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Optimizing of Polyploidization by *In-Vitro* methods for genetic improvements of Garlic (*Allium sativum L.*)

Pamela Sinha, Rajesh Kannan, and D Ganesh*.

Department of Biotechnology, Madurai Kamaraj University, Tamil Nadu,625021 India

ABSTRACT

In adverse environment, the physiological as well as biochemical flexibility enable polyploid plants to possess stronger tolerance and viability than the diploid progenitors or relative species, especially for the vegetatively propagated or perennial plants. In crop breeding strategies, widely polyploid breeding is adopted. Garlic explants were obtained from in vitro grown diploid (2n = 16) plantlets. Shoots and roots were regenerated by culturing the explants on MS basal medium with two cytokinins BAP and KN at varying concentration ranging from 1.0- 5.0 mg/l with eight different combinations. In addition to that different concentration of colchicine 0.1%, 0.25%, 0.50%, 0.75%, 1.0% and 2.0% were supplemented in the medium with selective concentration of BAP at 0.1mg/L then the root-tips of the regenerated shoots were sampled for count of chromosome number. Compare to the roots of treated explant (with colchicine) thicker shoots with more root branches then in control plants. Polyploidization was found to be induced by colchicine and the chromosomes doubling was achieved by different concentration of colchicine. Genomic DNA analysis have been done, were it was observed that plantlets obtained through in vitro polyploidization had more number of nucleic acids than normal one. This paper gives an overview of the progress in the methods of polyploidization and the development of garlic breeding technology for the coming years.

Keywords: Garlic, colchicine, Polyploidization, cytology, Nano drop technique.

Corresponding author

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INTRODUCTION

Garlic (*Allium sativum L*.) is one of the most important vegetable crops belongs to the family Alliaceae. The prominent genus of the *Allium* includes onions, shallots and leeks. The commercial types of garlic can be divided into four categories such as (1) violet or Asian, which is cultivated insubtropical regions, (2) pink, which needs long photoperiods and has low requirements for cold, (3) white, which needs longs periods, has medium to high requirements for cold, and (4) purple, which needs long photo periods and periods of cold [1]. They can be classified into hard-neck and soft-neck garlic.Hard-neck garlic forms a floral scape whose flowers normally abort and produces top sets, while soft necked garlic does not form a scape. The majority of garlic cultivated for commercial purposes is soft- neck type because it is easier to cultivate besides its long shelf life [2]. Garlic is grown all over the world from temperate to subtropical climates [3].Garlic prefer to grow in a soil containing high organic content under a wide range of soil condition and pH levels.

The diploid number of common garlic is (2n=16) with a karyotypic and 6 acrocentric chromosomes as reported. Tissue culture provides a useful technique for eliminating virus from the infected plantlets and for producing virus-free garlic plantlets and to improve the efficiency of plant micro- propagation utilizing apical meristem or shoot tip explants [4].Garlic is used for both culinary and medicinal purposes because of its ability to improve the taste of food and its biological activities that include antibiotics, antitumor, cholesterol lowering and antithrombic effects on animal cells. Sexual propagation of garlic is highly impossible due to failure in production of fertile seeds. Thus, nearly all of the garlic in cultivation is propagated asexually by planting individual cloves in the ground. Cultivated garlic is a vegetative propagated plant due to its sexual sterility and about 10% of the harvest crop is stored and utilized as the source of propagule for subsequent cultivation [5].

Garlic is characterized by pungent, spicy flavour that mellows and sweetens the processed foods. Garlic is used for cardiovascular associated problems in human as it reduces the accumulation of cholesterol on the vascular walls of animals and humans [6]. Medical properties of garlic showed that garlic extract inhibit vascular calcification in human patients having high blood cholesterol [7]. It is also reported that garlic is highly useful inregulation of blood sugar level. Regular use of garlic extracts lower blood homocysteine levels and prevents diabetes. It can be used as a disinfectant because of its bacteriostatic and bactericidal properties [8].

Garlic is grown globally, but china is by far the largest producer of garlic with 10.5 million tonnes grown annually, accounting for over 77% of world production. India's share in the world market is only 4.1 %, followed by South Korea (2%), Egypt and Russia (1.6%) and the United Statesin sixth place (1.4%).India has been exporting garlic for many years. Major importing countries of Indian garlic have been Qatar, Saudi Arabia, UAE, Kuwait, Bangladesh and Sri Lanka. The export has been 2-3% of the total production but fluctuating due to frequent and sudden change in the policy of garlic- importing countries.

Development of polyploids is one of the important activities in genetic improvement of any crop plants. Development of polyploidy in garlic (*Allium sativum L.*) was reported in china. Polyploidization is one of the important tools as alternative approaches for genetic improvement of plant species. In the present study, systematic experiments were carried out to generate polyploidy lines of *Allium sativum L.*

MATERIALS AND METHODS

Source of the material

Healthy and fresh garlic cloves of local variety were collected from the farmer's field at Kodaikanal, Dindugal District, Tamil Nadu. The underground parts of the garlic were thoroughly washed in order to remove the soil adhering on the cloves as well as to reduce the microbial contamination. These samples were stored and used as a source of explants for the present study.

Preparation of Explants

Clumps of garlic bulbs were restored from the storage and separated into individual bulb before thoroughly washing under running tap water for 5-10 mints. The out most layer of the bulbs were carefully peeled and surface sterilized under aseptic condition with 0.1% $HgCl_2$ for 5-8 mints, depending upon the

nature and maturity of the garlic bulbs. Disinfected bulbs were thoroughly rinsed with sterile distilled water atleast 4-5 times in order to remove the traces of mercury. Each bulbwas taken out and chopped using a sterile surgical blade. The size of the explant was measuring about 0.5cm length with intact meristem. These segments were used as explants for optimization of culture conditions as well as for induction of polyploids.

Medium and Culture Condition

The culture medium used for the present work includes Murashige and Skoog's (1962) medium (MS) Supplemented with sucrose (3%). The medium was further augmented with two commonly used cytokinins BAP and KN at varying concentration ranging from 1.0-5.0 mg/l with eight different combinations. In addition, different concentration of colchicine (0.1%, 0.25%, 0.05%, 0.75%, 1.0%, 1.5% and 2.0%) were supplemented in the medium with a selective concentration of BAP at 0.1 mg/l. The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar (Hi-media, India). All the chemicals used in the present study were of analytical grade (British Drug House, Sigma, Merck and Hi-media). Molten medium was dispensed into culture tubes before sterilization at 121^{o}Cfor 20min. Explants were implanted vertically on the culture medium and incubated at 25+ 1^{o}C under 16 hours photoperiod. The number of explants cultured in each treatment was 20.However, the final observation with regard to the response of the explant was varying based on the recovery of the explants.

Optimization of colchicine treatment

Garlic explants were inoculated on MS liquid medium supplemented with various concentrations of colchicine such as 0.1%, 0.25%, 0.50%, 0.75%, 1.0%, 1.5%and 2%. Cultures were loaded onto the orbital shaker with a gentle agitation at 120rpm and maintained overnight. These explants were restored from the liquid medium and explants were transferred to MS solid medium supplemented with BAP (0.1 mg/l) but without any colchicine. Cultures were incubated at 25+1^{o}C at 16 hours photoperiod under white florescent light for one month. The final observation on the response of explants with regard to shoot regeneration was carried out and analysed in order to identify the optimal concentration of colchicine for induction of polyploids in garlic.

Optimization of hormonal concentration for shoot regeneration

Processed explants were cultured on MS medium supplemented with BAP and KN at various concentrations ranging from 1.0 mg/l- 5.0 mg/l either alone or in combination. Cultures were incubated at 25+1^{o}C at 16 hours photoperiod under white florescent light for one month. The final observation on the response of explants with regard to shoot regeneration was carried out after the total culture period of 30 days. Responses with regard to shoot regeneration was observed and recorded. Based on the above experiments, one of the concentrations of colchicine (1%) and with an optimal concentration of KN (1.0mg/l) BAP (5.0 mg/l) was chosen and routinely used for inducing polyploidization.

Restoration of polyploidy lines

Plantlets grown on the medium supplemented with colchicine (1%) and KN (1.0mg/l) BAP (5.0 mg/l) were taken out from the culture vessels and grown on semi- sterile substrate (vermiculite) for further growth and development. Plantlets with adequate shoot and root system weremaintained at 25+1^{o}C at 16 hours photoperiod under white florescent light with frequent watering. Control plants (plants without colchicine treatment) were also maintained in order to compare the growth pattern of colchicine treated and normal plants.

Preparation of samples for cytological observations

Plantlets regenerated from the colchicine treated explants were uprooted and the roots were washed with sterile distilled water. Actively growing root tips measuring 1-1.5cm length were randomly collected for processing the samples for cytological observation. Actively growing root tip segments were pre-treated with 2mM 8-hydroxyquinoline for 20 hrs. These segments were fixed in Carnoy fixative (95% ethanol; acetic acid 3:1) for 24 hrs. Then hydrolysed with 1N HCL and kept it in hot water bath at 60^{o}C for 11 mints and then dissociated in 45% of acetic for 1 hour. The treated root tips were gently squashed and stained with fleugen

staining before making the observation under the microscope. Root tip of untreated plants were served as control (Figure: 1)

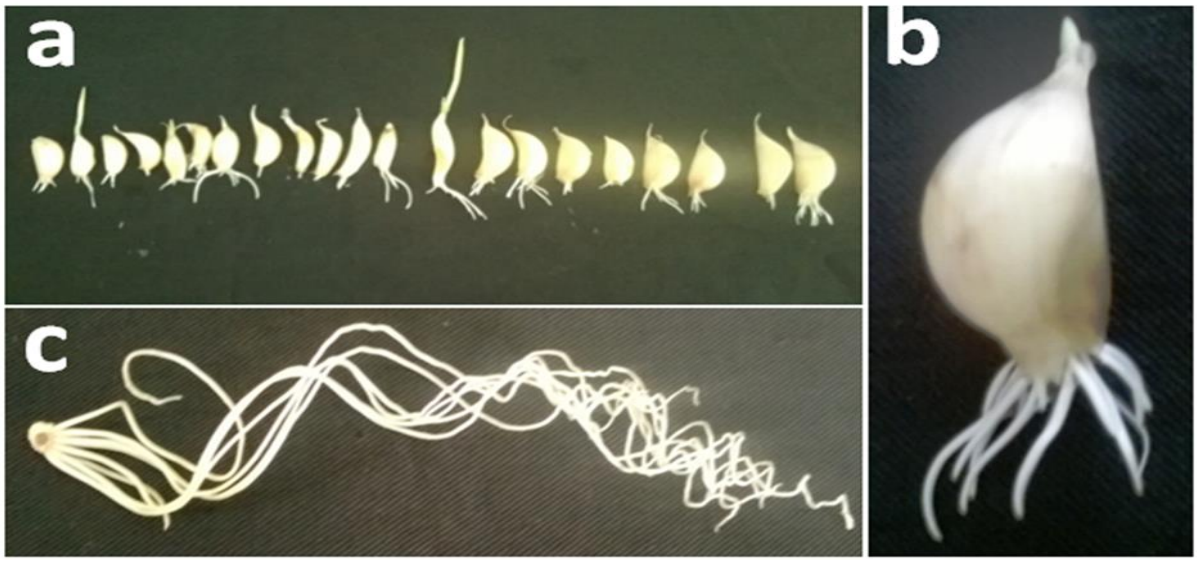

Fig 1: Induction of root from garlic bulb, a, b) initiation of root from the bulb of garlic for cytological studies; c) long root developed by the explants of colchicine treated bulb.

Isolation of genomic DNA

Genomic DNA was isolated from fresh young leaves using a modified CATB method. About 0.1g of frozen leaf tissue of garlic was ground into fine powder in liquid N_2using sterile/chilled mortar and pestle. The powder samples were then transferred to centrifuge tube containing 1ml of extraction buffer. The tubes were incubated at 60^{o}Cfor 1 hr. and samples were cooled to the room temperature and equal volumes of chloroform and isoamylalchol (24:1) were added and gently mixed to form an emulsion. Sample was centrifuged into a new tube gently without disturbing the debris at bottom layer. The supernatant was extracted once again with chloroform and isoamyl alcohol in order to improve the quality of DNA by removing the contaminants like carbohydrates and protein and subjected to another round of centrifugation at 12000 rpm for 10mint at 4^{o}C. The supernatant was taken out and precipitated with 2/3 volume of the isopropanol and incubated the samples at (-20^{o}C) for an hour.

Determination of DNA quantity by agarose gel electrophoresis

Equal volume of DNA samples (10µl) was added to 0.8% agarose gel in order to determine the quantity of DNA approximately. The difference between normal and polyploidy lines of garlic with respect to overall quantity of DNA was determined based on the thickness of DNA bands in the agarose gel.

DNA quantification

Genomic DNA isolated from the leaf tissues of polyploidy lines and control plants were quantified by dropping 1µl of DNA sample on Nano drop and the quantity of DNA from the polyploidy lines were compared with normal plant.

RESULT AND DISCUSSION

Garlic is one of the most important spice crops in agricultural community. Production of garlic is completely relaying upon asexual method of propagation and thus cultivation of this crop is wholly depends on the availability of high quality bulbs. Since propagation of seeds is a well- known crop either as medicinal and vegetable crops in many developing countries including India. The leading garlic producers in the world include Africa, Asia, North and Central America, South America and other smaller parts of the world [9].

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Genetic improvement of garlic is totally relaying upon the natural selection of superior and high yielding clones. Despite of its high degree of genetic variability, genetic improvement of this precious crop seriously hampered due to non-availability of superior clones in the natural population [10]. Most of the reports on the characterization of garlic were based on the vegetative and reproductive parameters. This characteristic feature includes bulb colour, bulb size, number of bulbs per plant etc. Lack of seed production in these crop demands the intervention of biotechnological approaches for genetic improvement of garlic of the various approaches for genetic improvement of garlic. Polyploids are generally classified either as allo or auto polyploids depends on the mode of origin or degree of divergence between the parental genomes involved.

In the present study, a different concentration of colchicine ranging from 0.1 to 2% was used in the liquid media for exposing the garlic bulbs to colchicine. Fortunately dissected garlic bulb did not show any phytotoxic effect against colchicine treatment. These could be possibly due to larger size of explant coupled with inherent ability of garlic tissue to withstand against colchicine. High incident of bacterial and fungal contamination was commonly observed during the initiation of culture. The recovery of explants were ranging from 40 to 73% in various concentration of colchicine used. In both control as well as treated explants with colchicine, healthy plantlets were obtained (Figure: 2).

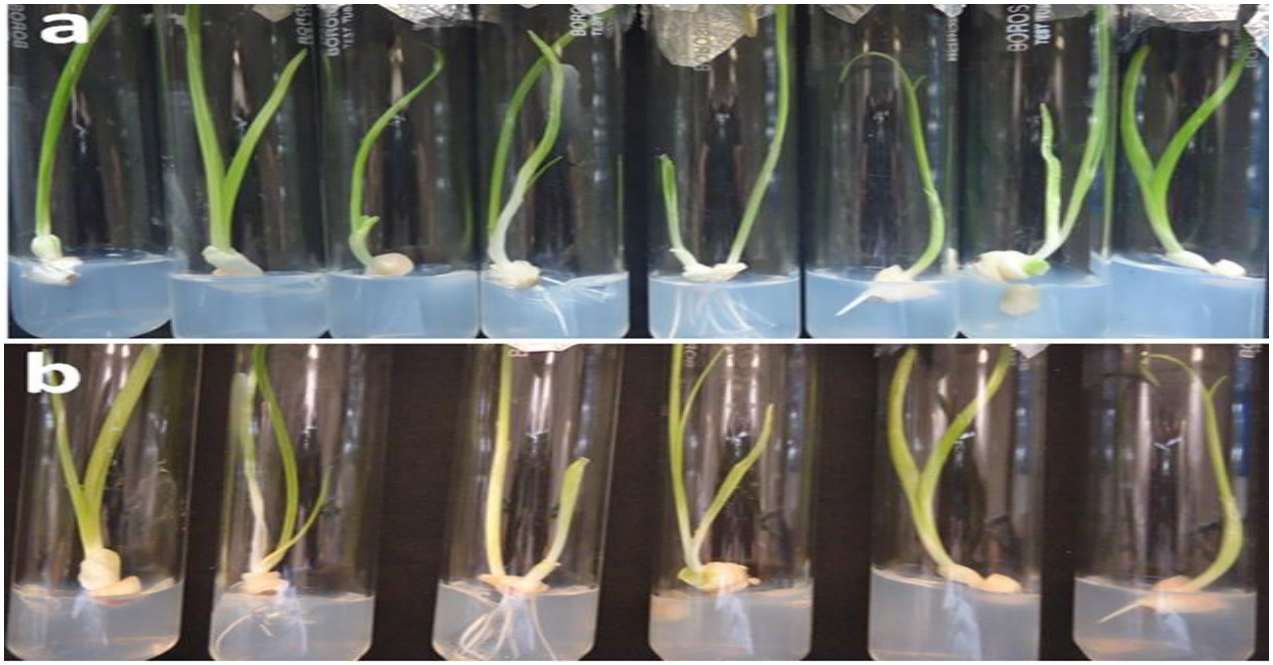

Fig 2: Regeneration of plantlets: a0 colchicine treated explants producing healthy shoot and root system on MS medium supplemented with BAP (0.1 mg/l); b) Untreated explant showing shoot and root development in presence of BAP (0.1 mg/l)

The response of the culture was also varying from 37.5 to 77%. Pre- treatment of explant with colchicine under liquid media did not cause any browning of tissue even after transferring them to solid media. Cultures grown under colchicine had developed healthy micro shoot with long shoot and root systems. It was observed that all the bulbs had produces many adventitious roots with varying length. From these experiments it was inferred that the high concentration of colchicine (1to 2%) can be used for inducing polyploids in garlic. In general the response of the tissue was faster as evidences by the development of healthy plantlets within one month of culture.

Experiments carried out with 9 different combinations of growthregulators involving two cytokinins, namely BAP and KN had produced interesting result with regard to shoot initiation. Plantlets cultured in different combination of media had induced multiple shoots ranging from 2 to 8 depends upon the hormonal combinations used. It was observed that explant cultured on the media supplemented with KN (1.0 mg/l) and BAP (5.0 mg/l) had developedmaximum number of shoots (7.6 shoot/explant) contrastingly, explant cultured on the media supplemented with KN (1.0 mg/l) had developed only 2 shoot/explant. Interestingly, explant cultured on the basal medium also developed about 7 shoots/explant. This could be possibly due to the

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influence of endogenous growth regulator present in the tissue. The length of the roots obtain in various combination was ranging from 0.5 to 3.5 cm (Figure 3 and Graph 1).

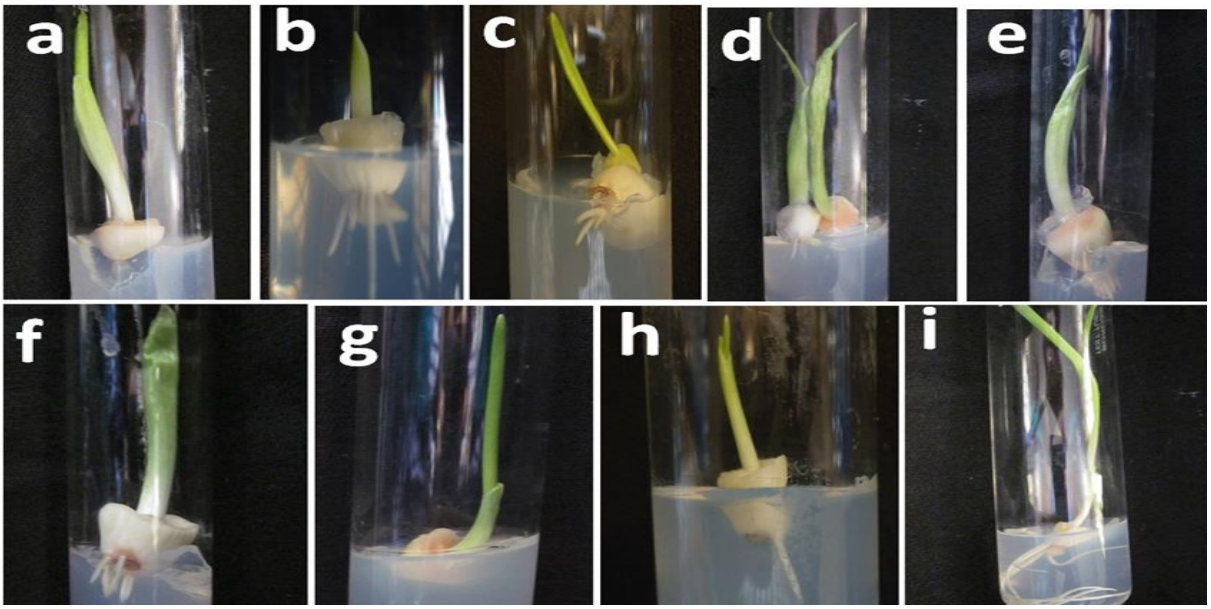
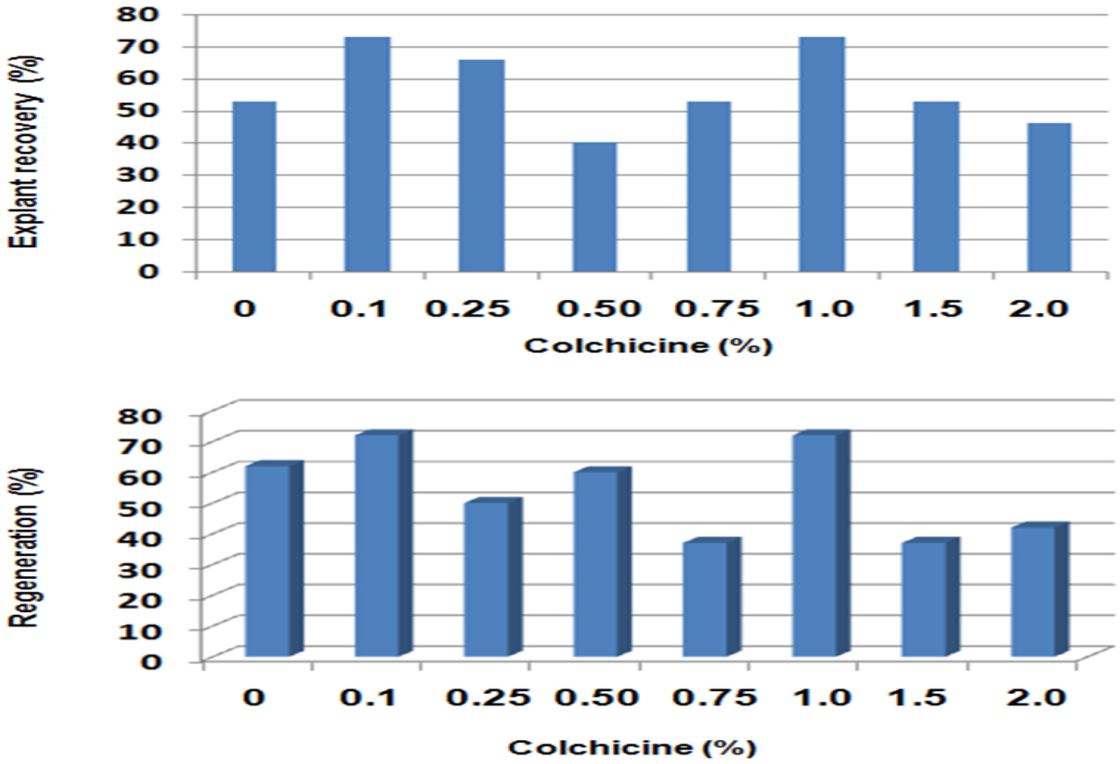


Fig 3: Development of plantlets in different combinations of BAP and KN: a) single long shoot development in MS basal medium, b) regeneration of shoots in presence of BAP (1 mg/l); c) development of lean and lanky shoots on MS medium with KN (1 mg/l); d) stunted shoot growth in medium supplemented with KN (1 mg/l) and BAP (2.5 mg/l); e) healthy shoot development in presence of KN (2.5 mg/l) and BAP (1 mg/l); f) response of shoots on MS medium containing KN (1 mg/l) and BAP (5 mg/l); g) shoot development in medium containing KN (5 mg/l) and BAP (1 mg/l); h, i) healthy shoot development in higher concentration of BAP and KN.



Graph 1: Response of explants in different concentration of BAP. Varying percentage of explants recovery and regeneration of plantlets was shown separately.

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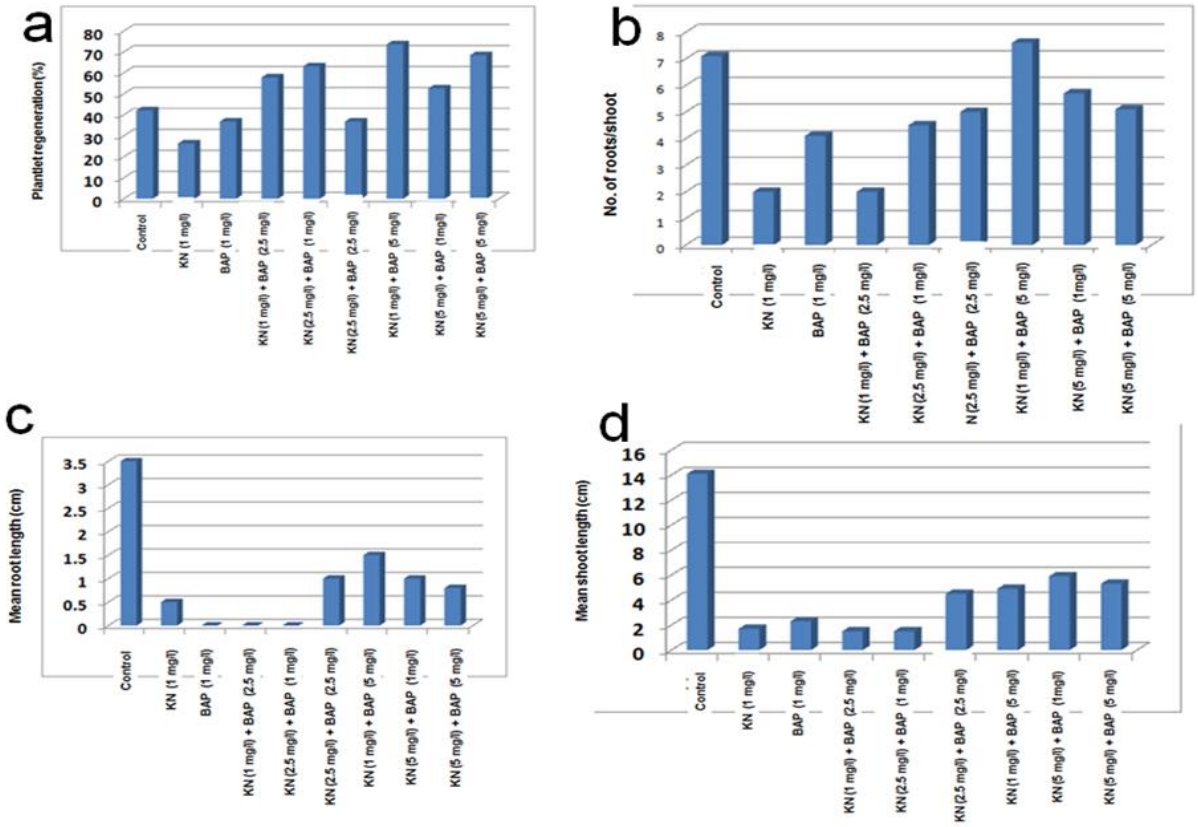
In the present study, the optimal concentration of colchicine and BAP was determined by inducing polyploidy under in vitro culture conditions. Polyploidization is an important event, leading to increase to increase in chromosome number in a cell by arresting the cell division during the metaphase. This phenomenon occurs naturally. However, polyploidization can be achieved under in vitro condition using colchicine. In general, polyploidy enhance the overall vigour of plants in addition to enhancement of various agronomic features to bring genetic improvement. Polyploids are generally tolerant to various biotic and abiotic stresses. Therefore they are expected to perform better than normal plants.(Graph 2)



Graph 2: Effect of BAP and KN on shoot regeneration; a) percentage of regeneration, b) number of shoot/explants, c) mean root length and d) mean shoot length of micro shoots developed in various combinations of medium with lower and higher levels of cytokinins.

Cytological Studies

Actively growing root tips when analysed for the overall size of nucleus and chromosome number, plantlets regenerated from colchicine treated garlic explants was found to have enlarged nucleus as compared to normal plants. However, the extract number of chromosome. But it appears that the number of in vitro polyploidy line was more than 16 which indicated that in vitro polyploidization occurred after treating the meristem tissues of garlic under suspension culture followed by regeneration of plantlets (Figure 4)

Genomic DNA analysis

Genomic DNA isolated from four randomly collected control plants and three plantlets obtained from the in vitro induced polyploidy lines when subjected to agarose gel electrophoresis, clear differences could be observed between normal and polyploidy lines. Based on the quantity and intensity of DNA bands between these samples, it was confirmed that plantlets obtained through in vitro polyploidization had more number of nucleic acids than normal one. In support of this observation, root tips of colchicine induced plantlets had significantly larger number of nucleus with more number of chromosomes than the normal plants (Figure 5).

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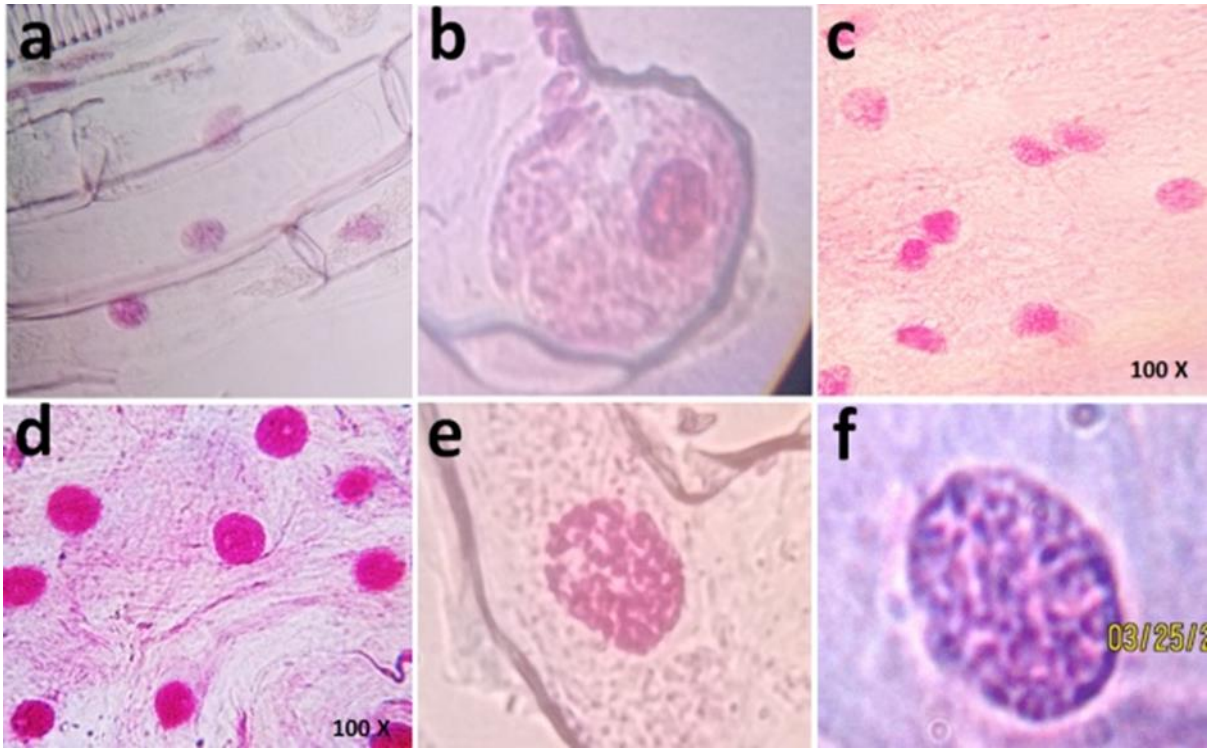

Fig 4: Microscopic observation of nucleus. A) root tip showing the evenly distribution of nucleus, b) single cell showing the prominent nucleus, c) nucleus of normal plant, d) larger size of nucleus in polyploidy, e) and f) enlarge view of the nucleus containing more than 16 number of chromosome. Determination of DNA Quantity in polyploids.

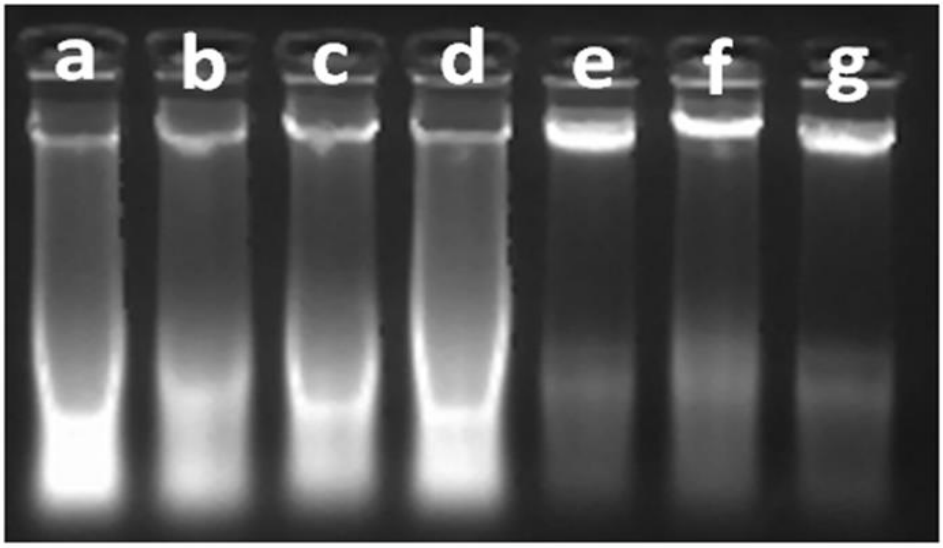

Fig 5: Agarose gel electrophoresis of genomic DNA of control and polyploidy lines. A-d) genomic DNA of control plants; e-g) genomic DNA of polyploidy lines. Note the many fold increase of nucleic acid in polyploids as compared to normal plants.

Nano Drops analysis

Quantification of DNA showed that the plants treated with colchicine presented higher contents of genomic DNA than the untreated one. 160.2 ng/µl was recorded for untreated plants were as 368.9ng/µl for

untreated and treated plants respectively, which showing the variation in the DNA quantity and indicated that the prepared DNA solution were pure (Figure 6 and Table 1).

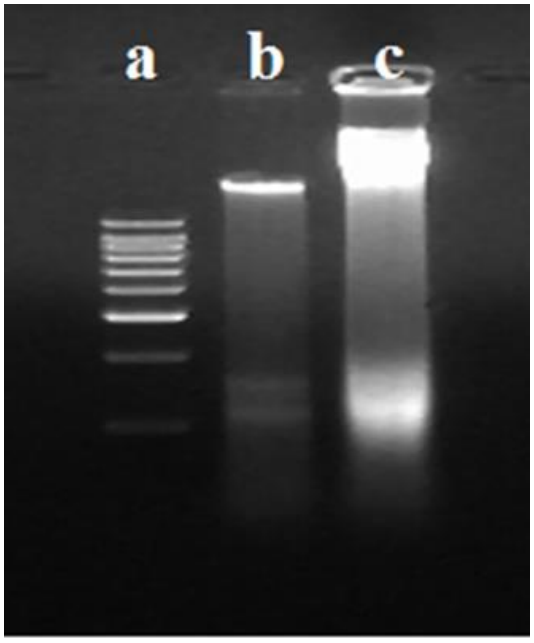

Fig 6: Migration profile of DNA extractionfrom leaves of control & Polyploidy line.

Table 1: Quantification of genomic DNA of untreated & treated plant by Nano Drop technique

Sample	260/230nm	260/280nm	ng/µl
Control	1.19	1.47	160.2
Test	1.51	1.96	368.9

CONCLUSION

Since garlic is propagated by asexual method, genetic improvement of garlic is very limited. Therefore the method of polyploidization in expected to be helpful for the genetic improvement of garlic by development of different polyploidy lines with desirable agronomic features.

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