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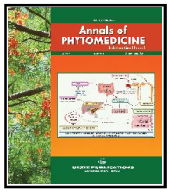

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Effect of plant growth regulators on *in vitro* regeneration of Onion (*Allium cepa* L. var. *aggregatum* Don.)

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Abstract

In vitro plant regeneration was achieved in Onion (*Allium cepa* L. var. *aggregatum* Don.) cv. CO (On.) 5. Basal plate explants were cultured on MS medium fortified with different concentrations of benzyl amino purine (BAP; 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l), naphthalene acetic acid (NAA; 0.2, 04, 0.6, 0.8, 1.0, 1.2 and 1.4 mg/l) either alone or in combination for multiple shoot induction. Pre-sterilization of the basal plate of onion in 0.5% solution of bavistin, followed by treatment with 0.1% mercuric chloride for 10 min produced the highest rate of survival of explants. Basal plate explants produced a greater number of shoots when cultured with MS medium containing different combinations of BAP and NAA. The highest rate of multiple shoot induction (12/explant) was observed under basal plate explants cultured in MS medium with 3.0 mg/l of BAP and 0.4 mg/l of NAA. Increasing BAP concentration up to 3.0 mg/l favored shoot initiation and multiplication, while shoot initiation and multiplication were decreased in MS medium containing BAP (4 mg/l). Shoots were allowed to root *in vitro* on MS medium containing IBA (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l) or NAA (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l). IBA was found to be superior to NAA for induction of root development *in vitro* in basal plate explants. Rooting of *in vitro* raised shoots was best induced on MS supplemented with 1 mg/l indole-3-butyric acid (IBA) with 10 roots per shoot and 3.10 cm root length. The rooted shoots were acclimatized with a success rate of 85%.

1. Introduction

Onion (*Allium cepa* L. var. *aggregatum* Don.) is economically important, most widely cultivated and consumed horticultural spicy vegetable belonging to the family Alliaceae. Onion is prized for its therapeutic uses and is common element in culinary preparations. Among onion growing countries, India ranks next to China and cultivated in about 1.6 million hectares (Rani and Singh, 2022). Onion productivity can be hindered by various factors including poor seed viability and dormancy in plantlets. These factors can limit the natural vegetative multiplication of onions in the open field, leading to lower productivity compared to other countries (Amit *et al*., 2023). Onions are typically propagated through seeds or sets, which involves a two-year cycle. However, it is important to mention that other plants in the *Allium* family can be propagated vegetatively. These plants have a high potential for regeneration both *in vivo* and *in vitro*. Although, traditional breeding and production methods have been extensively researched in India, *in vitro* studies on Indian genotypes are still in their early stages (Khar *et al*., 2005). Micropropagation as a promising tool is a viable alternative for production and continued supply of disease free quality planting materials to the farmers. Direct organogenesis approach is reliable and most often attempted way to regenerate plants under *in vitro* cultures. In the present study, attempts were made to optimize and establish a regeneration protocol through direct organogenesis in an elite onion cultivar CO (On) 5 through production of multiple shoots.

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2. Materials and Methods

2.1 Explants collection and sterilization of explants

The present study was conducted by the Plant Tissue Culture Laboratory of the Agricultural College and Research Institute, located at Eachangkottai, Thanjavur, Tamil Nadu from 2022 to 2023. A commercial grade of Onion CO (On) 5 bulbs was collected from the disease-free farmer's field in Perambalur district, Tamil Nadu, India. Medium-size bulbs were used for regeneration studies. Excised bulbs were treated with bavistin 0.5% for 10 min. Subsequently, sliced basal plates were sterilized with 70% ethanol for 30 sec, followed by mercuric chloride 0.1% for 5 min. The explants were washed with sterile water 3-4 times to remove excessive disinfectants.

2.2 Culture initiation and multiplication

Individual explants were cultured in MS (Murashige and Skoog, 1962) medium supplemented with various concentrations of BAP

(0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mg/l) and NAA (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 mg/l) either alone or in combination. The culture was incubated at approximately 25ºC with a 16 h photoperiod. The light source used for illumination was a 40 W white fluorescent light. This was followed by an 8 h dark period. The media was adjusted to pH of 5.8 for before autoclaving. The inoculated materials were placed in a culture room with a relative humidity of 80%. The shoots were initiated within 21 days of inoculation which shows the good response of explants to the medium. Multiple shoots were observed 8 weeks after inoculation. Subcultures were carried out every 14 days interval. Each experiment was replicated three times.

2.3 Root induction and hardening

Shoots were inoculated in MS medium contains various concentrations of IBA (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l) or NAA (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l) for root induction. Inoculated culture was maintained for four weeks to promote optimal growth. Rooted plantlets were delicately removed and washed thoroughly with distilled water. After obtaining the plantlets, they were transplanted into plastic pots filled with a mixture of autoclaved garden soil, farmyard manure, and sand in a ratio of 2:1:1. The plantlets were then kept under these conditions for a period of 4-5 days. Following this, the hardened plants were transferred to a glass house and kept there for 8-10 weeks to monitor their continued growth and development.

2.4 Data collection and analysis

During the experiment, several data points were meticulously recorded. These included the number of days needed for shoot emergence, the number of shoots generated per explants, the number of roots formed per shoot, the length of the roots and the survival rate of the plantlets. These measurements were taken at specific time intervals to monitor the progress of the regeneration process. To derive meaningful conclusions from the collected data, a comprehensive statistical analysis was performed. The collected data was analyzed using Analysis of Variance (ANOVA) with the assistance of SPSS Version 10 software (SPSS, Chicago, IL). Duncan's Multiple Range test (DMRT) was employed at a 5% probability level. This test allows for comparisons between treatments means to identify statistically significant variations. The resulting data were represented as mean ± Standard error (SE). This robust statistical analysis helped to validate the findings and draw reliable conclusions from the experimental results.

3. Results

In the present investigation, the focus was on the *in vitro* regeneration of Onion cv. CO (On) 5. Different concentrations of cytokinins and auxins were added to MS medium for the purpose of shoot initiation, multiplication and root induction. The protocol for this process was successfully standardized by using basal plates as explants.

3.1 Effect of BAP and NAA for shoot initiation and multiplication onion

In the present investigation, the basal plate was inoculated in an MS medium containing various concentrations of BAP (0.5-4.0 mg/l) and NAA (0.2-1.4 mg/l) with the aim of inducing multiple shoots (Table 1; Figure 1a and 1b). Among different plant growth regulators used for shoot initiation and multiplication, MS medium containing 3.0 mg/l BAP was significantly efficient for shoot initiation and produced higher multiple shoots (12 shoots/explant) followed by BAP 2.5 mg/l (7 shoots/explant) (Figures 1c and 1d), than other BAP and NAA combination tried for shoot initiation and multiplication. The observation from this study shows the effectiveness of BAP on shoot initiation over other PGRs. NAA exhibited the very minimal effect on shoot development from onion basal plates. The MS medium containing BAP raised up to 3.0 mg/l was favored for producing more multiple shoots induction then further increased the concentration of BAP reduced the number of multiple shoots. The present study also revealed that the increased concentration of BAP above 3.0 mg/l did not influence the production of higher multiple shoots induction in onion cv. CO (On.) 5. (Table 1). MS medium containing any concentration of NAA did not favor shoot induction and multiplication in onion. Further, increasing the concentration of both BAP and NAA also does not favor the production of multiple shoots in onion cv. CO (On.) 5. BAP 3.0 mg/l combined with 0.4 mg/l of NAA produced higher shooting regeneration then BAP alone. The present study revealed that the addition of low concentration of NAA along with BAP in shooting medium rapid the shoot development and produced higher shoots (12 shoots/explant) (Table 2). The study found that increasing the concentration of NAA above 0.4 mg/l in combination did not have a significant effect on shoot initiation and development. Additionally, it was observed that higher concentrations of NAA showed a lesser effect on shoot regeneration.

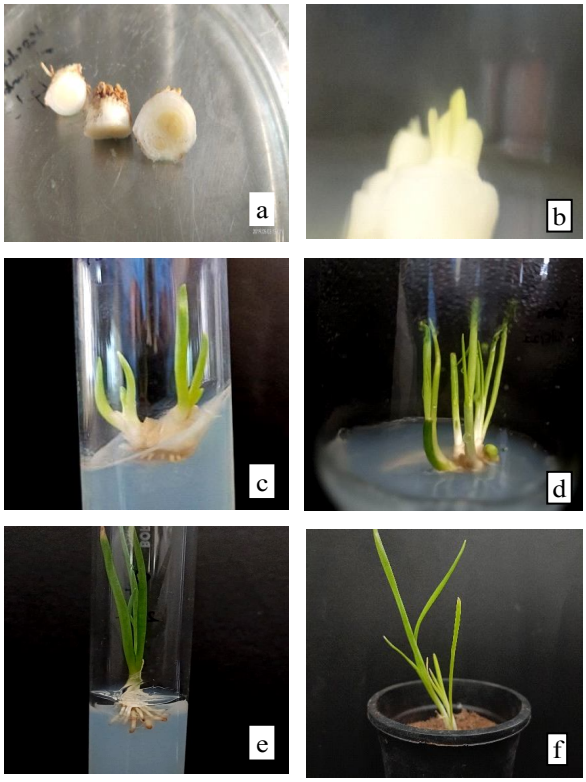

Figure 1: ***In vitro*** **regeneration of Onion cv. CO (On.) 5 using basal plate. a. Basal plate; b. Multiple shoot initiation; c. Multiple shoots induced from MS medium with 3.0 mg/l of BAP and 0.4 mg/l of NAA; d. Elongation of multiple shoots; e. Rooting was induced in MS supplemented with 1 mg/l IBA; f. Hardening of plants in plastic pot containing autoclaved garden soil, farmyard manure, and sand (2:1:1).**

Table 1: Effect of BAP and NAA either alone on shoot regeneration from basal plate explants of Onion cv. CO (On.) 5

Plant growth regulators		Number of shoots regenerated from explants*
BAP (mg/l)	NAA (mg/l)	
-	-	0.00 ± 0.00^{g}
0.5	-	4.00 ± 0.23^{cd}
1.0	-	4.00 ± 0.23^{cd}
1.5	-	5.00 ± 0.17^{c}
2.0	-	6.00 ± 0.11^{bc}
2.5	-	7.00 ± 0.08^{b}
3.0	-	9.00 ± 0.96^{a}
3.5	-	7.00 ± 0.56^{b}
4.0	-	6.00 ± 0.15^{bc}
-	0.2	3.00 ± 0.36^{d}
-	0.4	4.00 ± 0.41^{cd}
-	0.6	3.00 ± 0.64^{d}
-	0.8	2.00 ± 0.39^{e}
-	1.0	2.00 ± 0.44^{e}
-	1.2	2.00 ± 0.17^{e}
-	1.4	1.00 ± 0.04^{f}

* Means value within same column bearing different letters a, b, c, d is significantly different (p<0.05) on application of Duncan's Multiple Range test.

Table 2: Effect of BAP and NAA in combination on shoot regeneration from basal plate explants of Onion cv. CO (On.) 5

Plant growth regulators		Number of shoots regenerated from explants*
BAP (mg/l)	NAA (mg/l)	
-	-	0.00 ± 0.00^{e}
2.5	0.2	7.00 ± 0.45^{c}
3.0	0.2	8.00 ± 0.49^{bc}
3.5	0.2	8.00 ± 0.55^{bc}
4.0	0.2	7.00 ± 0.92^{c}
2.5	0.4	9.00 ± 0.88^{b}
3.0	0.4	12.00 ± 0.95^{a}
3.5	0.4	9.00 ± 0.66^{b}
4.0	0.4	8.00 ± 0.78^{bc}
2.5	0.6	7.00 ± 0.33^{c}
3.0	0.6	7.00 ± 0.35^{c}
3.5	0.6	6.00 ± 0.29^{cd}
4.0	0.6	6.00 ± 0.29^{cd}

* Means value within same column bearing different letters a, b, c, d is significantly different (p<0.05) on application of Duncan's Multiple Range test.

3.2 Effect of auxins for root induction in onion

In vitro root induction and root length are presented in Table 3 and Figure 1d. Root initiation was observed for a period of 14 days after transferring the shoots onto the MS basal medium. This medium contained various concentrations of IBA (0.5-3.0 mg/l) and NAA (0.5-3.0 mg/l). After three weeks of transferring the shoots to the rooting medium, the growth and development of roots were assessed. In the present study, it was observed that the addition of 1.0 mg/l of IBA to the basal media resulted in early and good rooting performance, with an average of 10.61 roots per shoot. Following that, the supplementation of 0.5 mg/l of IBA also showed a favorable rooting response, with an average of 9.12 roots per shoot. It was also found that a concentration of 1.0 mg/l IBA resulted in the maximum length of roots from shoots compared to any other concentration used. Further increased concentration of IBA above the 1.0 mg/l, it had a negative effect and did not significantly influence the production of the number of roots per shoot and root length. The shoots, which were cultured in a rooting medium, had an average root length of 3.10 cm (Table 3). The MS medium containing 3.0 mg/l of IBA produced only six roots from the shoot. The present study also revealed that the MS medium containing any concentration of NAA did not favor enhancing the production of roots as well as root length in Onion CV Co (On) 5. The medium supplemented with NAA, a concentration of 1.0 mg/l resulted in a higher number of roots per explant (8.53). Additionally, a concentration of 0.5 mg/l NAA led to a longer root length of 2.60 cm. (Table 3).

Table 3: Effect of IBA and NAA on root generation from regenerated shoots of Onion cv. CO (On.) 5

Plant growth regulators		Number of roots per explants	Root length(cm)*
IBA (mg/l)	NAA (mg/l)		
-	-	0.00 ± 0.00^{i}	0.00 ± 0.00^{f}
0.5	-	9.12 ± 0.34^{b}	2.70 ± 0.21^{b}
1.0	-	10.61 ± 0.82^{a}	3.10 ± 0.34^{a}
1.5	-	8.53 ± 0.32^{c}	2.90 ± 0.44^{ab}
2.0	-	7.68 ± 0.22^{df}	$2.90. \pm 0.82^{ab}$
2.5	-	7.68 ± 0.22^{df}	2.40 ± 0.63^{b}
3.0	-	6.11 ± 0.09^{h}	2.20 ± 0.37^{bc}
-	0.5	7.68 ± 0.46^{df}	2.60 ± 0.65^{ab}
-	1.0	8.53 ± 0.11^{c}	2.40 ± 0.78^{b}
-	1.5	7.11 ± 0.39^{f}	2.20 ± 0.45^{bc}
-	2.0	6.11 ± 0.29^{h}	2.00 ± 0.33^{c}
-	2.5	6.89 ± 0.65^{gh}	1.60 ± 0.29^{d}
-	3.0	6.11 ± 0.41^{h}	1.40 ± 0.18^{e}

* Means value within same column bearing different letters a, b, c, d is significantly different (p<0.05) on application of Duncan's Multiple Range test.

3.3 Hardening and field transfer

The well-developed roots of the plants were gently taken out from the test tube and the washed roots were then transferred to a plastic pot. The pot used for planting the regenerated plantlets contained a mixture of autoclaved garden soil, farmyard manure, and sand in a ratio of 2:1:1 (Figure 1e). The plantlets were successfully established in the field and displayed morphological traits that were similar to the mother plants. The reported success rate for this establishment process was 85%.

4. Discussion

BAP plays an efficient role in promoting the multiple shoots from the basal plates was observed from the current study. Dustan and Short (1978) reported that BAP 1.5 mg/l was superior for culture response, these results agree with the present findings on the primary establishment of shoot in culture. Similarly, higher shoot regeneration was obtained in 1.5 mg/l BAP and 2.0 mg/l glycine supplemented medium (Ramakrishnan *et al*., 2013). According to Malla *et al*. (2015), it was found that the *in vitro* bulb production response was observed when using MS medium supplemented with B5 vitamins and 2.0 mg/l of BAP. Similarly, the highest shoot formation was observed on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA (Marlin *et al.,* 2021). Similar to the previous findings, increasing the concentration of the BAP up to 3.0 mg/l positively correlated with the shoot initiation and further increasing the BAP concentration significantly reduces the development of shoot numbers (Shailaja *et al*., 2020).

It is indeed reported that the hormonal response of BAP and NAA for shoot development in different cultivars of onion has been studied (Pike and Yoo, 1990; Rodrigues *et al.,* 1996; Gems and Martinovitch, 1998; Kamastaityte and Stanys, 2004). According to the study conducted by Rodrigues *et al*. (1996), the highest multiplication rate in onion was reported using MS medium containing 2.0 mg/l of BAP and 0.25 mg/l of NAA. While Malla *et al*. (2015) used kinetin (0.5 mg/l) along with 0.5 mg/l of BAP and 0.1 mg/l NAA for regeneration. Pan *et al*. (2012) found that the onion variety 'Zaochunhuang' in the culture medium of MS + 6.0 mg/l 6-BA + 0.1 mg/l NAA had the best proliferation effect. The optimum hormone concentration for getting

high-frequency regeneration of shoot, with 78.13%, from callus was 1.0 mg/l thidiazuron (TDZ) on B5 medium (Wang *et al*., 2012). The highest *in vitro* shoot proliferation (20 shoots/explant) by direct organogenesis was obtained from NAA and BA supplemented BDS medium reported by Marinangeli (2013). According to present and previous experiments BAP was found to be an effective PGR for shoot initiation and BAP used along with lower level of other PGRs exhibited the enhanced shoot production.

The effectiveness of IBA on root initiation and development was described by earlier studies supported to result which obtained from current study produced highest number of roots (Boonerjee *et al*., 2006; Ramakrishnan *et al*., 2013; Deepak *et al*., 2015; Rani *et al*., 2021). And maximum root length was achieved in IBA supplemented medium. Similar to the current findings' highest percentage of shoot let produced root on MS medium containing 2.0 mg/l IBA for onion the cultivars (Taherpuri 90% and Indian 80%) (Boonerjee *et al*., 2006). NAA at any concentration produces a lower number of roots when compared to IBA were observed in this study. NAA at 1.0 mg/l produced the third most rooting response and further increasing the concentration of NAA did not show the effective rooting response. The present study revealed that IBA was more effective than NAA for root induction. In contrary to the present observations, NAA was found to be superior to IBA for better root induction (Panduang and Kanchanapoom, 1996; Rashid *et al*., 2009; Wang *et al*., 2012, Sharma *et al*., 2021; Selvaraj *et al*., 2022). According to the study conducted by Rashid *et al.* (2009), the highest percentage (100%) of root induction in the Taherpuri cultivar of onion was achieved using medium supplemented with 1.0 mg/l of NAA from *in vitro* raised shoots. The observations from this study also revealed that 1.0 mg/l of NAA has produced higher roots when comparing to other concentrations of NAA. All shoots were rooted on half MS medium with 0.01 mg/l NAA as reported by Wang *et al*. (2012). Ramakrishnan *et al*. (2013) studies shown the effective root induction was obtained in medium containing IBA after 2 weeks of culture. The addition of IBA has been observed to have an influence on and increase the formation of roots and shoots in onion (Zare *et al.*, 2023).

5. Conclusion

The present study successfully standardized an *in vitro* regeneration protocol for onion production using basal plates as explants. The highest rate of multiple shoot induction (12/explant) was achieved when basal plate explants were cultured in MS medium with 3.0 mg/l of BAP and 0.4 mg/l of NAA. Rooting of the *in vitro* raised shoots was best induced on MS medium supplemented with 1.0 mg/l IBA, resulting in 10 roots per shoot. Additionally, an 85% success rate was observed for the establishment of regenerated plantlets in field conditions. The standardized protocol for onion production through micropropagation has the potential to be highly helpful in increasing the number of plants produced.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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