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Effects of genotypes, explants and plant growth regulators on *In vitro* callogenesis of garlic (*Allium satium* L)

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Abstract

Garlic (*Allium satium* L.*)* is categorized as an important medicinal plant worldwide which was known for its very high therapeutic value. Several biotic and abiotic factors may affect its sustainable yield and quality. Viral diseases of garlic Asexual mode of propagation may cause of spread of viral diseases in garlic which may be eliminated through *in vitro* techniques. Therefore, research was carried out to determine effect of different sterilization methods, carbon source and explant on *in vitro* production of disease-free garlic. The highest numbers of clean cultures were obtained using treatment combination of 70% ethanol 1 min^{-1} and 55% Sodium Hypochlorite (NaOCl) 20 $minutes^{-1}$. All other treatments either fail to produce clear cultures or reduced the efficiency of germination. Sugar and maltose were used as carbon source and no significant variation was observed among them during germination of cloves Two commercial varieties NARC G1 and White garlic were used as source of explant. Calli mass were assessed after interval of every 10-15 days and subsequently sub-cultured. Data was obtained after 60 days. Both accessions produced maximum callus (59.73% and 58.39% respectively) when used shoots were used explant. The calli were yellowish white in hue and had a dense and nodular morphology. Different concentrations of 2, 4-D showed significant variations in the average frequency of induced calli, germinated shoots explant of NARC G1 variety of garlic produced the highest calli at plant growth regulator concentration of 3.0 mg L^{-1} of 2,4-D. The result of present studies may provide a source of disease-free material for genetic manipulation of garlic genotypes.

Keywords: Accessions. Disease free plants, 2-4,-D, Sterilization

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Introduction

Garlic (*Allium sativum* L.*)* is a monocot, edible bulbous crop in the Alliaceae family that has long been cultivated. It was originated in Central Asia based on historical and archaeological evidence (Endalew et al., 2021). Garlic has been used as a medical herb and as a spice and condiment in many different cultures (Ahmada et al., 2021). Each garlic variety has its own characteristics in terms of clove size, smell, and taste. Commercially grown garlic is diploid with (2n=16) and has various accession that varies in the number of cloves per bulb, the color and size of bulb and storage duration (Shahzad et al., 2019). The bulb and leaves are mostly used as an edible part. Each bulb has four to twenty cloves that are separated by thin white or indigo covering and grouped by membranous skin (Stewart, 2018). In garlic breeding, mutations have been induced for clonal selection. The cloves are asexually propagated and its yield is affected by variety, agronomic practices, environmental conditions, soil texture and fertility level (Sun et al*.,* 2020). Garlic improvement through conventional breeding approach was difficult due to sterile flowers and vegetative propagation (Benke et al., 2019). Establishment of *In vitro* protocol for garlic improvement was the potential alternative. It was a rapid propagation technique to produce virus free plants through somatic embryogenesis (Toor et al., 2021).

The main objective of this study was to optimize various steps involved in the *in vitro* propagation in commercial garlic varieties named "NARC G1" and "White Garlic " by using explants such as garlic cloves and *in vitro* germinated shoot leaves.

Materials and Methods

The research work was carried out at the Center of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture Faisalabad during the year 2020-2021. Different varieties of garlic (*Allium satium L.*) were compared for their callus induction and regeneration potential. Cloves of two varieties "NARC G-1" and "White garlic" were collected from Ayub Agricultural Research Institute (AARI). Bulbs were initially selected based on their physical appearance, only healthy and disease-free bulbs were chosen for *in vitro* propagation.

Sterilization of cloves

Mature cloves of both varieties of garlic were washed and skin was removed before sterilization. To avoid contamination cloves were surface sterilized in laminar air flow hood. Cloves of both varieties were surface sterilized by using different treatment combinations of ethanol (50, 70 and 90 percent) and Sodium hypochlorite (NaOCl, 50%, 55% and 60%) as describe in Table 1. Exposure time for each treatment combination was kept constant. Tween 20 was used as surfactant and after placing the cloves in sterilizing solution they were continuously stirred to improve the sterilization efficiency. Cloves were continuously stirred to increase the efficiency of sterilization. Cloves were thoroughly washed with autoclaved d_3H_2O to remove traces of sterilizing agents.

Cloves were dried on autoclaved filter paper to remove excessive moisture. They were sterilized, after 20 min of drying the cloves and were placed on MS (Murashige and Skoog, 1962) media. Two types of explants were used which include cloves and leaf discs and propagated on simple MS medium.

Media preparation

Commercially available ready to use MS-media mix containing all macro and micro salts was used for *In vitro* culture. 4.33 g L^{-1} of MS salt was added in autoclaved distilled water. MS salts were completely dissolved in water 10 ml vitamin and 10ml iron from the stock solution were added. For solidification of media agar, 9g/L of media was used. After sterilization of cloves and MS media, one clove test $tube^{-1}$ was placed for germination. Tubes were placed in growth chamber where temperature was maintained continuously at 25±2˚C, fluorescent light emitting 3000 Lux for 16 h light period was used.

In this experiment different types of carbon sources were used to study their influence on callus induction. Sugar and maltose (30g each) were used as two different treatments.

Table 1. List of treatment combinations used for sterilization

Code #	Pre-sterilization		Sterilization	
	Disinfectant	**Exposure time(min)**	**Active compound**	**Exposure time (min)**
1A	50%ethanol	1	NaOCl 50%	20
2A	50%ethanol	1	NaOCl 55%	20
3A	50%ethanol	1	NaOCl 60%	20
1B	70%ethanol	1	NaOCl 50%	20
2B	70%ethanol	1	NaOCl 55%	20
3B	70%ethanol	1	NaOCl 60%	20
1C	90%ethanol	1	NaOCl 50%	20
2C	90%ethanol	1	NaOCl 55%	20
3C	90%ethanol	1	NaOCl 60%	20

Preparation of 2, 4-D stock solution

For preparation of 50ml stock solution, pH of water was maintained at > 10 using 1M, NaOH. 50 mg of 2, 4-D was mixed in 1ml of ethanol using vortex mixer. Dissolved 2, 4-D was added drop by drop in water with basic pH. After mixing 2, 4-D the volume of solution was made upto 50 ml.

The effect of carbon sources on callus induction was evaluated by using sugar and maltose (30g each) in MS media. Variable concentration of 2,4-D (0, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L) was used to determine the callus induction %. 9g L^{-1} of agar was added before adjusting solution through volumetric flask. Media was poured in petri plates (10 ml $plate^{-1}$). Petri dishes were wrapped properly and autoclaved (Temperature 121ºC and pressure was maintained at15 psi for 20 minutes) for sterilization. After autoclaving media was placed in growth room for one week.

Culturing of explant for germination

The cloves as well as germinated leaves of both varieties of garlic were cut into 2cm

pieces and placed on petri plates using laminar air flow cabinet. Petri plates were incubated at 25˚C in dark.

Plant regeneration

Friable and healthy calli were transferred to plant regeneration medium (MS medium having pH 5.8 with various combinations and concentrations of the two growth hormones i.e., 6-Benzylaminopurine (BAP) as cytokinin and Indole-3-acetic acid (IAA) as auxin for shoot and root regeneration. An ambient temperature of 25 ± 1°C with a 16/8 h photoperiod was maintained in growth room. The cultures were sub-cultured after every four weeks to fresh medium for avoidance of any contamination.

Data Analysis

Data were collected for callus growth parameters on regular intervals. Callus initiation frequency was recorded and measured under aseptic conditions using sterile petri plates at different intervals. Data were recorded for callus induction frequency of both varieties "NARC G-1" and "White Garlic" after 2, 4, 6 weeks.

The experiment was carried out under controlled environment in a completely randomized design (CRD) using three replications. Variation among treatment means was evaluated using analysis of variance and the difference between means were recorded using Duncan's Multiple Range Test (DMRT). All analyses was computed using statistical package of SPSS.

Results

Effect of sterilization protocol in callus induction:

Nine treatment combinations were tested to optimize the sterilization process. 16 explants of each combination were inoculated on MS medium to check the contamination and growth efficiency. The efficacy of sterilization procedure was evaluated based on number of clean cultures obtained and their germination. 95% cultures were free from contamination and germination efficiency of explants was also maximum as shown in Fig. 1.

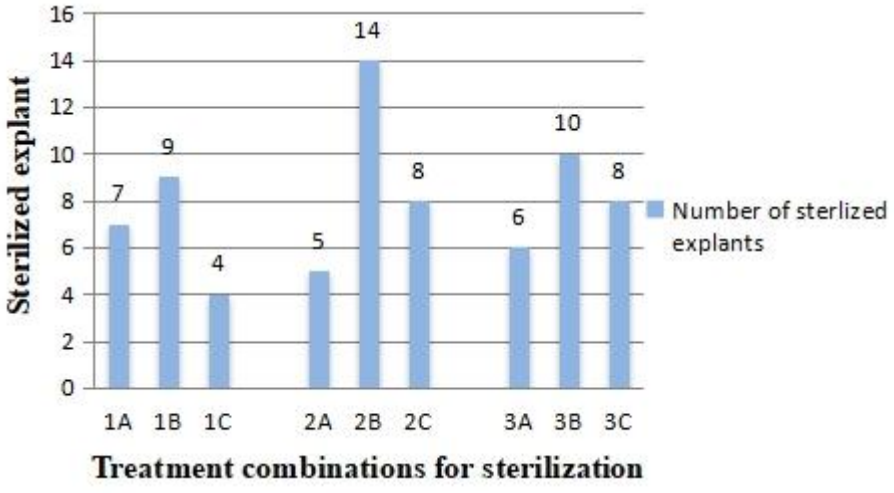

Figure 1. Growth of both varieties of garlic on various sterilization treatments

Effect of different carbon sources on germination

Commercially available sugar and maltose were used as carbon source. Garlic cloves were cultured on MS medium without growth regulator. Shoots and roots started to regenerate in "NARC-G1" after 2 days while "White Garlic" shoots started to regenerate after 10 days. No difference was observed based on variation in carbon source as shown in Fig. 2. Initially small protuberance appeared on the nodal part of cloves. The highest shoot and root development the cloves were examined at regular interval of 2 days. The highest shoots and roots germinated were observed in NARC-G1 at the end of first week. White garlic performance was quite slow, shoot initiation was delayed till end of first week, maximum shoots and roots were grown in 3 weeks as shown in Fig. 3.

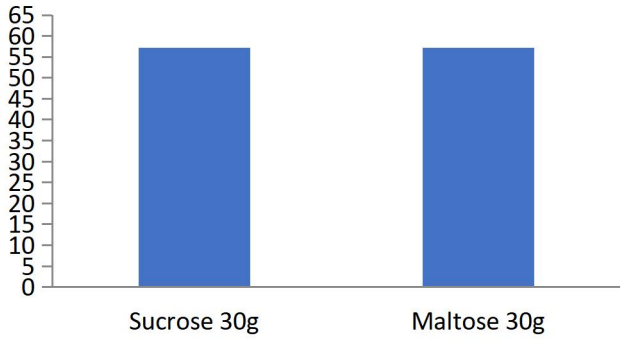

Figure 2. Effect of sucrose and maltose on culturing of explant

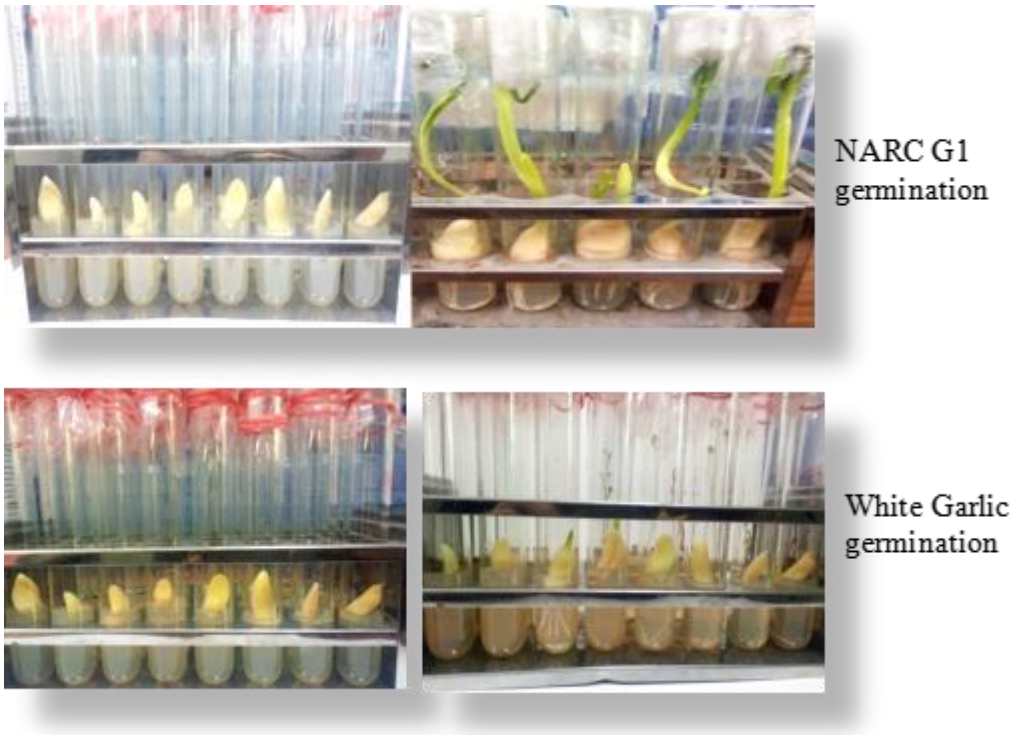

Figure 3. *In vitro* growth of explant of "NARC G1" and "White Garlic"

Effect of various concentration of 2, 4-D on callus induction

Cloves and *In vitro* regenerated shoots were used as explant for callus induction. The callus was initiated within one week after inoculation with various doses of 2, 4-D. There was no callus development in the absence of 2,4-D. Callus was elicited at all concentration. However, its frequency and proliferation varied depending on the concentration.

Callus started to appear after two weeks as edges of the explants swell. The calli obtained at 3.0 mg L^{-1} were compact and nodular in morphology and yellowish white in color as shown in Fig. 4. The highest callus induction frequency was obtained from clove explant was 52.85% and from shoot explants was 59.73% at 3.0 mg L^{-1} concentration of 2,4-D as shown in Table 2. The sub-culturing was carried out every 10-15 days. Higher or lower concentration of 3.0 mg L^{-1} 2,4-D reduced callus mass, size, and frequency of fresh weight. Results showed that that the best callus induction was obtained from germinated shoots of NARC G1 variety (Table 2).

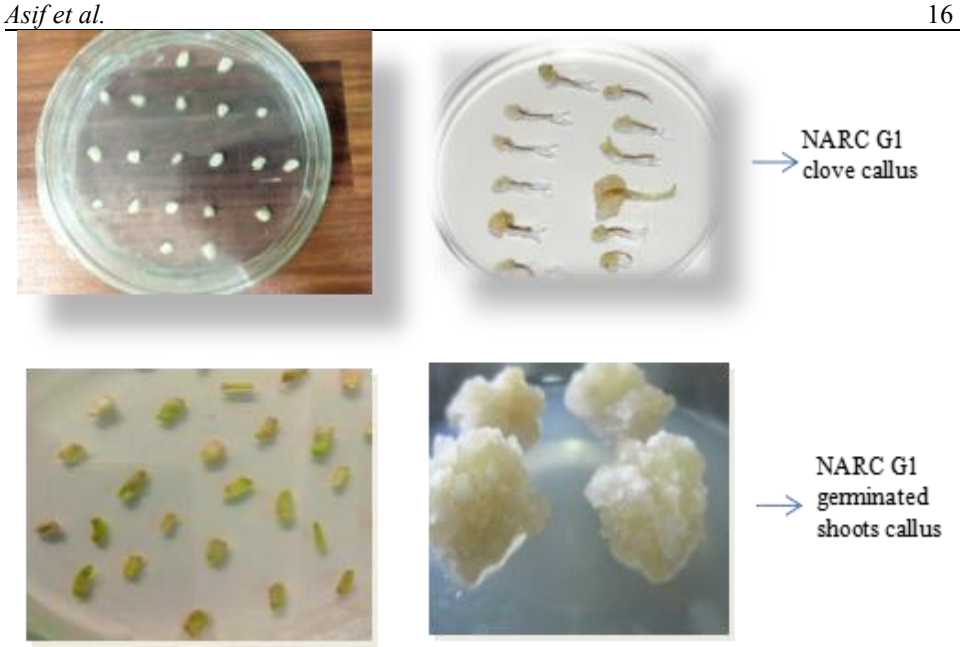

Figure 4. Callus induction after 60 days of NARC G1 cultured in MS medium at 3.0 mg L^{-1} 2,4-D

The callus of "White garlic" was initiated after two weeks inoculation with various doses of 2, 4-D. The concentration of 3.0 mg L^{-1} for callus inductionwas shown to be optimal (Table 3). The calli obtained were compact and nodular in morphology and yellowish white in color as shown in Fig. 5. Calli mass was observed under sterile conditions. Calli mass was assessed, data were collected after 60 days. The frequency of maximum callus obtained from clove explant was 42.15% and from shoot explants was 58.39%. All other concentrations showed reduced callus mass. The highest callus induction may be obtained from germinated shoots of "White Garlic".

The figure 5 describe the optimum result of the designed research. The highest callus was induced from the NARC-G1 variety using *in-vitro* germinated shoots as an explant with MS medium having 2, 4-D dose of 3.0 mg L^{-1}. Plant growth regulator (PGR) concentration produced compact, nodular and yellowish white disease-free callus. The percentage of callus induced was 59.73% within 60 days under sterile conditions. The results showed that accessions variation, source of explant and PGR Concentration influenced the amount and type of callus produced.

Table 2. Effect of various concentration of 2,4-D on callus induction in NARC G-1

NARC G-1 from clove explant				
Concentration mg/L	% of explant induced callus	Days to induce callus (frequency %)	Callus morphology	Degree of callus initiation
Control (0.0)	---	---	---	---
1	37.14	56	Compact yellowish white	++
2	41.42	56	Compact, nodular, yellowish white	++
3	52.85	60	Compact, white	+++
4	28.57	63	White	++
5	21.28	63	White	+
NARC-G1 from germinated shoots explant				
Control (0.0)	---	---	---	---
1	35.37	56	Compact yellowish white	++
2	43.52	56	Compact, nodular, yellowish white	++
3	59.73	60	Compact, white	+++
4	32.47	63	White	++
5	28.31	63	White	+

No Callogenic Response; +: 10-20 g; ++: 20-40 g; +++: 40-60 g; ++++: 60-80 g

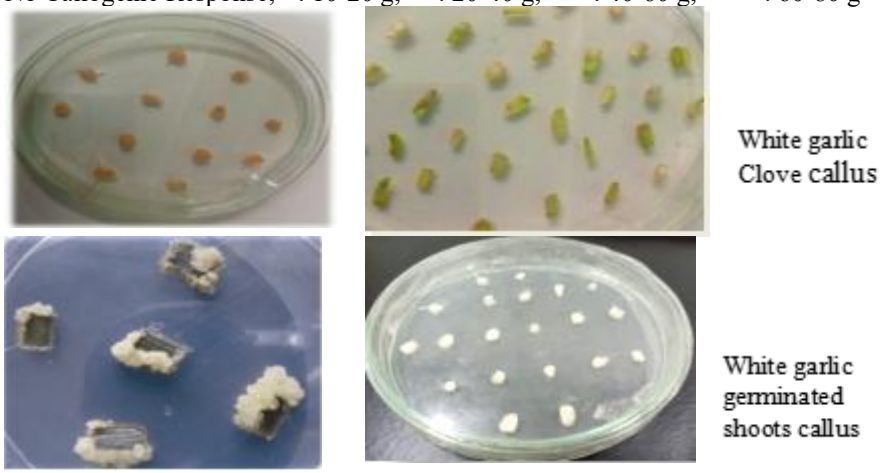

Figure 4: Callus induction after 60 days of White garlic cultured in MS medium at 3.0mg/L 2,4-D

Table 3. Effect of various concentration of 2,4-D on callus induction in White Garlic

White Garlic from clove explant				
Concentration mg/L	**% of explant induced callus**	**Days to induce callus (frequency %)**	**Callus morphology**	**Degree of callus initiation**
Control (0.0)	---	---	---	---
1	31.13	56	Compact yellowish white	++
2	39.63	56	Compact, nodular, yellowish white	++
3	42.15	60	Compact, white	+++
4	14.26	63	White	++
5	11.59	63	White	+
White Garlic from shoot explant				
Control (0.0)	---	---	---	---
1	39.19	56	Compact yellowish white	++
2	43.52	56	Compact, nodular, yellowish white	++
3	58.39	60	Compact, white	+++
4	34.71	63	White	++
5	23.18	63	White	+

No Callogenic Response; +: 10-20 g; ++: 20-40 g; +++: 40-60 g; ++++: 60-80 g

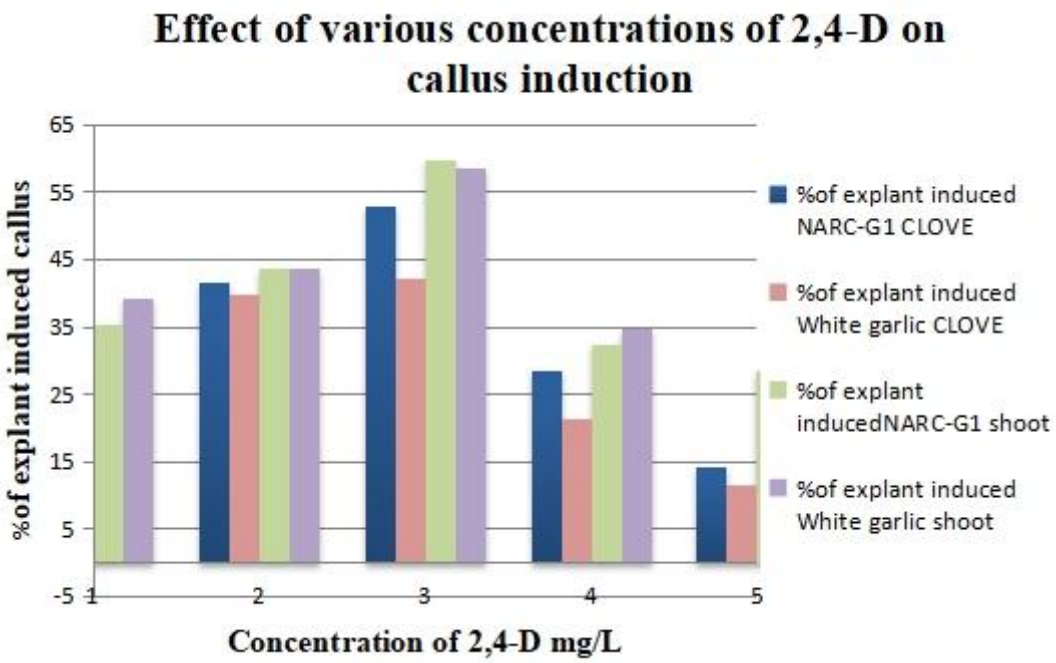

Figure 5. Effect of various concentrations of 2,4-D on callus induction

Discussion

The physiological state of plant, seasonal clock, nature of tissue used, genotype and the nutritional components of the medium all play a role in invitro callogenesis. The hormonal balance in the tissue culture medium has a major impact on differentiation at each stage of growth. The creation of an effective regeneration mechanism is critical for improving garlic characteristics. The designed experiment was carried out in order to discover a highly efficient, cost-effective, and high-yielding callus induction strategy in garlic plants in order to increase the output and mass of garlic cloves.

Type and concentration of phytohormone determine the path explant will follow (Metwally *et al*., 2014). Auxins and cytokinins may be used alone or in combination for somatic embryogenesis or direct organogenesis depending on the concentration of hormones (Moosavian *et al.*, 2020). Growth hormones and the kind of medium both have a role in callus induction and subsequent regeneration. 2,4-D, commonly known as synthetic acid, was used in the current study for callogenesis. It is also a broad-spectrum weedicide used to eliminate weeds and undesirable herbs, this herbicide was originally utilized for callus induction (Zahm *et al*., 1990). The selection of explant sources with the ability to regenerate into a full plant is a prerequisite for effective crop advances using modern biotechnology approaches (Zhang *et al*., 2010). In this study, 2,4-D concentrations were examined to determine their effects on callus production. (Yan *et al*., 2009). Various explant sources can be used in garlic to produce callus, each with varying

potential for callogenesis (Salam *et al.*, 2010). Findings of our research are inline with previous observations, Callus may be used as a source of variation in case of asexually propagated plants for genetic improvement (Parrano *et al*., 2012), this mass of undifferentiated tissue lack cellular connections and rapid multiplication rate do not allow the pathogenic organism to establish /multiply in these tissues. Calli may be considered as a disease-free source for plant propagation (Lu *et al*., 2021).

Genotypic effect is dominant factor that determine plants response toward callogenesis (Luciani *et al*., 2006) our findings are in agreement with previous findings as both varieties response was different toward different treatment combinations. Optimal 2,4-D induced callogenesis was observed in NARC G1 garlic variety using the germinated leaf as the explant. Increase or decrease in concentration of hormone from 3.0 mg/L resulted decrease in callus induction frequency of explant below 59.73 percent. It was found that the minimum calli mass was produced in white garlic genotype when cloves were used as explant, 5 mg/L 2,4-D concentration. In the present investigation, young garlic leaves were found to be best for callus induction. The difference was not only in the callus induction frequency but also quality of callus produced. It was revealed that there is no callus induction in the medium when 2,4-D is not present.

Development of efficient callus induction protocol may help conserve biodiversity in endangered landraces of garlic. Somatic embryogenesis/ callogenesis may prove to be a useful source to create variation in asexually propagated plants. *In vitro* techniques provide healthy and disease-free source material for propagation.

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