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Production of virus free planting material through meristem culture in short day garlic cultivars Bhima Omkar and Bhima Purple

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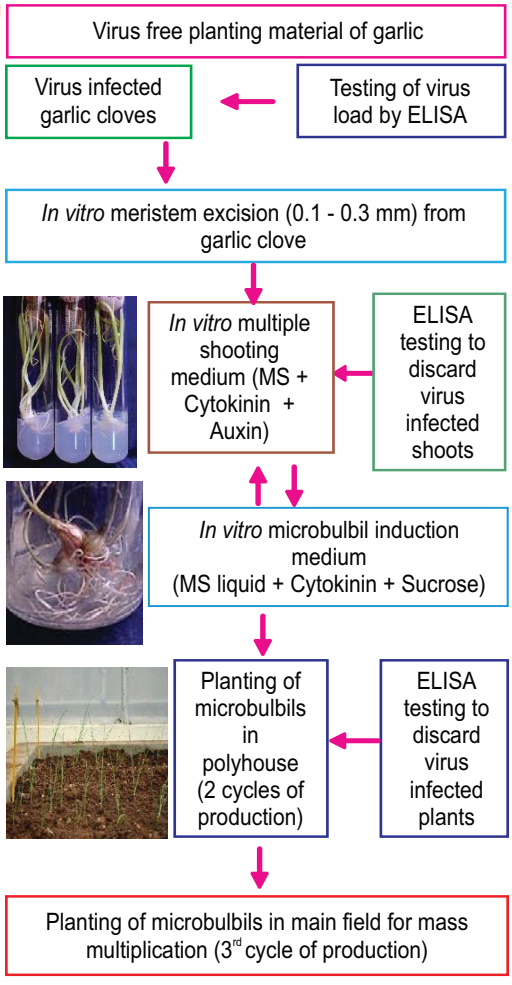
Abstract

Aim : Low productivity of garlic in India has been attributed to viral infestation of planting material. *In vitro* techniques offer a suitable alternative; hence, standardization of protocol to raise virus free planting material through meristem culture *in vitro* and its multiplication was planned.

Methodology : Response of different cytokinins viz. BAP, Kin (1 and 1.5 mgl^{-1}) in combination with NAA (0.1, 0.5 mgl^{-1}) on basal MS medium was evaluated for inducing multiple shooting through meristem tip (0.1 – 0.3 mm) *in vitro* in two garlic cultivars viz. Bhima Purple and Bhima Omkar. Higher sucrose (6 to 11 %) along with cytokinins was evaluated for *in vitro* microbulbils induction. Viral load was tested at different stages using potyvirus specific alkaline phosphatase based direct antigen coating ELISA kit. *In vitro* raised microbulbils were transferred to three production cycles to get normal size garlic bulb.

Results : MS + 1 mgl^{-1} Kin + 0.1 mgl^{-1} NAA medium was the best performing medium for induction of multiple shooting in both the cultivars. About 70% mericlones were free from viruses in both the cultivars. Liquid medium MS + 1 mgl^{-1} Kin + 6% sucrose produced the highest number of microbulbils than other treatments. Normal garlic bulbs (9-12 cloves/bulb) were produced in field from *in vitro* raised microbulbils after two cycles of production in polyhouse followed by field transplanting.

Interpretation : *In vitro* meristem culture is an efficient method for producing virus free planting material of garlic. Protocol for production of higher number of microbulbils *in vitro* has also been standardized. Using standardized protocol a normal size garlic bulb can be produced in three production cycles.



Introduction

Garlic is a world famous spice commodity commonly consumed in daily diet and used for its medicinal properties. In India, 12.21 lakh tons of garlic was grown on 2.38 lakh ha area in 2013-14 (NHRDF, 2014). Madhya Pradesh is the leading producer of garlic (2.70 lakh ton) followed by Gujarat (2.50 lakh ton) and Rajasthan (2.25 lakh ton) in 2013-14. Despite second largest garlic production after China in the world, productivity in India is far meager (5.13 t ha^{-1} in 2013-14), as compared to 16.71 tha^{-1} of world average. Requirement of garlic to satisfy domestic consumption will increase from 1.23 million tons at present to 1.66 million tons for projected 1.7 billion population of India of by 2050 (DOGR, 2014). This demands the increase in productivity to 6.77 t ha^{-1} against the existing 5.13 t ha^{-1}. The world trade of garlic through export is 1.98 million tons of worth 2834.78 million dollars. However, India once an exporter of garlic is now importing it to meet the domestic requirement.

Low productivity of garlic is attributed to its susceptibility to diseases caused by viruses (Kim *et al*., 2003; Metwally *et al*., 2013). The *in vitro* meristem-tip culture is a preferred alternative for effective exclusion of viruses and maintain clonal fidelity (Verbeek *et al*., 1995). Several *in vitro* regeneration protocols were developed for mass multiplication of garlic (Luciani *et al*., 2006). Albeit, most of the studies followed indirect shoot proliferation through callus, which is not advisable for maintaining clonal fidelity. Other protocols using different ex-plants (Haque *et al*., 1997, 2003; Kim *et al*., 2003) do not elaborate about status and extent of elimination of viral infestation. Media and culture conditions for different genotypes need to be standardized separately due to specific requirements of different cultivars (Barandiaran *et al*., 1999). Further, establishment of transplanted tissue-cultured garlic plants to field continues to be a bottleneck in commercialization of *in vitro* propagation of garlic (Metwally *et al*., 2012). Meager studies unravel *ex vitro* hardening and production of regular garlic bulb cycles. Since, majority of reports are pertaining to long day cultivars of garlic and Indian garlic is predominantly short day cultivar, thus have separate culture requirements, which ought to be standardized. Therefore, the present study was undertaken to standardize a protocol for *in vitro* induction of virus free multiple shooting to produce mericlones followed by induction of microbulbils in two promising cultivars Bhima Omkar and Bhima Purple.

Materials and Methods

The present study was conducted using two garlic cultivars viz. Bhima Omkar and Bhima Purple. The stored garlic bulbs (2 – 3 months old) were used in this study. Bulbs were given cold treatment (4°C) for about a month and the separated cloves were peeled to remove dry skin. Further, all other activities were performed in Laminar Air Flow cabinet. Cloves were surface sterilized by 70% (v/v) alcohol followed by sodium hypochlorite (2% w/v) for 15 min. The cloves then cleaned by rinsing in sterilized double distilled water 3-4 times and dried with sterilized blotting paper. Basal MS (Murashige and Skoog, 1962) medium was used with fortification by either 6-Benzylaminopurine (BAP), Kinetin (Kin) (1 and 1.5 mgl^{-1}) alone or in combination with 1-Naphthalene Acetic Acid (NAA) (0.1, 0.5 mgl^{-1}). All media consisted of 3% sucrose and gelled using 0.8% agar. pH of the media were adjusted at 5.7 prior to autoclave, which was done at 121°C and 15 lb/psi for 20 min.

Meristems (0.1 – 0.3 mm) were aseptically excised in Laminar Air Flow and inoculated in petri plates on different media. All the cultures were provided photoperiod of 16/8 hrs light and dark with white fluorescent tube lights at 40µmoles $m^{-1}s^{-1}$. The well grown mericlones (7 – 10 days old) were subcultured after 12 – 15 days in individual test tubes (25 x 150 mm, Borosil Make) on same media. Parameters pertaining to days required for multiple shooting, number of shoots/ culture, shoot length were recorded. The 50–55 day old cultures were transferred to MS basal medium having cytokinins *i.e*., BAP or Kinetin (0.5, 1.0 and 1.5 mgl^{-1}) with 3% sucrose for microbulbil induction. The best performing treatment for microbulbil induction was fortified with different sucrose concentrations (6, 7, 8, 9, 10 and 11%) in solid medium (agar 0.8%). The cultures were maintained on the similar medium until harvest. At harvest number of microbulbils/ culture, weight of microbulbils etc. were recoded. The best performing treatment identified for microbulbil induction and development was used to test the effect of liquid medium than solid medium under light (16/8 hrs). Harvested microbulbils were subjected to cold treatment at 4°C for about a month and planted in polyhouse for two cycles of production and third cycle was planted in main field. Statistical analysis was done using three replications per treatment, in which each replication comprised of ten-twelve cultures and mean were compared by LSD.

ELISA kit (Agdia, Elkhart, Indiana, USA) was used for identification of Onion yellow dwarf virus (OYDV) and Leek yellow stripe virus first for excising meristems and secondly to ensure elimination. Viral load was also assessed in polyhouse raised plants before harvesting of garlic bulbs in first production cycle.

Results and Discussion

In experiments pertaining to induction of multiple shooting, results revealed that number of shoots/ culture and days required for shoot proliferation were significantly affected by fortification of basal MS medium with phytohormones (Table 1). Kinetin treatments along with NAA (0.1 mgl^{-1}) was superior to BAP. Callusing in explants was observed when concentration of NAA was increased to 0.5 mgl^{-1}. Callus formation can induce somaclonal variation in the regenerants, thus a method of regeneration involving no callus formation is preferred for production of clones of true to type. (Haque *et al*., 1997). Hence, these treatments were discontinued further. Cytokinin and auxin as 1.5 mgl^{-1} BAP + 0.5 mgl^{-1} NAA was found essential (90% explants responded) for shoot-root multiplication and proliferation

in garlic (Roksana *et al*., 2002). Also, combination of 6-benzyladenine (BA) (10µM) and NAA (1µM) was the best combination for multiple shoot proliferation (75%) in garlic (Haque *et al*., 1997).

When cultures were subjected to microbulbil induction, formation of microbulbils initiated after about 30 days of subculturing of mericlones (Table 2). The media composition with MS + 1 mgl^{-1} Kinetin produced more number (4.16 in Bhima Omkar and 5.69 in Bhima Purple) of microbulbils than other treatments. Rooting in cultures started simultaneously in all the shoots, which were subcultured for microbulbil induction without any additional endeavors. In earlier study, MS + 1.5 mgl^{-1} kinetin had the lowest frequency of bulb formation (3.2), but addition of NAA (0.5 mgl^{-1}) improved the response to 4.8 bulbs/culture (Roksana *et al*., 2002).

Although microbulbil development was observed with the regular sugar concentration (3%) of basal medium, increase in sugar concentration of medium responded favorably for microbulbil induction. It was observed that 6% sucrose yielded the highest number of microbulbils (4.23 bulbils/culture in Bhima Omkar and 5.19 bulbils/culture in Bhima Purple) than other treatments (Table 3) and on par with 7% sucrose concentration. Rooting was initiated in all shoots along with microbulbil induction. Higher concentrations (> 7%) showed ill effects and at the highest concentration (11%) the cultures dried out. Liquid medium had significant increase in weight of microbulbil in similar duration over solid medium (0.8% agar). In liquid medium, the average weight (20-30 microbulbils) increased form 122 mg to 815 mg in cv. Bhima Omkar and from 188 mg to 804 mg in cv. Bhima Purple. Although, Kim *et al*. (2003) found that 11% sucrose had higher bulbing response than lower dosage (3, 5, 7 and 8%). Dixit *et al*. (2013) corroborated to 6% sucrose concentration form higher mass gain of bulbils than higher concentration (9 and 12%). In the present study, the bulbil development was started after about 80 days of inoculation of explants. In different Indian cultivars,

Table 1 : Effect of phytohormones on *in vitro* multiple shoot proliferation from meristem (0.1-0.3 mm) explant in two garlic cultivars

Medium (MS +)	Bhima Omkar			Bhima Purple		
	Success rate (%)	Shoot initiation (days)	Multiple shoots/ culture (No.)	Success rate (%)	Shoot initiation (days)	Multiple shoots/ culture (No.)
MS	100^{a}	9.31^{c}	2.62^{a}	100^{a}	9.8^{c}	1.9^{a}
0.1 mgl^{-1} NAA + 1 mgl^{-1} BAP	100^{a}	8.3^{b}	3.13^{b}	100^{a}	7.4^{b}	3.9^{b}
0.1 mgl^{-1} NAA + 1.5 mgl^{-1} BAP	100^{a}	8.7^{b}	2.68^{a}	100^{a}	7.9^{b}	3.7^{b}
0.1 mgl^{-1} NAA + 1 mgl^{-1} Kin	100^{a}	6.9^{a}	4.43^{c}	100^{a}	6.5^{a}	5.8^{c}
0.1 mgl^{-1} NAA + 1.5 mgl^{-1} Kin	100^{a}	6.6^{a}	4.48^{c}	100^{a}	6.1^{a}	5.5^{c}

Mean values with different letters in a column differ significantly at p <0.05 according to LSD

Table 2 : Effect of phytohormones on *in vitro* microbulbil induction and development in two garlic cultivars

Medium (MS +)	Bhima Omkar			Bhima Purple		
	#Days for microbulbils induction	Bulbils/ culture (No)	[1]Wt / bulbil (mg)	#Days for microbulbils induction	Bulbils/ culture (No)	[1]Wt / bulbil (mg)
0.5 BAP mgl^{-1}	32.31^{c}	2.16^{a}	93.1^{a}	31.12^{b}	2.69^{a}	105^{a}
1.0 BAP mgl^{-1}	30.12^{b}	3.89^{b}	94.2^{a}	31.69^{b}	3.16^{b}	109^{a}
1.5 BAP mgl^{-1}	29.26^{b}	3.16^{b}	89.1^{a}	30.12^{b}	3.36^{b}	108^{a}
0.5 Kin mgl^{-1}	30.19^{b}	2.49^{a}	91.3^{a}	30.36^{b}	2.69^{a}	106^{a}
1.0 Kin mgl^{-1}	27.69^{a}	4.16^{c}	92.3^{a}	27.89^{a}	5.69^{c}	110^{a}
1.5 Kin mgl^{-1}	27.36^{a}	4.45^{c}	90.4^{a}	27.56^{a}	5.76^{c}	112^{a}

#50 - 55 days old cultures were transferred for microbulbil induction; [1]Average weight recorded about 120 days of after initial culturing. Mean values with different letters in a column differ significantly at p < 0.05 according to LSD

100% bulbing was achieved after about 120 days of culture which required further 30 days to mature fully (Devi *et al*., 2007).

Total 100 mericlones of each cultivar Bhima Purple and Bhima Omkar derived from the test – positive material were again tested for potyvirus group. Meristem tip culture was found to eliminate viruses and 76% mericlones of Bhima Purple and 72% mericlones of Bhima Omkar were found free of virus infestation. Virus load in the plantlets raised in polyhouse was 3% in Bhima Omkar and 4% in Bhima Purple. Although, garlic is infected by various other viruses such as Carla and Allexiviruses, but in the present study mericlones were tested for single group because potyviruses are widespread in garlic (Gawande *et al*., 2013; 2014). Due to high titer potyviruses have been used as indicator virus, this mean elimination of these viruses from infected mother bulbs simultaneously eliminates other viruses too. Earlier, Conci and Nome (1991) reported 50-51% virus free mericlones using a combination of thermo and chemotherapy. The better results in present study could be attributed to fidelity in excision of meristem and modifications in protocol. Meristem culture was found to eliminate all viruses including OYDV and LYSV in garlic (Taskin *et al*. 2013). Sugarcane Yellow Leaf Virus (SCYLV) was also effectively excluded using meristem tip culture (Madugula and Gali, 2017). Average virus elimination rate using *in vitro* meristem tip culture was 42.9% in apple cultivars cv. HF ans A123 (Hu *et al*., 2017).

Establishment of microbulbils developed *in vitro* was easier and 95% of Bhima Purple and 93% of Bhima Omkar germinated in net house (Table 4). However, the garlic bulb had a single bulb (without differentiated cloves) at the end of first production cycle (90 – 95 days). The clove separation started in second production cycle (90 – 95 days) in some bulbs. In the third production cycle, which was planted in the main field (110 – 120 days), 84% of Bhima Omkar and 89% of Bhima Purple plants produced a regular size of garlic bulb with more than two cloves. Maximum in Bhima Omkar 14 cloves per bulb (avg. 9.28 cloves per bulb) and Bhima Purple 17 cloves per bulb (avg. 12.24 cloves per

Table 3 : Effect of different sucrose concentrationson *in vitro* microbulbil induction and development in two garlic cultivars

*Medium + Sucrose	Bhima Omkar			Bhima Purple		
	#Days for microbulbils induction	Bulbils/ culture (No)	[1]Wt / bulbil (mg)	#Days for microbulbils induction	Bulbils/ culture (No)	[1]Wt / bulbil (mg)
3%	27.2[b]	3.11[c]	90[a]	27.5[b]	3.13[b]	112[a]
6%	24.4[a]	4.23[d]	122[d]	23.7[a]	5.39[c]	128[c]
7%	24.1[a]	4.61[d]	124[d]	23.2[a]	5.73[c]	130[c]
8%	26.8[b]	3.15[c]	118[c]	26.3[b]	3.56[b]	124[c]
9%	27.1[b]	2.17[d]	115[c]	28.6[b]	1.13[a]	129[c]
10%	28.8[b]	1.13[a]	109[b]	28.5[b]	1.74[a]	121[b]
11%	Dried out	--	--	--	--	--

*MS + 1 mgl-1Kin + Agar 0.8% + Sucrose; # 50 - 55 days old cultures were transferred for microbulbil induction; [1]Average weight recorded about 120 days of after initial culturing Mean values with different letters in a column differ significantly at $p < 0.05$ according to LSD

Table 4 : Field performance of microbulbils when planted *ex vitro* (production cycles in polyhouse followed by field transplanting) in two garlic cultivars

Parameters	Bhima Omkar				Bhima Purple			
	FG	SG	TG	OP	FG	SG	TG	OP
Germination (%)	93[a]	90[a]	90[a]	91[a]	95[a]	92[a]	94[a]	94[a]
Bulbs having more than two cloves (%)	-	25[a]	84[c]	96[c]	-	40[b]	89[c]	98[c]
#Highest number of cloves per bulb	1	5	14	13	1	9	17	18
*Avg. cloves/ bulbs	1[a]	3.12[b]	9.28[c]	11.7[c]	1[a]	4.24[b]	12.24[c]	13.36[c]
*Avg. wt/ bulbs (g)	1.06[a]	4.23[b]	17.13[c]	20.2[c]	1.29[a]	6.13[b]	22.26[d]	24.56[d]

#Mere representation of highest values without statistical analysis; * Average value of 20 – 30 bulbs; FG - First generation, SG -Second generation, TG -Third generation, OP – Original performance of garlic bulb when it is grown traditionally through planting a garlic clove in a field. Mean values with different letters in a row differ significantly at $p < 0.05$ according to LSD

bulb) were produced. Metawally *et al*. (2012) also reported non-differentiation of garlic into multiple cloves at first production cycle and normal growth in second and subsequent production cycles.

Thus, the present study proposes an efficient protocol for exclusion of viruses from virus infested planting material though *in vitro* meristem culture. The microbulbil induction protocol standardized in present study will enable easy handling of planting material and exclude the traditional cumbersome phase of acclimatization/hardening of *in vitro* raised platelets. Production of normal size garlic bulb in field multiplication from microbulbil has also been elaborated in this study.

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