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Callus Induction and Regeneration of Indigenous Garlic (*Allium sativum* L.)*

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Abstract: The experiment was conducted at plant Biotechnology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh to study the regeneration of garlic at various combinations and concentrations of growth regulators and also to develop an efficient protocol for regeneration of garlic via callus culture. Vigorously growing leaf discs of garlic strains were used as explants. The explants were collected from cloves germinated in a MS basal medium. Different combinations and concentrations of growth regulators like 2, 4-D, NAA and BA were used in MS medium to observe the callus induction, proliferation and organ formation. The highest callusing was recorded at the best concentration of 2, 4-D (1.0 mg L^{-1}) in MS medium (71.42%). Highest regeneration and highest number of shoots per callus were found in combinations and concentrations of 1.0 mg L^{-1} NAA plus 1.0 mg L^{-1} BA (71.42%).

Key words: Callus, regeneration, MS medium, 2, 4-D, BAP

INTRODUCTION

Garlic (*Allium sativum* L.) is a member of the Alliaceae family. The family is taxonomically intermediate between the Liliaceae and the Amaryllidaceae and was formerly included in the Amaryllidaceae (Collins, 1964). Asia is the primary center of origin and the Mediterranean region as the secondary center. Presently the major garlic producing countries are China, South Korea, Spain, India, Egypt, United States, Thailand and Turkey. China leads in garlic cultivation both in world production and acreage, 4.65 million tons and 5.57 million hectare respectively (Anonymous, 1998). Considering the total annual requirement (85000 mt), there exists a great deficit in garlic production (40000 mt). The yield rate of garlic is 7.9 t ha^{-1} in China, whereas in Bangladesh is only 3.80 t ha^{-1} (Anonymous, 2003). Locally, the cloves with their noses just below the soil surface are planted in winter and harvested in summer season.

Garlic (*Allium sativum* L.) is an important and widely cultivated plant with both culinary and medical uses stemming from its biological activities, which include anticancer (Chung *et al*., 2004), antibiotic, an antiviral agent, antithrombotic (Abdullah *et al*., 1989), cardiovascular diseases and diabetes (Collin, 2004), sickle cell anemia patients (Takasu *et al*., 2002). In obtaining a large quantity of regenerated shoots and secondary metabolites (Ziv and Shemesh, 1996) for commercial use.

Garlic is mainly propagated through vegetative means. Owing to the reported chromosomal irregularities occurring in this material, sexual reproduction is restricted. Further, during the evolutionary process some genetic modifications occurred, resulting in the flower production in general (Zhang *et al*., 1999). Therefore improvement of garlic production can be achieved through chromosomal manipulation and selecting the desirable variants e.g., large bulb size, number of cloves/bulb, storability etc. The alteration of chromosome constitute in a vegetative propagated plant like garlic can be made

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possible through *in vitro* techniques (Novak, 1990). The regeneration of plants from tissue culture is an important and essential component of biotechnological research and sometimes is required for the genetic manipulation of plants.

High frequency regeneration of plants from *in vitro* culture is a pre-requisite for successful application of tissue culture technique for crop improvement. More recently many plant breeders are getting interest in tissue culture techniques, especially in crops where sexual reproduction is relatively complicated or difficult. Considering the aspects mentioned above the present research program has been undertaken with the following objectives:

- To development *in vitro* callus induction regeneration protocol of indigenous garlic of Bangladesh.
- To compare the efficiency in optimum concentration of 2, 4-D and 2, 4-D with coconut water for *in vitro* callus induction of indigenous garlic variety.
- To compare the efficiency in optimum concentration of BA and NAA plus BA for *in vitro* regeneration of indigenous garlic variety.

MATERIALS AND METHODS

Leaf discs of local garlic variety were used as explants. Garlic was collected from elite farmer at Khulna region in Bangladesh. The cloves were washed 3 times with tap water and subsequently with distilled water and then immersed into 70% ethanol for one minute and washed thoroughly. The ethanol treated cloves were sterilized in 0.1% $HgCl_2$ solution for 5 min with agitation followed by 3-4 rinses in sterile water to remove trace of $HgCl_2$. The cloves were then cultured onto MS basal media (Murashige and Skoog, 1962). The explants were collected from cloves germinated of MS basal medium. Leaf disc from each germinated clove was separated by sterile scalpel. Each disc was divided into 3-4 pieces (0.2-0.3 cm) and then placed into the culture vessel.

The concentration of callus induction media were MS and supplemented with different concentration of 2, 4-D (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg L^{-1}) and MS and supplemented with different concentration of 2,4-D (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg L^{-1}) in combination with a constant 10% CW.

The concentration of regeneration media were a) MS and supplemented with different concentration of BA (0.0, 5.0, 10, 15, 20 mg L^{-1}) and MS and supplemented with different concentration of NAA (0.5, 1.0, 1.5, 2.0 mg L^{-1}) plus constant concentration of 1.0 mg L^{-1} BA.

Incubation

Cultures were incubated in dark (10 days) and then transferred to light conditions under controlled temperature (25±2°C). The photoperiod was maintained 16 h light intensity of 2000-3000 lux provided from white cool fluorescent tube. Observations were carried out daily to note the response.

Subculture

When the calli attained a convenient size they were removed aseptically from the culture vessels and placed on a sterilized petridish inside the laminar airflow cabinet. The calli were cut into small pieces and were placed into freshly prepared media with appropriate combinations and concentrations. These were again sub-cultured to freshly prepared medium containing different hormonal supplements BA and NAA plus BA for regeneration. The culture vessels showing signs of contamination were discarded. Repeated sub-culturing was done at 15 days interval.

Data Collection and Analysis

Seven replications were used per treatment for callus induction. Visual observation of culture was taken every week. Duration of callus induction and its color, texture was recorded after 8 weeks. Also seven replications were used per treatment for regeneration. Regeneration initiation, percentage were recorded after 6-7 weeks.

RESULTS AND DISCUSSION

A detailed investigation was conducted to examine the appropriate growth conditions that may help in callus induction, proliferation, plantlets regeneration in garlic.

Callus Induction

Response at Differences Concentration of 2, 4-D

Leaf discs of the garlic strains were cultured on MS medium supplemented with different concentration of 2, 4-D and callusing response was evaluated. Callusing from leaf discs of garlic was observed between 56-63 days which gives off white color and friable. The results indicated that concentration 1.0 mg L^{-1} of 2, 4-D showed highest percentage (71.42%) and degree of callus inductions after 56 days of leaf discs and of garlic (Fig. 1). It was evident from the experiment that callusing days increased with the increasing concentration of 2,4-D but decreased the degree of callus. MS medium supplemented with zero concentration of 2,4-D showed no response for callus induction (Table 1). These findings showed similarities to the finding of Khan *et al*. (2004), Haque *et al*. (2003) and Kodou *et al*. (1995) but duration of callus induction was different due to genotypic variation.

Response at Differences Combinations and Concentrations of 2, 4-D with 10% Coconut Water (CW)

Different concentrations of 2, 4-D in combination with CW were used in MS medium to observe the callus formation ability using leaf discs of garlic. Callusing from leaf discs of garlic was observed between 55-62 days which gives off white color and friable. It was observed that MS medium of callusing ability in leaf discs garlic (Fig. 2). It was evident from the experiment that callusing days

Table 1: The effect of concentrations of 2, 4-D on callus induction for local garlic (*Allium sativum* L.)

Auxin	Concentration (mg L^{-1})	No. of inoculation tube	No. of callus induction tube	Rate of callus induction (%)	Days to callus induction	Color	Degree of callus
2,4-D	0.0	14	-	-	-	-	-
	0.5	14	8	57.14	56	Off white	++
	1.0	14	10	71.42	56	Off white	+++
	1.5	14	6	42.85	60	Off white	++
	2.0	14	4	28.57	63	Off white	+
	2.5	14	2	14.28	63	Off white	+

+++ = Very good, ++ = Good, + = Poor, - = Indicate no result

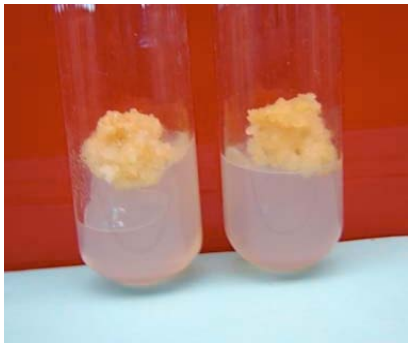

Fig. 1: Callus induction from leaf discs of indigenous garlic (*Allium sativm* L.) cultivar in MS medium supplemented with 2, 4-D (1.0 mg L^{-1})

Fig. 2: Callus induction from leaf discs of indigenous garlic (*Allium sativum* L.) variety in MS medium supplemented with 2, 4-D (1.0 mg L^{-1}) + 10% CW

Table 2: The effect of concentration of 2,4-D plus 10% coconut water on callus induction for local garlic (*Allium sativum* L.)

Auxin	Conc. of 2,4-D (mg L^{-1}) with 10% CW	No. of inoculation tube	No. of callus induction tube	Rate of callus induction (%)	Days of callusing	Color	Degree of callus
2,4-D + 0% CW	0.5	14	8	57.14	55	Off white	+++
	1.0	14	8	57.14	55	Off white	+++
	1.5	14	6	43.85	58	Off white	++
	2.0	14	4	28.57	60	Off white	+
	2.5	14	2	15.28	63	Off white	+
	3.0	14	-	-	-	-	-

+++ = Very good, ++ = Good, + = Poor, - = Indicate no result

Table 3: The effect of different concentration of BA on percent shoots induction from callus

Cytocinine	Conc. of BA (mg L^{-1})	No. of inoculation tube	No. of shooting induction tube	Rate of shoots induction (%)	Days of shoot initiation
BA	0.0	14	-	-	-
	5.0	14	2	14.28	55
	10.0	14	6	42.85	52
	15.0	14	2	14.28	54
	20.0	14	-	-	-

increased with the increasing concentration of 2,4-D plus fixed amount of coconut water but decreased the degree of callus. This result was not more efficiencies than 1.0 mg L^{-1} of 2, 4-D treatment producing callus (Table 2).

Maintenance of Calli

The calli were obtained from MS + 1.0 mg L^{-1} 2, 4-D and MS + 2, 4-D +10% CW; which are maintenance by subculture to applied similar concentrations. The cultured calli were proliferated within 18-24 days.

Regeneration of Plantlets

Response at Differences Concentration of BA

Different concentration of BA was used to observe the regenerating capacity of proliferated calli in MS medium. It was evident from the experiment that percent of shoot regeneration and number of shoots per callus increased with the increasing concentrations of BA up to 10 mg L^{-1} (Table 3). Highest regeneration and highest number of shoots per callus were found in 10 mg L^{-1} BA (Fig. 3).

Fig. 3: Shoot regeneration through callus, derived from garlic leaf disc in MS medium supplemented with 10 mg L^{-1} BA

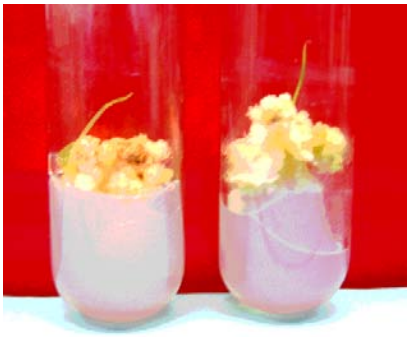

Fig. 4: Shoot regeneration through callus, derived from garlic leaf disc in MS medium supplemented with combinations concentrations of NAA plus BA

Table 4: The effect of different concentration of NAA plus constant concentration of 1.0 mg L^{-1} BA on percent shoots formation from callus

Cytocinine	Conc. of NAA (mg L^{-1}) with 1.0 mg L^{-1} BA	No. of inoculation tube	No. of shooting induction tube	Rate of shoots induction (%)	Days of shoot initiation
NAA+BA	0.0	14	-	-	-
	0.5	14	6	42.85	55
	1.0	14	10	71.42	52
	1.5	14	8	57.14	54
	2.0	14	4	28.57	53

Response at Different Combinations and Concentrations of NAA with Plus BA

Different concentrations of NAA in combination with a constant BA were used to observe the shoot formation ability in MS medium using callus derived from garlic leaf discs. MS medium supplemented with 1.0 mg L^{-1} NAA plus 1.0 mg L^{-1} BA exhibited with the highest (71.42%) regenerating ability (Fig. 4 and Table 4). This result was more efficiencies than 10 mg L^{-1} of BA treatment. These findings were in agreement with that of Haque *et al.* (2003), Seabrook (1994) and Suh and Park (1995).

It was worth noting that plant regeneration depended on diverse factors including the genotypes, hormone supplements and so on. Statistical analysis revealed that significant variation was presented

among varieties, hormonal concentrations and their interaction for percent shoot regeneration and days required for shoot regeneration and number of shoots per callus. In the present study, we observed that use of BA helped in shoot regeneration, this result corroborated with the findings of Kodou *et al*. (1995) who reported that BA was the most effective stimulator for shoot formation and increased percentage of shoot regeneration.

A number of treatment of 2,4-D and its combination with 10% coconut water were used for callus induction from leaf discs of indigenous garlic (*Allium sativum* L.) variety. The callus was started 53-57 days after incubation. It was noticed that MS media supplemented with 1.0 mg L^{-1} produced highest (71.42%) callus for indigenous garlic variety. It was also observed that MS medium supplemented with 2, 4-D (0.5 mg L^{-1}) + 10% CW exhibited 57% callusing ability in leaf discs garlic. The color of all callus were off white and the texture of those friable. The calli were obtained from MS + 1.0 mg L^{-1} 2, 4-D and MS + 2, 4-D +10% CW; which are maintenance by subculture to applied similar concentrations. The cultured calli were proliferated within 18-24 days. *In vitro* produced calli were transferred to the regeneration media containing MS supplemented with BA and NAA plus fixed amount of BA. It was evident from the experiment that MS medium supplemented with 1.0 mg L^{-1} NAA plus 1.0 mg L^{-1} BA exhibited with the highest (71.42%) regenerating ability. It was worth noting that plant regeneration depended on diverse factors including the genotypes, hormone supplements and so on.

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