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Rapid *in vitro* plant regeneration of *Allium Sativum*

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Abstract
Garlic (*Allium sativum)* is an important spice crop which is widely used for culinary and medicinal purposes. In this experimental work of *in vitro* micropropagation the protocol has been established for regeneration of garlic (*Allium sativum* L.) plants subjected to MS media with different concentrations of hormones. The study has reliable and reproducible protocols to get healthy and good quality plants from cloves as explants. Leaf discs and root tips were used for callus induction and plantlet regeneration. Satisfactory callus induction was observed in root tips inoculated in MS media supplemented with 2,4-D (1mg/l) + Kn (0.5mg/l). Excellent quality callus of root tips was seen in MS media supplemented with 2,4-D (1.5mg/l) + Kn (5mg/l) weighing 60.98 mg. Shoot regeneration was achieved via callus on MS medium supplemented with combination of 3mg/l BAP and 0.5mg/l NAA. The highest number of shoots that is 5 shoots / callus were observed when the culture is inoculated on to the MS media supplied with 3mg/l BAP and 0.5mg/l NAA. Regenerated plants were transferred on rooting medium. 2.5 mg/l IBA gives highest rooting percentage (80 %) in regenerated plants. *In-vitro* plantlets were planted in pots containing autoclaved mixture of soil, coco peat and vericompost (1:1:1) and grown in the green house for hardening. Higher survival rate (70 %) was observed when planted in field.

Keywords: *Allium sativum* L., callus, 2,4-D, Kn, regeneration

Introduction
Garlic (*Allium sativum)* is an aromatic monocotyledonous bulbous plant and an annual spice belonging to the subfamily *Allioideae* under family *Alliaceae* (Haider *et al*., 2015). It is used worldwide as a condiment and for its medicinal properties. Garlic is world's oldest cultivated crop. It is about 5000 years old indigenous herb having origin in central Asia (Kazakhstan), south Asia or southwestern Siberia. Garlic is closely related to the onion, shallot, leek, and chive. It is easy to grow and can be grown all year round. It is cultivated in temperate and tropical climates (Gaffar *et al.*, 2102). Garlic has a high amount of nutritional value also it has shown a variety of biological activities including antioxidant, anti microbial, cancer prevention, liver protection and reduction of cardiovascular disease risk factor (Khan *et al*., 2004). Garlic has high concentration of sulfur containing compounds, including allicin (first reported in 1994 by Cavallito and bailey) (thio-2-propene-1-sulfinic acid S-allyl ester) which was confirmed to be the main active component of garlic. It has wide range of biological and pharmacological activities, such as anticoagulation, antihypertensive, antimicrobial, antibiotic, antiparasitic, antimycotic, antiviral, antitumoral, anti-oxidant, anti-aging, antiplatelet, detoxifies heavy metals, fibrinolusis, hypolipidaemic (lipid-lowering) and immune enhancer and modulator (Mehta *et al*., 2013). The propagation of garlic is vegetative because of sexual sterility and it almost never produces fertile seeds. This mode of reproduction facilitates disease transfer (Novak *et al*., 1990). Also the propagation rate of garlic in the field is low, and therefore it takes many years to produce a sufficient number of seed bulbs for practical cultivation of a new variety. Many of the garlic cultivars are susceptible to diseases caused by viruses, nematodes, fungi and suffer from insect pests (Verbeek *et al*., 1995). *In vitro* regeneration or micro propagation is the best alternative to overcome these hurdles and it holds tremendous potential for rapid multiplication and production of high quality, uniform and disease free planting material (Bhojwani, 1980). This paper describes a method for the micro propagation of garlic using cloves and root tips explants in MS medium along with the supplementation of plant growth hormones.

Materials and Methods
The 2 varieties of garlic Bheema Omkar and Bheema Purple were obtained from ICAR-Directorate of Onion and Garlic Research, Rajgurunagar, Pune, Maharashtra. Garlic cloves were excised and used as explants for *in vitro* culture. The excised explants were initially surface sterilized with ethanol (70% v/v) for 30 sec and then dipped in 0.1% (m/v) aqueous mercuric chloride ($HgCl_2$) solution for 5 min and then rinsed 3 times with sterile double

distilled water. All this sterilization techniques were performed under laminar air-flow cabinet. The surface sterilized explants were inoculated into culture bottles with Murashige and Skoog (1962) medium (MS) (pH=5.8) containing 3% sucrose and solidified with 0.8% agar, supplemented with various concentrations of 6-Benzylaminopurine (BAP), and indole-3-butyric acid (IBA) either alone or with combination for shooting and rooting respectively. Different concentrations of the BAP and IBA hormone were used. The cultures were maintained at (25±2 ºC) temperature and a photoperiod of 16/8 light/dark with a light intensity of 2000 – 3000 lux in growth room. Further after the growth of shoots and roots the leaf discs and root tips were cut by the surgical blade and sub cultured on MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylamino purine (BAP), kinetin (Kn) hormones alone or in combination for 8 weeks for callus induction. Inoculated explants were incubated both in dark and light condition under controlled temperature. The number of explants forming calli was scored to calculate callus formation frequency after 5 - 6 weeks of culture. The regenerated calli of optimum size was removed aseptically and then cultured for shoot regeneration in MS medium supplemented with 1-naphthaleneacetic acid (NAA) and BAP. Sub culturing was carried out after 3 to 4 weeks of culture; the number of shoots per explants, shoots length, regeneration frequency, rooting percentage was recorded. Then the shoots were inoculated in IBA supplemented MS medium for proper root induction. The well rooted plantlet was removed from the media and was washed with double distilled water properly to get rid of agar sticking to the roots. These well shooted and rooted plantlets were transplanted into plastic cups containing autoclaved mixture of soil, coco peat and vericompost (1:1:1) and kept in the green house for hardening. Watering of plants was done at regular intervals and acclimatized plantlets were cultivated in the field.

Results and Discussion

The clove explants inoculated on MS medium containing BAP and IBA in the range of 1.0-2.0 mg/l showed appropriate and good growth of shoots and roots respectively. Callus formation was initiated from root tips explants on MS medium supplemented with different concentrations of BAP, 2, 4-D and Kn. The callus formation was not observed in MS medium without hormones. Difference in morphology was observed in callus growth on different growth hormones. We performed callus induction from two different methods. And from the results it was observed that the leaf disks showes slow or no induction of callus in both the hormone combinations 2,4-D + Kn and 2,4-D + BAP. Whereas satisfactory callus induction was observed in root tips inoculated on MS media supplemented with 2,4-D (1mg/l) + BAP (0.5mg/l) (Table 1 , Fig1D). Excellent quality callus of root tips was seen in MS media supplemented with (1.5mg/l) + Kn (5mg/l) (Table 1, Fig1D). Khan *et al*., 2004 and Robledo *et al*., 2000 also reported that good callus induction was observed using root tips in MS media supplemented with kinetin and 2,4-D. Creamish brown callus was subjected for shoot regeneration on new media supplemented with plant growth hormones.

Shoot regeneration was achieved via callus on MS medium supplemented with combination of BAP and NAA. Different concentrations of BAP and NAA were checked in media for achieving highest regeneration frequency and multiple shoots per callus. Shoot initiation was observed after 3 weeks of inoculated callus tissue on regeneration medium. The medium supplemented with 3mg/l BAP and 0.5mg/l NAA shows maximum regeneration frequency (70 %) with highest number of shoots per callus (5 shoots/callus) (Table 2, Fig. 1F) followed by medium supplemented by 2.0 mg/l BAP with 0.5 mg/l NAA shows regeneration frequency (60 %) with (4 shoots/callus) (Table 2, Fig F) . The maximum shoot length (6.9 cm) was also observed in MS medium fortified with 3.0 mg/l BAP with 0.5 mg/l NAA (Table 2). These results were corroborated with the findings of Kudou *et al*., 1995 and Choi *et al*., 1993 who reported that BAP was the most effective stimulator for shoot formation and increased percentage of shoot regeneration. The shoots were then subjected on MS media supplemented with IBA ranging from 1 to 3.0 mg/l. The maximum growth was seen in 2.5mg/l IBA having 8 roots/callus of about 2.7 cm (Table 3, Fig. 1G). Then further the well shooted and rooted plant was kept for hardening (Fig. 1H)

Table 1: Regeneration of callus from root tips

Hormone (mg/l)		Type of explants used	Callus	Colour of callus	Callus type	Weight of callus (mg)	Performance
2,4-D	Kn						
1.5	1.0	Root tips	observed	Creamish brown	Compact	31.45	++
1.5	2.0	Root tips	observed	Creamish brown	Compact	44.32	++
1.5	3.0	Root tips	observed	Creamish brown	Compact	49.22	++
1.5	4.0	Root tips	observed	Creamish brown	Friable	58.33	++++
1.5	**5.0**	**Root tips**	**observed**	**Creamish brown**	**Friable**	**60.98**	+++++

(+++++ - excellent, ++++ - very good, +++ - good, ++ - fair)

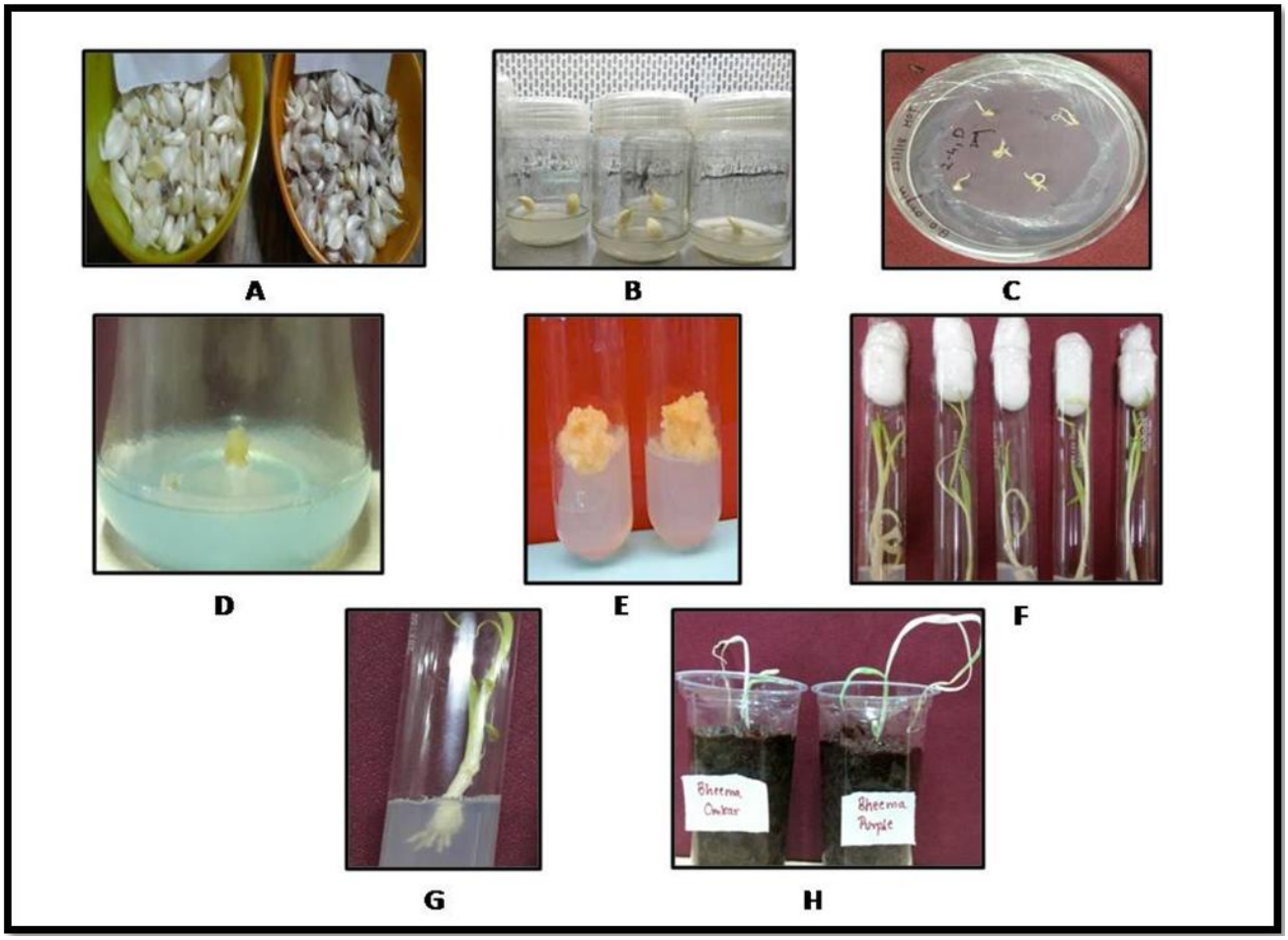
Table 2: Regeneration of shoots from callus

Hormone (mg/l)		Regeneration frequency (%)	No. of shoots per callus	Shoot length (cm)	Performance
BAP	NAA				
1.0	0.5	60	03	4.6	++
2.0	0.5	60	05	6.2	+++
3.0	**0.5**	**70**	**05**	**6.9**	++++
4.0	0.5	50	03	5.0	++
1.0	0.5	40	04	4.3	++
2.0	0.5	50	04	5 .1	++

(+++++ - excellent, ++++ - very good, +++ - good, ++ - fair)

Table 3: Regeneration of roots from callus

Hormone (mg/l) IBA	Regeneration frequency (%)	No. of roots per callus	Root length (cm)	Performance
1.0	50	05	1.0	++
1.5	60	06	1.4	++
2.0	70	07	2.0	+++
2.5	**80**	**08**	**2.7**	++++
3.0	50	05	1.3	++



(+++++ - excellent, ++++ - very good, +++ - good, ++ - fair)

Fig: *In-vitro* plant regeneration of *Allium sativum* – **(A)** 2 varieties of garlic Bheem Omkar and Bheema Purple **(B)** Cloves inoculated on MS media for root regeneration **(C)** Root tips inoculated on MS medium supplemented with 2,4-D + Kn, **(D)** Compact callus obtained on MS medium supplemented with 2,4-D(1.5mg/l) + Kn(3mg/l) **(E)** Friable creamish brown callus obtained on MS medium supplemented with 2,4-D(1.5mg/l) + Kn(5mg/l) **(F)** Shoots proliferation from callus on MS medium supplemented with BAP(3mg/l) + NAA(0.5mg/l) (G) Root proliferation from callus on MS medium supplemented with IBA(2.5 mg/l) **(H)** Primary hardening of both the varieties.

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