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Comparative effect of different pyhtohormones on the micropropagation of *Allium sativum*

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Abstract: The purpose of the study was to produce pathogen free plants of *Allium sativum* by micropropagation. To achieve the best results different plant growth regulators (6-Benzylaminopurine, Kinetin, α-Naphthaleneacetic acid, Indole-3-acetic acid, Indole-3-butyric acid) were used for shoot formation, root induction and bulb formation. Shoot meristem was used as explant. Among the different concentrations and combinations of phytohormones, 1.5mg/l kinetin was found optimal for both the shoot (93%) and root formation (70%).At optimal concentration, garlic plantlets with the maximum height of 15.4 cm and 5.7 roots per plant were obtained after 15 days. For the bulb formation different concentration of sucrose were tested and at a concentration of 12% a small, non-dividable, 1 cm, white and purple colored bulb was obtained after 1 month.

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INTRODUCTION

Allium sativum also called as garlic is a monocotyledonous spice plant and belongs to the onion family *Alliaceae.* It is an extensively used crop due to its culinary and medicinal uses[1]. The study of early documented history highlights the possible advantages of *Allium sativum,* as it was one of the plants which were used for the treatment of diseases in the beginning of recorded age[2,3]. Previous research studies have revealed the fact that garlic is involved in numerous health benefits i.e., lowers the cardiac disease risk factor, can act as an immunomodulator, anticancer agent, antioxidant agent and also involved in liver protection[4-6]. More than 2000 compounds having therapeutic properties are present in garlic[8] and among all concentrations of sulphur containing compounds are remarkably elevated. A raised level of allicin (sulphur compound) was confirmed in the root bulb of *Allium sativum* having numerous pharmaceutical and biological functions such as an immune enhancer and modulator, hypolipidaemic (lipid-lowering) role, fibrinolusis, detoxifies heavy metals, antiplatelet, anti-aging, antiparasitic, anti-oxidant, antitumoral, antimycotic, antiviral, antibiotic, antimicrobial, antihypertensive and anticoagulation[8,5,9].

Sexual sterility of garlic allows its extensive vegetative propagation[10]. However, there are some problems in the vegetative propagation of garlic like decreased multiplication rate and difficulties in flowering induction, which are the limiting factors in the progress of this crop[11]. Another drawback in vegetative breeding is that garlic is highly prone to many viral, fungal nematodes and insects attack[12,13]. All these difficulties have stimulated the advancement of numerous *in vitro* propagation protocols for improving multiplication rate and providing pathogen free garlic crops[14-20].

Several reports on *in vitro* propagation of garlic has been presented during some last years using different explants. To improve the health quality of garlic plants *in vitro* tissue culturing is an alternative beneficial approach. In this study, we have used shoot meristem with basal portion as a suitable explant for obtaining pathogen free garlic cloves *in vitro*.

MATERIALS AND METHODS

Surface sterilization and explant preparation

The cloves from a garlic bulb were separated and outer dry and papery leaves were removed. Now healthy cloves were selected for surface sterilization and culture inoculation.

Firstly, all the cloves were placed under running tap water for 15 min to remove all the dust particles. Then soaked in household detergent for 10 min and finally washed several times with tap water to remove all detergent content from the clove surfaces. These cloves were surface sterilized by the treatment with 70% ethanol for 3 min with continuous agitation. Following ethanol disinfection explants were immersed in 5% sodium hypochlorite (NaOCl) solution for 20 min with continuous shaking. Finally, for obtaining disinfectant free explant, all the cloves were washed three times with double distilled sterile water.

Sterile garlic cloves were transversely dissected under aseptic conditions to remove all the dome like tissue from shoot meristem. Now all the foliage leaves were excised one by one for obtaining shoot tip with basal stem disc of almost 1mm. These shoot tips were cultured as explant for shoot formation.

Culture conditions

For culturing, sterilized MS medium[21] pH 5 having 30g/l sucrose and 7g/l sucrose and fortified with different phytohormones was used. The temperature of the culture room was maintained at 25°C with a fluorescent light of 2500 lux under photoperiod of 16/8 hours.

Shoot induction

Well excised shoot tips were inoculated on MS medium containing varying concentrations of different phytohormones for shoot induction. Two different cytokinins (BAP and Kinetin) were used separately with concentrations ranging from 0.5-2.0 mg/l and also in combination with varying concentration of BAP (0.5-2.0 mg/l) and 0.25 mg/l kinetin. After 15 days optimum shoot length and number of days required for the shoot induction was recorded to analyze statistically.

Rooting

In vitro established shoots were shifted to MS medium supplemented with two different auxins (NAA and IAA) alone with concentrations ranging from 0.5-2.0 mg/l. Average number of roots were recorded for ANOVA after 15 days.

Bulb formation

Hormone free MS medium was used for bulb formation from *in vitro* developed garlic shoots. Healthy shoots of garlic was transplanted in MS medium with higher concentrations of sucrose ranging from 4%-16%. Optimum concentration for the bulbing was analyzed statistically.

Data analysis

For describing level of significance statistical analysis of the documented data was carried out using ANOVA and Duncan's multiple range test[22].

RESULTS AND DISCUSSION

For healthy and pathogen free shoot induction of garlic *in vitro* culturing technique (micropropagation) was adopted. To obtain shoots with optimum height and colour different cytokinins (BAP and Kinetin) alone and in combination with varying levels were tested (Table 1). Among all these culture media, MS medium having 1.5 mg/l of kinetin provided the most early (5 days) and maximum shoot length of 15.4 cm after 15 days with 93% survival rate (Table 1, Figure 1A). In our study kinetin was found as an ideal phytohormone for the shoot formation of garlic as it was noted that by increasing the concentration of kinetin to 1.5 mg/l in MS media significant increase in shoot length was observed but further increase in the concentration showed a decline in the shoot length (Table 1, Figure 1B). Findings of Sharma and Sharma[23] was also in favour of our study in this regard. They also described that among all cytokinins kinetin is the most appropriate shoot enhancer in garlic. Kim *et al.*[19], also supported the use kinetin for the shoot regeneration of garlic but at higher concentration (3.0 mg/l) as compared to our protocol and also in combination with 3.0 mg/l NAA. On the other hand Robledo-paz *et al.*[18], found that the combination of 4.6 mM kinetin and 4.5 mM 2,4-dichlorophenoxyacetic stimulated multiple shoot formation in garlic. BAP was also a potent phytohormone for shoot induction in garlic but the combination of BAP and kinetin was not as much suitable (Table 1).

For the rooting of *in vitro* grown plantlets different auxins were tried. Root induction was observed in almost all the compositions even in the hormone free full strength MS medium. But in all experiments response was quite delayed and number of roots were also significantly low (Table 2). In our study a cytokinin was proved more effective as compared to media fortified with different auxins because *in vitro* established garlic plantlets in MS medium containing kinetin started root formation along with the shoot formation in the same medium after 7-8 days of inoculation. Optimal response (70%) was obtained at 1.5 mg/l kinetin with 5.7 roots per plant after 15 days and by comparing all the results this response was considerably higher than all other auxins (Table 2, Figure 1C). In contrary to our results some reports have showed that for the rooting of garlic there is no need for any phytohormone, it can be easily achieved on hormone free MS medium[24,25]. However our results are strengthened by the study of Tapia[26] as he reported that for the best response of garlic a composition of MS medium containing Kinetin and IAA is ideal.

After rooting of plantlets the bulblet formation of garlic was tested by using different concentrations of sucrose (4-16%) in MS medium (Table 3). Best response (80%) was observed at 12% sucrose in MS medium with a small non divided bulb of 0.5 g in white and purple color. It was also noted that by shifting bulblet in liquid MS media containing 12% sucrose significant increase in size and weight was observed (Table 3, Figure D and E). Kim *et al.*[19], also described the same concentration of sucrose but in combination with 2.0 mg/l jasmonic acid. In our study only sucrose is for optimal bulb formation. Haque *et al.*[17], also supported our finding as they found heaviest bulblet on 12% sucrose and maximum number of bulblets (small sized) on 6% sucrose.

Table 1: Comparative Effect of different cytokinins on rate of shoot formation of *Allium sativum* after 15 days.

Cytokinins	Concentration (mg/l)	Days for shoot induction	Average shoot length After 15days (cm)	Rate of shoot formation (%)
BAP	0	9.2± 0.982^{a}	3.9± 0.982^{h}	67%
	0.5	5.9± 0.721^{defg}	5.4± 0.471^{fg}	77%
	1.0	5.2± 0.942^{fg}	8.1± 0.942^{d}	90%
	1.5	6.7± 0.982^{cde}	6.9± 0.981^{e}	80%
	2.0	6.9± 0.982^{cd}	5.8± 0.471^{f}	77%
Kinetin	0.5	6.5± 0.471^{cde}	11.6± 0.981^{c}	73%
	1.0	5.5± 0.545^{efg}	13.3± 0.981^{b}	77%
	1.5	5.0± 0.720^{g}	15.4± 0.720^{a}	93%
	2.0	6.3± 0.471^{def}	14.7± 0.981^{a}	77%
BAP + kinetin	0.5+0.25	8.2± 0.274^{b}	4.0± 0.720^{h}	50%
	1.0+0.25	7.9± 0.981^{bc}	4.6± 0.272^{gh}	57%
	1.5+0.25	7.0± 0.272^{bcd}	5.6± 0.720^{fg}	70%
	2.0+0.25	7.7± 0.720^{bc}	4.2± 0.471^{h}	57%

Table 2: Comparative effect of different Auxins and cytokinins on rate of root induction in *Allium sativum*

Phytohormones	Conc. (mg/l)	Days for root induction	No. of roots per culture	Rate of root formation
NAA	0.0	16.5± 1.186^{a}	1.0± 0.371^{e}	30%
	0.5	12.8± 0.981^{b}	1.7± 0.471^{de}	43%
	1.0	7.8± 0.720^{gh}	4.0± 0.274^{b}	67%
	1.5	8.7± 0.721^{efg}	3.0± 0.471^{bcd}	53%
	2.0	10± 0.274^{cd}	2.5± 0.471^{cd}	43%
IAA	0.5	7.5± 0.471^{h}	4.3± 0.942^{b}	60%
	1.0	8.6± 0.272^{fg}	3.3± 0.720^{bc}	57%
	1.5	9.8± 0.272^{cde}	2.3± 0.471^{cde}	47%
	2.0	10.8± 0.720^{c}	2.0± 0.371^{cde}	33%
Kinetin	0.5	12.1± 0.982^{b}	2.0± 0.471^{cde}	43%
	1.0	9.4± 0.471^{def}	3.0± 0.471^{bcd}	50%
	1.5	7.3± 0.471^{h}	5.7± 0.272^{a}	70%
	2.0	8.2± 0.721^{gh}	4.3± 0.942^{b}	60%

In conclusion an effective protocol for mass propagation of pathogen free *Allium sativum* has been established. Moreover, in this protocol *in vitro* bulblet formation further reduce the chances of pathogen attack on plantlets.

Table 3: Bulb formation of well-established *in vitro Allium sativum* plants.

No	Medium Composition	Days for bulb formation	Rate of plant survival
1	MS+4% sucrose	45.0±0.654^{a}	10%
2	MS+8% sucrose	39.3±0.334^{a}	45%
3	MS+12% sucrose	30±0.321^{b}	80%
4	MS+16% sucrose	40.2±0.272	51%

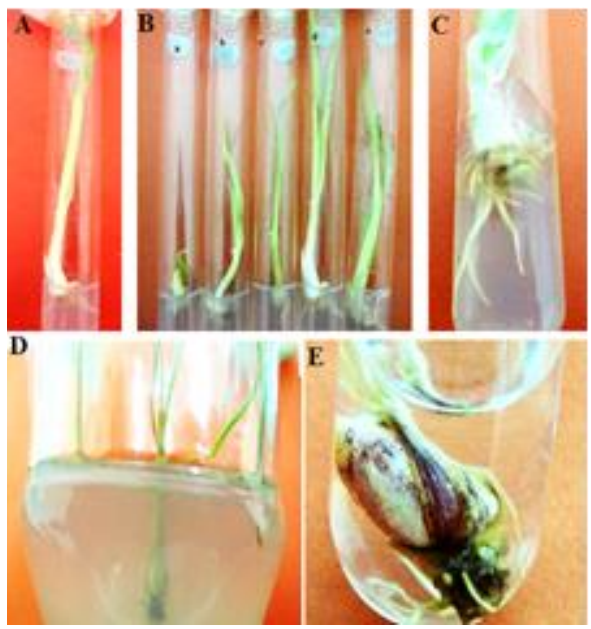

Figure 1: Different stages of pathogen free micropropagation of *Allium sativum*. (A) Shoot formation on MS medium supplemented with 1.5mg/l kinetin after 15 days of meristem inoculation, (B) Shoot formation at different concentrations of kinetin(a: MS media, b: 0.5mg/l , c: 1.0 mg/l , d: 1.5mg/l, e: 2.0mg/l), (C) Root formation of *in vitro* established *Allium sativum* plantlet inoculated on MS medium supplemented with 1.5mg/l kinetin, (D) Initiation of bulb formation in MS medium containing 12% sucrose after 20 days, (E) Significant increase in size of bulblet after subculturing in liquid MS medium containing 12% sucrose.

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