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INDIRECT REGENERATION OF *CURCUMA LONGA* L (VARIETY SUGUNA) FROM*IN-VITRO*DERIVED LEAFCALLUS

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Abstract

Turmeric (*Curcuma longa*) is one of the oldest and most important spices known for its distinctflavour and its numerous medicinal properties. This vital spice is inflicted by various diseases and pests; and none of the cultivars are resistant. Therefore, the conservation of the *Curcuma longa*gene pool is important. In response to this requirement, an attempt has been made in the present study, to induce callus formation using *in-vitro* derived leaf of *Curcuma longa* (variety suguna) on Linsmaier and Skoog basal medium(LSBM) fortified with 2,4-Dichlorophenoxy acetic acid (2,4-D).The callus was then cultured and sub culturedoverLSBM supplemented with combinations of 2 Isopentenyl adenine (2-iP), 6-Benzylaminopurine(BAP)with 2,4-Dichlorophenoxy acetic acid to obtain multiple shoots. Our results delineatedthat 2-iP and BAP exhibited a synergistic effect on the indirect regeneration of *Curcuma longa* (varietysuguna) derived from *in-vitro*leafcallus.

Keywords: leaf callus, plant regeneration,*Curcuma longa*,6-Benzylaminopurine(BAP) and 2,4-Dichlorophenoxy acetic acid(2,4-D)

Introduction

Curcuma longa, belonging to the Zingiberaceae family, commonly called turmeric (in English) or*haldi*(in Hindi), is an important spice celebrated since ancient times for its medicinal properties,

and distinct flavour. Furthermore, turmeric is also used in pharmaceutical and cosmetic industries as antimicrobial and skin lightening agent.[1]

Indian Ayurveda prescribes the use of turmeric against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis.[2]While in China, it is used to cure abdominal pains and icterus. In modern medicine, turmeric is used for wound healing, treating gastric ulcers, problems in bile secretion and pancreatic secretion, lipid metabolism, and for its anticoagulant, antifertility, antispasmodic, antibacterial antifungal and antitumor activities.[1,3]

Previous researchers have reported clonal propagation by axillary bud proliferation and plant regeneration from leaf base derived callus, but no information is available on indirect regeneration from callus cultures so far.[4]While micropropagation from isolated shoot tips, somatic embryogenesis or shoot formations through organogenesis are known to lead to genetic alterations.[4]The introduction of valuable variation through somaclones may help in programmes designed to improve the physical and genetic features of the crop. In the present investigation, we have initiated callus formation from an invitro derived leaf explant and subsequent regeneration of turmeric plantlets.

Materials and Methods

Non-embryogenic vegetative buds measuring 0.5 to 1 cm were excised from the rhizome of *Curcuma longa*(variety suguna). The scales were removed from the buds and washed thoroughly under tap water, surface sterilized using liquid detergent labolene (5% v/v), rinsed in 70% ethanol for 30 seconds and disinfected in 0.1% $HgCl_2$ for 3 minutes. The buds were then treated with 0.1% streptomycin for 1 minute and washed several times in double distilled water. The sterilised vegetative buds were cultured on LSBM supplemented with BAP (4mg/L) to obtain *invitro* derived plantlets. All the growth hormones and reagents used in the experiment were procured from HiMedia Laboratories,Mumbai, India.

A leaf segment, measuring 0.5-1cm was excised from 6-8 weeks-old *in-vitro* raised plants that was derived from vegetative bud explants, through direct regeneration of *Curcuma longa*(variety suguna). This was then cultured on Linsmaier and Skoog basal medium (3% sucrose and 0.8% agar) fortified with different concentrationsof NAA and 2,4-D ranging from 0.5 - 4mg/l for callus induction.The callus initiated from *in-vitro* leaf explant was further sub cultured on the same medium to harvest a profusely formed callus.[5] Further initiation of shoot primordia was obtained when the profuse callus was transferred to LSBM fortified with different concentrations and combinations of 2-ip, BAP and 2,4-D *viz*. 1-5mg/l. *In-vitro* grown plantlets were carefully taken out from the culture tubes and washed with water to remove the traces of agar.

They were later transferred to plastic pots containing different combinations of peat, perlite and sand (v/v). It was found that peat, sand and perlite in the ratio of 50:25:25 (v/v) was found to be the best for hardening the tissue culture-raised *Curcuma longa* plants (variety suguna).About 92% of the plants were hardened and transferred to the pots. The potted plants were kept in the green house, covered with polythene bags to maintain high humidity.After 21 days, the covers were removed in phases to expose the plants to less humid conditions. After seven weeks of

hardening the *in-vitro*-raised rooted plants were transferred to the field. About 92% of plants survived in the field. The data obtained from the results of all the experiments carried out were analysed statistically by one-way Analysis of Variance (ANOVA) to determine the variation between the treatments using SPSS9.

Results

Tissue culture investigations were conducted to induce callus from leaf segments excised from four weeks old ***in vitro*** raised plants from the vegetative bud of turmeric variety Suguna. The leaf explant measuring 0.5-1 cm (Plate 1; Fig. 1)was inoculated separately on LS basal medium supplemented with 2,4-D(0.5 mg l^{-1}, 1.0 mg l^{-1}, 2.0 mg l^{-1}, 3.0 mg l^{-1} and 4.0 mg l^{-1}) and NAA (0.5 mg l^{-1}, 1.0 mg l^{-1}, 2.0 mg l^{-1}, 3.0 mg l^{-1} and 4.0 mg l^{-1}).

Initial proliferation was observed from the cut ends of the explants on the above medium (Plate 1;Fig. 2)after 20 days of culture on all the concentrations. But further growth of the callus was noticed only on LS basal medium fortified with 2,4-D (3.0 mgl^{-1}) after 30 days of culture (Plate 1;Fig. 3)However, it was found that profuse, compact and creamish white callus was observed by 45 days (Plate 1;Fig. 4),60 days (Plate 1;Fig. 5)and 80 days (Plate 1;Fig. 6)of culture on LS basal medium supplemented with 2,4-D 3.0 mgl^{-1}.

In the present study, it was found that the LS basal medium supplemented with 2,4-D 3.0 mgl^{-1} was found to be the best medium for leaf callus induction and LS basal medium supplemented with NAA resulted in poor callusing. The compact and creamish white callus was sub cultured on LS basal medium supplemented with different concentration and combinations of 2-ip, BAP and 2, 4-D to study the regeneration capacity of leaf callus.

After 21 days of culture root and shoot primordia were observed (Plate 1;Fig. 7 and 8). After 40 days of culture, further growth of callus and increase in the number of shoot primordia along with many thin roots were noticed (Plate 1;Fig. 9).By 75 and 96 days of culture, nearly 20 -24 well-developed shoots with roots were observed on LS basal medium supplemented with BAP 4.0 mgl^{-1} and 2,4-D 0.5 mgl^{-1}(Plate 1;Fig. 10 and11).

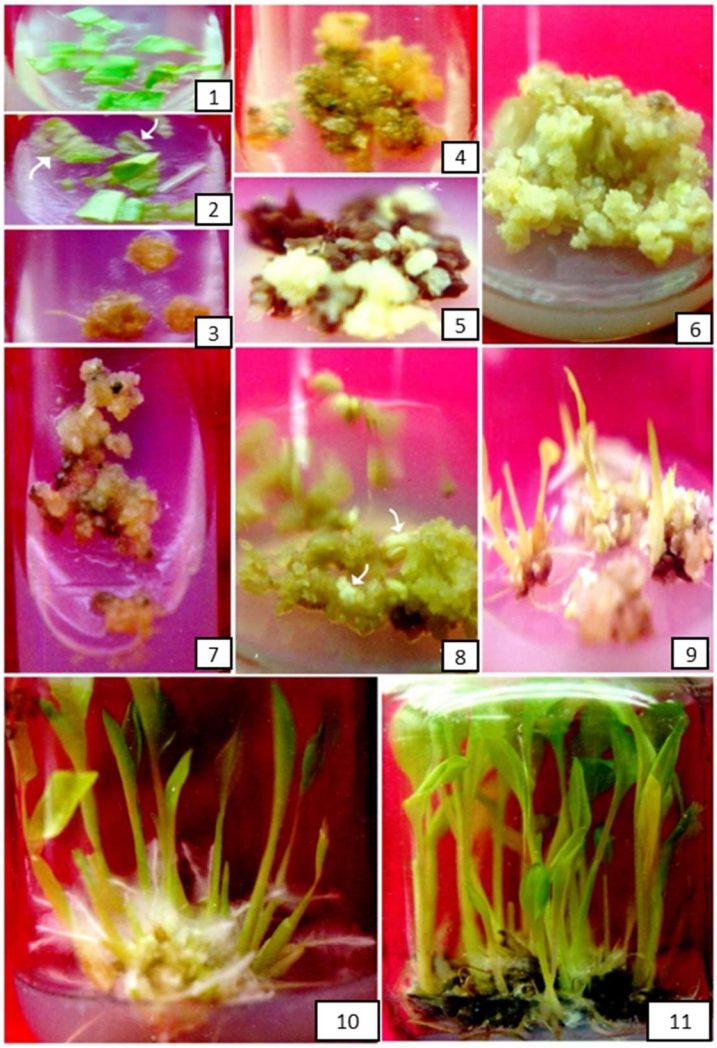

Plate 1. *Curcuma longa* L (Suguna variety) Indirect Regeneration and Shoot Differentiation and regeneration (Fig.1-11)

Plate 1: Fig.**1:**Leaf explants on LBSM+ 2,4-D 3.0 mgl^{-1},**Fig.2**:Explant at culture: 20 days old culture showing callus initiation; Fig.**3**: 30 days old culture showing callus **Fig.4**: Compactand creamish white callus after 45 days of culture; Fig.**5:**Further growth of callus after 60 days of culture; **Fig. 6:**Profuse callusing after 80 days of culture.**Fig. 7**: Sub cultured callus at culture; **Plate 8**: Callusshowing initiation of shoot primordia after 21 days of culture; **Fig.9**: Development of shoots after 40 days of culture; Fig.**10**: Multiple shoot formation and root initiation after 75 days of culture; **Fig.11**:Elongation of multiple shoots (20-24) with well-developed roots after 96 days of culture.

In the present investigation, when the cultures were sub cultured on LS basal medium supplemented with BAP 4.0 mgl^{-1} and 2,4-D 0.5 mgl^{-1}, further elongation of shoots (10-15 cm)

and good rooting was observed after 96 days of subculture.The statistical analysis of the data indicates that there exist highly significant differences within and between the treatments. The highest mean number of shoots obtained from leaf callus ranged from 2.10-24.40.The highest mean number (24.40) was recorded on LSBM +BAP (4.0 mgl^{-1}) + 2,4-D (0.5 mgl^{-1}) was the best medium for multiple shoot formation from *in vitro* derived leaf callus. The analysis of the results revealed that LSBM+BAP (4.0 mgl^{-1}) + 2,4-D (0.5 mgl^{-1}) was found to be significantly superior when compared to other concentration and combinations of 2-ip tested with respect to multiple shoot formation.

Discussion

The callus is a coherent and amorphous tissue, formed when plant cells multiply in a disorganized way. Callus can be initiated *in-vitro* by culturing small segments of explants on growth supporting medium. Upon the stimulus of endogenous and exogenous growth regulating factors, the metabolism of the quiescent cells are changed to highly active vegetative state. During this process, cell differentiation can occur in the intact plant tissue which gives rise to new tissues composed of meristems and unspecialized cells.[6]

Different parts of the entire plant have the potential to produce callus in *in-vitro*. But it is frequently found that callus culture can be established easily using some organs from juvenile parts of the plant as suggested by Green and Phillips,[7] and Dunstan *et al.*.[8]The exogenous supply of growth regulators bound to initiate callus formation. However, in monocots like turmeric, it is necessary to add auxin as a hormonal stimulus for callus induction. This depends strongly on the genotype and the endogenous hormonal content as suggested by Pierik.[9]Perusal of earlier literature reveals that callus induction has been achieved from different explants such as base of the plant and leaf base,[10]and slice of rhizome and shoot tips in different Curcuma species.[11]

In the present study, callus was induced successfully using vegetative bud, pesudostem, leaf, sheathing leaf base and root on LS basal medium fortified with 2, 4-D 3.0 mgl^{-1}. It was found that 2, 4-D 3.0mgl^{-1} concentration was effective in inducing callus in all explants tried when compared to NAA. This does not agrees with the findings of Yasuda *et al.,* who have used NAA alone and also NAA fortified with Kinetin on MS basal medium for callus induction from slice of rhizome and stem tip.[11] The present investigation is in agreement with the findings of Malamug *et al.,* and Babu *et al.,* who have used 2, 4-D 3.0mgl^{-1} for callus induction in ginger.[4,12]

It was found in the present study that two types of turmericCalli were obtained from explants; 1. loose and friable callus on LS basal medium supplemented with NAA and 2. compact and creamish white callus on LS basal medium supplemented with 2, 4-D and by subsequent subculture the compact creamish callus organized into “embryogenic lumps”. Further, the loose and friable callus failed to organize into morphogenetic callus.The organisation in callus can be brought about by controlled initiation of organ primordia through manipulation of nutrient and hormonal constituents in the culture media. The morphogenesis can be either through organogenesis or embryogenesis. Such freshly originating organs are called adventitious buds. These are frequently formed from single cell or from several cells.*In-vitro* regeneration in more than a thousand plant species has been reported.[13]The variety of factors controlling the

morphogenic response of a tissue is often unknown. But organogenesis in the majority of cultures is due to auxins and cytokinin ratio with in a particular range of concentrations.[14] It was shown by Kamada and Harada that phenoxy acetic acids are particularly active in stimulating the appearance of embryogenic cells, although growth regulators help to induce morphogenesis.[15]

In the present study, plants were successfully regenerated from, leaf, sheathing leaf explant of turmeric variety Suguna through callus phase. It was found that addition of cytokinin BAP4.0mgl^{-1} in the presence of auxin 2, 4-D 0.5 mgl^{-1} resulted in induction of organogenesis and subsequent development of plants. The plant regeneration in the present study was obtained after two passage of subculture on the same medium. The plant regeneration was only by organogenesis on the above medium in all the explants studied. Further, the experimental results found in the present study showed that LSBM + BAP (4.0 mgl^{-1}) + 2,4-D (0.5mgl^{-1}) was significantly superior when compared to other concentration and combinations of growth regulators tested with respect to multiple shoot formation from leaf callus.

The plant regeneration in ginger using young buds and leaf explants via organogenesis and embryogenesis was reported by Nadgauda *et al.,*Malamug *et al.,* and Kackar *et al.*[5,12,15] However, few reports are available on plant regeneration in turmeric through organogenesis.[10]

Efficient rooting of *in-vitro* regenerated plants and subsequent field establishment is the crucial stage of rapid micropropagation. In the present study, it was found that the regenerated plants produced roots on the same medium and therefore the additional step for rooting was eliminated. The results of the present study coincide with the report of Balachandran. ***et al.,***[17] and does not agree with the findings of Bhagyalakshmi and Singh, who have reported that the additional step for rooting was necessary in *Zingiber officinale*.[18]Acclimatization of regenerated plants to the external environment is an important stage and its success depends on different factors. It is a general observation that high humidity in *in-vitro* does not allow synthesis of cuticle, epicuticular wax, development of stomatal structures and their functions.[6] Consequently, when such plants are transferred to the natural conditions, they undergo death and desiccation.

In the present investigation, micro propagated as well as callus regenerated plants were transferred successfully to a potting mixture consisting of peat: sand: perlite (50:25:25 v/v). The Relative Humidity (RH) 90–95% was maintained for 3 weeks at 25 ± 2^0 C by keeping them covered with polythene bags and the humidity was reduced gradually for hardening and establishment. It was found that 92% of the plants survived in the above potting mixture.

Thus, the tissue cultured plants of turmeric variety Suguna can be hardened and acclimatized to the field conditions without any difficulty. This may be due to the genetic stability of the turmeric varieties, which are conventionally propagated vegetatively. This agrees with the findings of Nadagauda *et al.*, Bhagyalakshmi and Singh, Balachandran *et al.*,Yasuda *et al.,* and Mukhri and Yamaguchi.[5,11,17-19]

Conclusion

In conclusion, results of our study clearly demonstrated that 2 Isopentenyl adenine and 6-Benzylaminopurine exhibited a synergistic effect on the indirect regeneration of *Curcuma longa* (variety suguna) derived from *in-vitro* leaf callus.

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