M- 50bp ladder.

Table 2. ISSR primers used for fidelity test of *in vitro* generated elephant garlic clones, their sequences, anchoring, annealing temperature, mode of reaction, number and size of amplified fragments.

Oligo-name	Tm (°C)	5′-3′ motifs	Anchoring	Reaction to elephant garlic DNA	Number of scorable bands per primer	Total number of scorable bands	Size range (bp)
IS-6	52	$(GA)_8C$	3′anchor	Positive but not reproducible	-	-	-
IS-7	50	$(GT)_8A$	3′anchor	Positive but not reproducible	-	-	-
IS-8	52	$(AG)_8C$	3′anchor	Positive , reproducible, monomorphic	11	66	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, monomorphic	9	54	200-1450
IS-63	52	$(AG)_8C$	3′anchor	Positive but not reproducible	-	-	-
IS-65	50	$(AG)_8T$	3′anchor	Positive , reproducible, monomorphic	8	48	300-1050
Total					28	168	200-1850

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