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Genotypic response of short day garlic (*Allium sativum* L.) accessions to shoot multiplication

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Abstract

A simple protocol for *in vitro* shoot multiplication of 10 accessions including 8 varieties and 2 advanced lines of short day Indian garlic (*Allium sativum*) is described. Two shoot multiplication media (Murashige and Skoog 1 and Murashige and Skoog 2) were tried using basal plate explant from mature cloves for multiple shoot induction. The medium (Murashige and Skoog 2) consisting of 6-(γ,γ-dimethylallylamino)-purine (3 mg l^{-1}) was the best for induction of multiple shoots. Although genetic variability existed among the genotypes, shoot multiplication was obtained in all the accessions in Murashige and Skoog 2 medium along with almost 100% bulb formation in the shoots produced.

Keywords: *Allium sativum,* garlic, genetic variability, *in vitro* bulbil induction, shoot multiplication.

Abbreviations:2iP=6-(γ,γ-dimethylallylamino)-purine; MS medium=Murashige and Skoog medium.

Introduction

Garlic (*Allium sativum* L.) is sexually sterile and has been cultivated clonally through cloves. Even then, cultivated garlic displays considerable genetic variability in numerous characters ranging from bulb colour and shape to scape height, flower characteristics and adaptability to different day lengths (Messiaen *et al*. 1993). This variability is the result of hundreds of years of clonal selection, which forms the basis of many gene banks around the world (Maab & Klaas 1995). As garlic bulbs cannot be stored for more than 6–8 months, maintenance is usually done by planting them in the field every year. However, exposure to pests, diseases and other natural hazards, makes the maintenance of field gene banks a costly affair. Prolonged conservation in field gene banks also results in decreased yield and sometimes, total destruction due to accumulation of viruses present in this species. Tissue culture techniques allow for prolonged conservation of desirable genotypes. However, tissue culture protocols are greatly dependent upon the genotype used and hence, it may be necessary to standardize the media and culture conditions for different genotypes (Barandiaran *et al*. 1999b).

Numerous protocols for micropropagation of garlic have been described (Bhojwani 1980; Matsubara & Chen 1989; Seabrook 1994;

Ayabe & Sumi 1998; Khar *et al*. 2003a). However, maximum emphasis was given to the long day type garlic, which is common in the temperate regions. Indian garlic belongs to the subtropical group (Maab & Klass 1995) and work on this group is negligible (Koul *et al*. 1994; Sata *et al*. 2000). Only one earlier report has been made in India on genotypic studies in short day garlic (Khar *et al*. 2002; 2003a), where the responses to shoot multiplication of three Indian varieties was studied. Hence, an experiment was laid out to test the genotypic response of 10 different short day Indian garlic genotypes on *in vitro* shoot multiplication.

Materials and methods

Plant material

All the 10 accessions used in the investigation were obtained from the garlic germplasm bank of National Research Centre for Onion and Garlic, Pune (Maharashtra) (Table 1). The genotypes chosen represent maximum genetic variability available in the germplasm bank.

In vitro *culture*

Mature cloves grown during *rabi* 2003–04 were used as explant source. The outer dry skin of the cloves was removed and the cloves were washed thoroughly under running tap water to remove the surface contaminants. Surface sterilization was done using 70% alcohol for 1 min, 0.1% Bavistin and 10% Labolene (a neutral detergent - Qualigens, India) for 10–15 min followed by sterilization in sodium hypochlorite solution (2% available chlorine - Qualigens, India) for 10–15 min and thorough rinsing thereafter with double distilled water 3–4 times. Sterilized explants were blotted using sterile filter paper and basal plate (3–4 mm) was excised and inoculated on to shoot induction media.

The basal plate was individually cultured in sterile test tube containing 10 ml of MS medium (Murashige & Skoog 1962) supplemented with two concentrations of 2iP (1 and 3 mg l^{-1}, designated as MS1 and MS2, respectively). This medium was augmented with 3% (wv^{-1}) sucrose and solidified with 0.8% agar (HiMedia, India). Before autoclaving, the pH of the media was adjusted to 5.8. Cultures were maintained at 25 ± 1^0C under a 16 / 8 h light regime provided by cool white fluorescent tubes at 40 μmoles m^{-2} s^{-1}.

Three replications of basal plate explant for each multiplication media was maintained and each experiment was repeated thrice. Multiple shoot induction was initiated at approximately 30 days of culture. After 45 days, the first subculture was done after recording the number of shoots induced. The plantlets obtained from the initial meristematic explant were transferred as such without separation to 100 ml sterile flask containing 50 ml of the same medium for further proliferation. At the end of 90 days, the number of shoots induced was again recorded. On retaining the multiple shoots in the same multiplication media, *in vitro* bulbils were induced after about 120 days. *In vitro* bulbils were harvested upon maturity as indicated by the drying off of the vegetative parts and were analysed for bulbil characters namely, weight, polar and equatorial diameter and colour (white or purple).

Statistical analysis

The experimental set-up was two factorial randomized block design with three replications per accession for the analysis of shoot multiplication and randomized block design with five replications per accession for bulbil data. Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were conducted to analyse shoot multiplication and bulbil characters. The differences among means were analysed at 5% significance level.

Results and discussion

Many reports on the use of tissue culture as a biotechnological tool for the improvement of garlic are available (Abo El-Nil 1977; Khar *et al*. 2003b). Different explants have been successfully used for obtaining multiple shoots *in vitro* of which the basal plate is the

most commonly used explant (Bhojwani 1980; Seabrook 1994). Other explants such as shoot apex (Matsubara & Chen 1989) and stem disc (Ayabe & Sumi 1998) were also used successfully for the induction of multiple shoots. In the present study, basal plate explant was found to be highly responsive in all the genotypes.

Most protocols describe the use of different media for different stages of micropropagation namely, establishment, multiplication and *in vitro* bulbil induction with frequent subcultures in between. In the present study, with short day garlic genotypes, one subculture at 45 days after culture was sufficient during the growth period of 5 months. An almost similar approach was used by Barandiaran *et al*. (1999a) where they used three subcultures in 7 months. All the accessions produced mature bulbils by the end of 5 months.

Effect of media on shoot multiplication

Cytokinins, being adenine derivatives, are mainly concerned with cell division, modification of apical dominance and shoot differentiation in tissue culture. In the presence of a suitable cytokinin, apical dominance can be overcome and axillary buds can be made to grow into shoots (Stefaan *et al*. 1994). The ability of 2iP, a naturally occurring cytokinin, to give rise to multiple shoots in garlic has been documented by Koch & Salomon (1994). In the present study also, 2iP was very effective in inducing shoot multiplication in the wide range of genotypes studied. Among the two concentrations of 2iP tried, 2iP (3 mg l^{-1}) (MS2) was significantly superior at both 45 and 90 days of culture with a mean shoot multiplication rate of 2.8 and 5.2, respectively, as against 2.0 and 3.5 in 2iP (1 mg l^{-1}) (MS1) medium (Table 2). In general, the mean number of shoots declined in the MS1 medium, 2iP 1 mg l^{-1} both at 45 and 90 days after culture in most of the genotypes except for G1 (Fig. 1a), while there was a significant decline in Godavari at 90 days after culture. The advantage of using 2iP was that the shoots induced were healthy with no basal callusing from the basal plate explant.

Table 1. Garlic accessions used for *in vitro* studies

Genotype	Type	Bulb skin colour
G-1	Variety	White
G-41	Variety	White
G-50	Variety	White
GG-2	Variety	White
GG-3	Variety	White
G-282	Variety	White
G-323	Variety	White
Godavari	Variety	Purple
AC-50	Advanced line	White with purple streaks
AC-200	Advanced line	White

A majority of the accessions produced roots after 90 days of culture thus eliminating the need for a separate rooting medium. Further, *in vitro* bulbil induction was also seen in the same medium after about 120 days. Similar results of bulbing on shoot induction medium have been previously reported (Moriconi *et al*. 1990; Nagakubo *et al*. 1993; Barandiaran *et al*. 1999a). Formation of bulbil is an added advantage as they are good propagules that can be stored till the next season. Moreover, this overcomes the need for an intermediate hardening step. However, early bulbil induction reduced the multiplication rate. Similar observations were made by Barandiaran *et al*. (1999a) in long day genotypes.

Effect of genotypes

Genotypic variation in shoot multiplication has been reported in garlic by many authors (Moriconi *et al*. 1990; Barandiaran *et al*. 1999a; Khar *et al*. 2003a). However, most studies have analysed multiplication rate of long day type garlic only. Though Khar *et al*. (2003) have reported genotypic effect of short day garlic, only three genotypes were evaluated by them. Significant differences for multiplication rate after 45 days and 90 days respectively, among genotypes was observed indicating that genotype plays a very important role in response of culture under *in vitro* conditions.

Table 2. Effect of shoot induction media on shoot multiplication in 10 genotypes of garlic

Genotype	2iP (mg l^{-1})	No. of shoots (45 days)*	No. of shoots (90 days)*	Mean** 45 days	Mean** 90 days
G-41	1	3.7 ± 0.58	8.0 ± 1.00	5.0 ± 1.79^{a}	12.8 ± 5.34^{a}
	3	6.3 ± 1.53	17.7 ± 0.58		
GG-3	1	1.0 ± 0.00	1.0 ± 0.00	1.5 ± 0.84^{cd}	1.5 ± 0.84^{d}
	3	2.0 ± 1.00	2.0 ± 1.00		
AC-200	1	1.7 ± 0.58	1.7 ± 0.58	1.5 ± 0.55^{cd}	3.5 ± 2.26^{c}
	3	1.3 ± 0.58	5.3 ± 1.53		
G-1	1	3.0 ± 1.00	9.0 ± 1.73	2.2 ± 1.17^{cd}	5.2 ± 4.36^{b}
	3	1.3 ± 0.58	1.3 ± 0.58		
AC-50	1	1.0 ± 0.00	1.0 ± 0.00	2.7 ± 1.97^{bc}	3.8 ± 3.19^{c}
	3	4.3 ± 1.15	6.7 ± 1.15		
GG-2	1	1.3 ± 0.58	1.3 ± 0.58	2.2 ± 1.47^{cd}	2.3 ± 1.37^{d}
	3	3.0 ± 1.73	3.3 ± 1.15		
G-323	1	1.0 ± 0.00	1.0 ± 0.00	1.2 ± 0.41^{d}	1.3 ± 0.52^{d}
	3	1.3 ± 0.58	1.7 ± 0.58		
Godavari	1	3.0 ± 1.00	7.7 ± 0.58	2.5 ± 1.00b^{c}	5.7 ± 2.25^{b}
	3	2.0 ± 1.00	3.7 ± 0.58		
G-50	1	1.7 ± 0.58	2.0 ± 0.00	2.0 ± 0.89c^{d}	4.0 ± 2.28^{c}
	3	2.3 ± 1.15	6.0 ± 1.00		
G-282	1	2.7 ± 0.58	2.7 ± 0.58	3.3 ± 1.03^{b}	3.5 ± 1.04^{c}
	3	4.0 ± 1.00	4.3 ± 0.58		
Mean**	1	2.0 ± 1.08^{y}	3.5 ± 3.23^{x}		
	3	2.8 ± 1.83^{y}	5.2 ± 4.64^{x}		

CD (P=0.05) (45 days) Genotype x Medium 1.46 Within treatment 0.46 Among genotypes 1.03
CD (P=0.05) (90 days) Genotype x Medium 1.38 Within treatment 0.44 Among genotypes 0.97
* Means of three replications ± SE, Genotype x Medium **Means in each column followed by different lower-case letters are statistically different at P<0.05 as per Duncan's multiple range test.

DMRT distinguished four groups with a fairly good degree of overlap in multiplication rate after 45 days, but with clear distinction in groups after 90 days of culture. Variety G-41 was significantly superior at both 45 and 90 days after culture with a mean of 5.0 and 12.8 shoots (Fig. 1b), respectively. G-323 was the most inferior one at both 45 and 90 days after culture with a mean value of 1.2 and 1.3, respectively. However, it did not differ significantly from GG-2 and GG-3 at 45 and 90 days after culture, respectively.

Effect of genotype x *media*

Genotype x media interaction was significant in the ANOVA analysis both at 45 and 90 days after culture. Most of the genotypes exhibited better multiplication rates in MS2 medium.

Genotype G-41 was significantly superior in shoot multiplication rate in MS2 both at 45 and 90 days after culture exhibiting 5 x and 14 x higher mean number of shoots of 6.3 and 17.7, respectively, than the genotype exhibiting the lowest shoot number. However, MS1 medium was superior for the genotype G-1 at both 45 and 90 days after culture, while it was superior for Godavari at 90 days after culture.

In vitro *bulbil induction*

Bulbil induction *in vitro* is advantageous for garlic micropropagation as these structures are good propagules, easy to manage and does not require an intermediate hardening phase. *In vitro* bulbil induction in garlic *in vitro* cultures has been reported by Moriconi *et al*. (1990) and Nagakubo *et al*. (1993). However, many of them had to resort to complex media additives (Ravnikar *et al*. 1995; Robert *et al*. 1998; Kim *et al*. 2003) or temperature pre treatments (Ayabe & Sumi 1998) to achieve

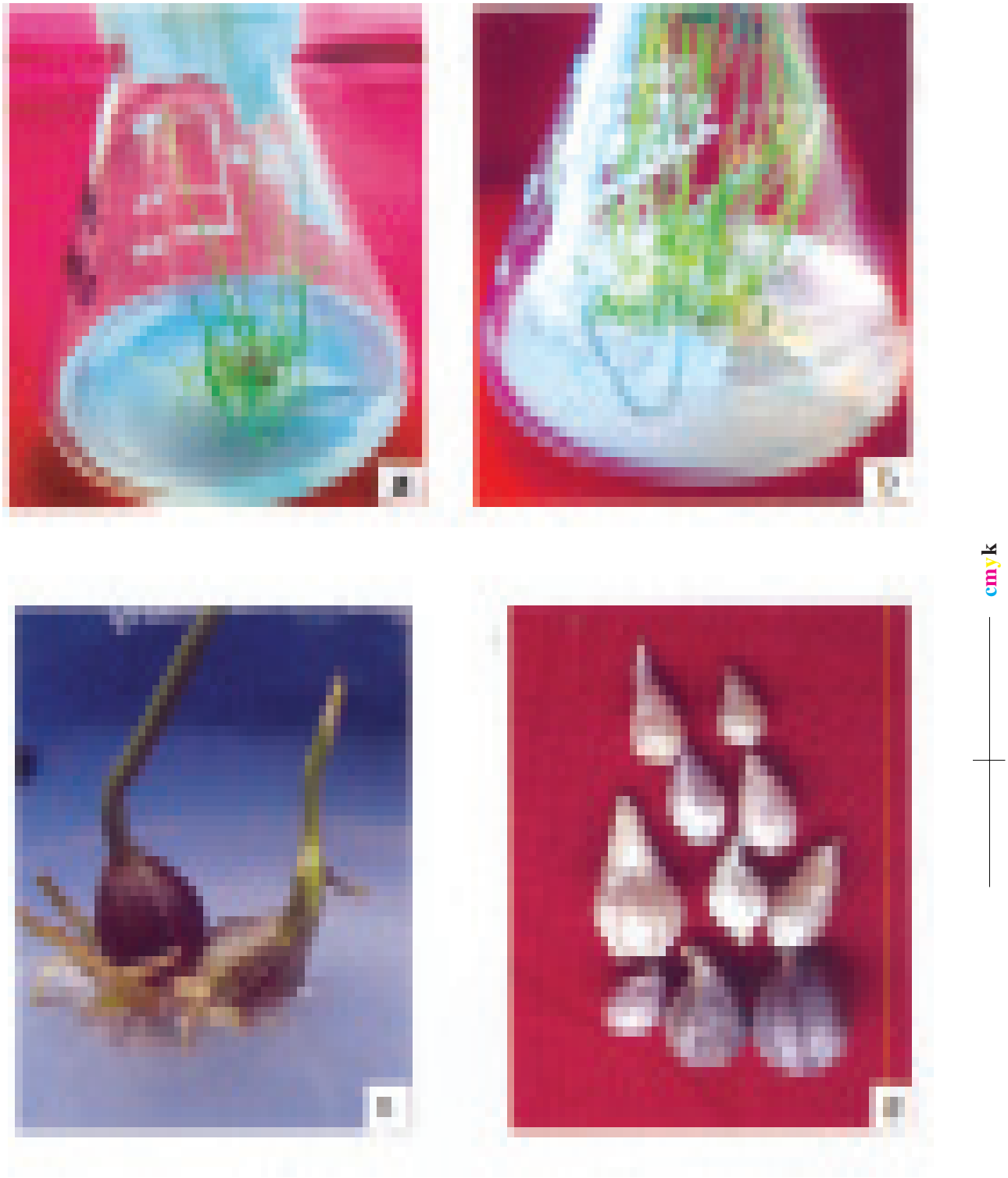

Fig. 1. Genotyic response of garlic accessions to shoot multiplication (a) Garlic var. G-1 showing 12–15 shoots from basal plate explant along with *in vitro* bulbils in 2iP (1 mg l^{-1}) (b) Garlic var. G-41 showing the induction of about 20 shoots in MS medium fortified with 2iP (3 mg l^{-1}) after 90 days of culture (c) Garlic var. G-282 showing well developed *in vitro* bulbils (d) Variability shown by *in vitro* bulbils in garlic with respect to colour and size

Table 3. *In vitro* bulbil (IVB) characters of 10 garlic genotypes

Genotype	Weight (mg)*	Polar diameter (mm)*	Equatorial diameter (mm)*	Colour (W/P)
G-41	304.8 ± 1.61^{bc}	9.10 ± 3.07^{abc}	6.74 ± 1.41^{a}	W/P
GG-3	126.0 ± 0.43^{d}	9.25 ± 1.27^{ab}	4.91 ± 0.96^{bc}	P
AC-200	63.6 ± 0.60^{d}	4.65 ± 0.95^{d}	4.02 ± 1.45^{cd}	P
G-1	647.0 ± 0.33^{a}	11.21 ± 1.15^{a}	7.21 ± 0.19^{a}	P
AC-50	355.8 ± 2.67^{b}	9.75 ± 3.09^{ab}	7.42 ± 1.99^{a}	P
GG-2	163.0 ± 0.88^{cd}	5.92 ± 4.21^{cd}	2.86 ± 1.27^{d}	P
G-323	376.0 ± 1.82^{b}	7.58 ± 1.31^{bcd}	6.03 ± 1.76^{ab}	P
Godavari	284.4 ± 2.00^{bc}	7.91 ± 1.84^{bc}	6.23 ± 1.38^{ab}	W/P
G-50	97.0 ± 0.39^{d}	8.06 ± 1.70^{abc}	4.04 ± 0.95^{cd}	P
G-282	197.2 ± 0.75^{cd}	6.80 ± 1.93^{bcd}	4.53 ± 1.22^{bcd}	W/P

*Means of five replications ± SE; Means in each column followed by different lower-case letters are statistically different at P<0.05 as per Duncan's multiple range test; W = White; P = Purple

the same. In this study, there was no need for a separate medium for bulbil induction. Almost 100% bulbil induction was achieved in the same shoot multiplication medium after about 120 days of culture which required a further 30 days to mature fully. However, in some genotypes namely, G-282, bulb induction started much earlier (Fig.1c), which in turn adversely affected shoot multiplication. Explants that failed to induce multiple shoots also produced *in vitro* bulbils, which were usually larger than the bulbils produced by the multiple shoots. This is in concurrence with the results of Barandiaran *et al*. (1999a).

Weight and size of the bulbil produced is important as it determines the quality and health of the plants in field condition. Many experiments show that clove size is an important factor for good yield in garlic (Sankar *et al*. 2001). Genotypes studied showed highly significant differences with respect to bulb weight and bulb size (polar and equatorial diameter). Even within a treatment, the bulbil size varied considerably (Fig.1d), which was dependent upon the size of the shoots induced *in vitro* with bigger shoots forming bigger bulbils and vice versa. DMRT grouped the 10 varieties into 4 groups with high degree of overlap (Table 3). Bulbil weight ranged from 63.6 (AC-200) to 647 mg (G-1) and bulb size ranged from 4.65 (AC-200) to 11.21 mm (G-1) for polar diameter and 2.86 (GG-2) to 7.42 mm (AC-50) for equatorial diameter. It was observed that the genotype G-1 exhibited all the superior characteristics of highest bulbil weight(647 mg), significantly higher bulb size of 11.2 mm for polar diameter and 7.2 mm for equatorial diameter. There was variation in the bulbil colour also with most of the *in vitro* bulbils showing purple colour.

The present protocol developed will be useful for multiplication and *in vitro* management of a large gene bank of short day type garlic.

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