Enhancement of *In Vitro* Regeneration of Several *Ocimum* Species and Varieties

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ABSTRACT

Tissue culture systems to optimize regeneration plant species of *Ocimum* spp were evaluated as a method to micropropagate individual plants and to better study their biology *in vitro*. *Ocimum* species were also evaluated for the production of natural plant products during and following the regeneration process. The primary goal of this project was to enhance the regeneration efficiency of basil. Several factors were examined using different *Ocimum* species and commercial varieties. The effect of cytokinin combination, activated charcoal, gelling agents, and different carbon sources were investigated. Anthocyanin callus spots were produced only in four varieties among six tested. 'Sweet Dani' showed the best results on anthocyanin accumulation, while 'African beauty', 'Tree basil' and 'Methylcinnamate' produced only a few spots. Shoot regeneration was only achieved from 'Sweet Dani' explants. As the activated charcoal concentration increased, callus formation rate decreased respectively compare to the controls for all varieties. There was a decrease in callus growth with increasing concentration of agar and phytagel.

Key words : basil, regeneration, tissue culture

INTRODUCTION

Sweet basil(*Ocimum basliicum L.*), annual herb of the *Lamiaceae* family is a popular, tender, and native to southeast Asia, sub-Sahara Africa. The genus *Ocimum*(Lamiaceae), which includes sweet basil, offers a wide diversity with more than 50 species(Darrah, 1980), particularly regarding plant growth, morphology, physical appearance and essential oil content and composition(Morales *et al.* ,1993, Simon *et al.*, 1984). Most basils are grown as culinary herbs primarily because of their unique aromas and fragrances (Simon *et al.*,1990). One of the limitations for the production of basil is the diseased susceptibility caused by pests and pathogens. Through conventional plant breeding techniques, host plant resistance to the pathogen,

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fusarium wilt(*Fusarium oxysporum* f.sp.*basilicum*) has been reported as a major disease(Reuveni *et al.*, 1997). Tissue culture techniques will allow for the application of gene manipulation techniques to address disease resistance in basil varieties. Several reports have been published on the *in vitro* propagation of basil meristems in tissue culture. *In vitro* plant regeneration from excised nodal segments and subsequent establishment of plantlets in soil has been reported in *Ocimum* gratissium and *O. viride*(Ahuja *et al.*, 1982). *In vitro* propagation using auxillary shoot buds are outlined for *Ocimum americanum* L.syn. *O. canum* Sims(hoary basil) and *O. sanctum* L.(holy basil)(Pattnaik and Chand, 1996). *In vitro* clonal propagation of *Ocimum basilicum* L.(sweet basil) has also been established by auxillary shoot proliferation(Sahoo *et al.*, 1997). An efficient plant regeneration protocol has also been developed for three basil varieties('Sweet Dani', 'Methylcinnamate', 'Green Purple Ruffles') using leaf segment culture(Phippen and Simon, 2000).

In that study, the authers observed that basil exhibited special characteristic of providing anthocyanin accumulation spots before shoot regeneration from cultured callus.

In this paper, we examined several different species and varities of basil for the enhanecement of a regeneration system from leaf segment culture.

MATERIALS AND METHODS

Plant material and explant sources

Seeds from several different species and varities of basil : 'Sweet Dani' lemon basil, 'African beauty', 'Genevese', 'Tree basil', 'Juicy fruit', and 'Methylcinnamate' basil were used for this study. Seeds were germinated under mist for two weeks and transferred to normal greenhouse conditions(28℃) under 18 hrs of supplemented light. Explant tissue was collected at young stage of development and consisted of only first true leaves. Leaf tissue was surface sterilized by immersion into a solution of 20% commercial bleach(1.05% sodium hypochlorite) for 20 min and rinsed 3 times with sterile deionized water.

Callus and shoot initiation

Explant materials were prepared in a sterile petri-dish by making two longitudinal cuts along side the mid-rib and a horizontal cut to remove the outer tissue including leaf margins and the basal portion of the stem. The basal part of the desected mid-lib section was then sliced into four pieces to the size of 5mm×5mm. Explants were inoculated abaxial side down on the callus and shoot induction medium(Phippen and Simon, 2000). Murashige and Skoog(1962) culture medium (MS) was supplemented with salts 4.32g/L, 100mg/L myo-inositol, 0.4mg/L thiamine-HCl, 4mg/L thidiazuron, 20g/L sucrose, 7.5g/L bacteriological grade agar. To enhance the regeneration efficiency of basil, several factors were examined. Cytokinins kinetin, zeatin and benzyl aminopurine(BAP) in combination with 4 mg/L thidiazuron(TDZ) were tested at concentrations of 0.1, 0.5, 1.0, 2.0, and 4.0mg/L. Activated charcoal was also investigated at concentrations of 0.1, 0.25, 0.5, and 1.0g/L. Concentrations of 7.5, 8.5,and 9.5g/L bacteriological grade agar and 3, 4, and 5g/L phytogel were evaluated as gelling agents. The pH of all nutrient media was adjusted to 5.8 before addition of agar and autoclaved for 20 min at 121℃. Cultured plates were wrapped with parafilm and maintained in darkness at 26℃ for 14 days in a culture room.

RESULTS AND DISCUSSION

Effect of three cytokinins and 4 mg/L TDZ on regeneration

In 'Sweet Dani', callus formation rate was good in general. Most treatments showed anthocyanin spots

from induced callus(Fig.3A). By addition of 2.0mg/L Zeatin and 1.0 mg/L BAP increased anthocyanin spot production as compared to 4 mg/L TDZ only. Shoots were obtained from the anthocyanin spot callus but frequency was very low(Fig.3B,C,D). 'Sweet Dani' did not differentiate into any type of root (Fig.1).

'African Beauty' showed high callus formation rate for most treatments. Most callus developed from the distal edge of leaf segments was white in color and friable. The callus growing condition was best on 1.0 mg/L kinetin,0.5 mg/L zeatin, and BAP with 4 mg/L TDZ. Anthocyanin callus producing rate was very low, varies from 1 to 3, and came from the combination of Zeatin and TDZ mostly. Even though it had anthocyanin formation, no shoots were observed from the explants. Root formation occured less than 6% (Fig.3D).

In case of 'Genevese', the callus formation rate was low and callus didn't grow well on all treatments. The callus had a brown color and wet appearance. No anthocyanin production was noticed on callus with a production of few roots.

In 'Tree basil', callus formation rate and growth condition was good except those of high concentrations of cytokinins. The callus was moist and very shinny. Pink spots appeared only on 1-2 mg/L Zeatin but formed neither shoots nor roots.

'Juicy fruit' demonstrated some interesting characteristics during *in vitro* culture. Callus formation rate reached almost a hundred percent from all treatments and callus growth also showed best. Juicy fruit callus starts to grow vigorously within 2 weeks, and only the basal vein formed callus at the high concentrations of cytokinins (Fig.3E). However, no anthocyanin spots or shoots were produced with Juicy fruit explants.

The 'Methylcinnamate' variety produced callus by a extremely slow rate. Likewise, only 5 to 13% of pink spots observed in the zeatin treatments. With an addition of BAP, callus grew slowly and had only a little swelling and twisting of leaf segments.

Anthocyanin callus spots were produced only in 4 varieties among 6 tested. 'Sweet Dani' showed the best anthocyanin accumulation, while 'African beauty', 'Tree basil' and 'Methylcinnamate' produced only a few spots. Shoot regeneration was only achieved from 'Sweet Dani' explants (Fig.2).

In vitro micropropagation of holy basil (*Ocimum sanctum* L.) has been accomplished on Murashige and Skoog (MS) medium utilizing young inflorescence explants. Direct multiple shoots were differentiated within 2-3 weeks when explants were cultured on MS media containing 6-benzyl aminopurine (BAP). Of the various levels of BAP tested, MS media supplemented

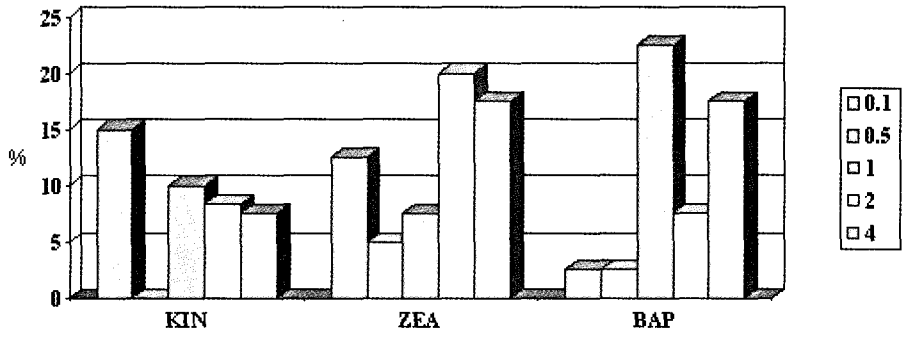

Fig. 1. Cytokinin combination effect with 4 mg/L of thidiazuron(TDZ) for anthocyanin spots accumulation of cultured Sweet Dani.

with 1.0mg/L BAP produced the maximum number of shoots (Singh and Sehgal, 1999)

High frequency bud break and maximum number of axillary shoot formation were induced in the nodal explants on Murashige and Skoog medium (MS) containing N-6-benzyladenine (BA). The nodal explants required the presence of BA at a higher concentration (1.0 mg/L) at the initial stage of bud break. However, further growth and proliferation required transfer to a medium containing BA at a relatively low concentration (0.25 mg/L). Rooted plantlets were successfully acclimated in vermi-compost inside a growth chamber and eventually established in soil. All regenerated plants were identical to the donor plants with respect to vegetative and floral morphology(Sahoo *et al.*, 1997).

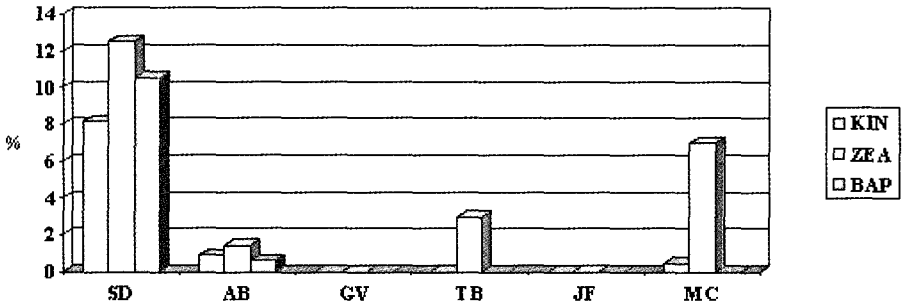

Fig. 2. Anthocyanin spot formation across basil species from the leaf segment culture ; SD:Sweet Dani lemon basil, AB:African beauty, GV:Genevese, TB:Tree basil, JF:Juicy fruit, and MC : Methylcinnamate basil.

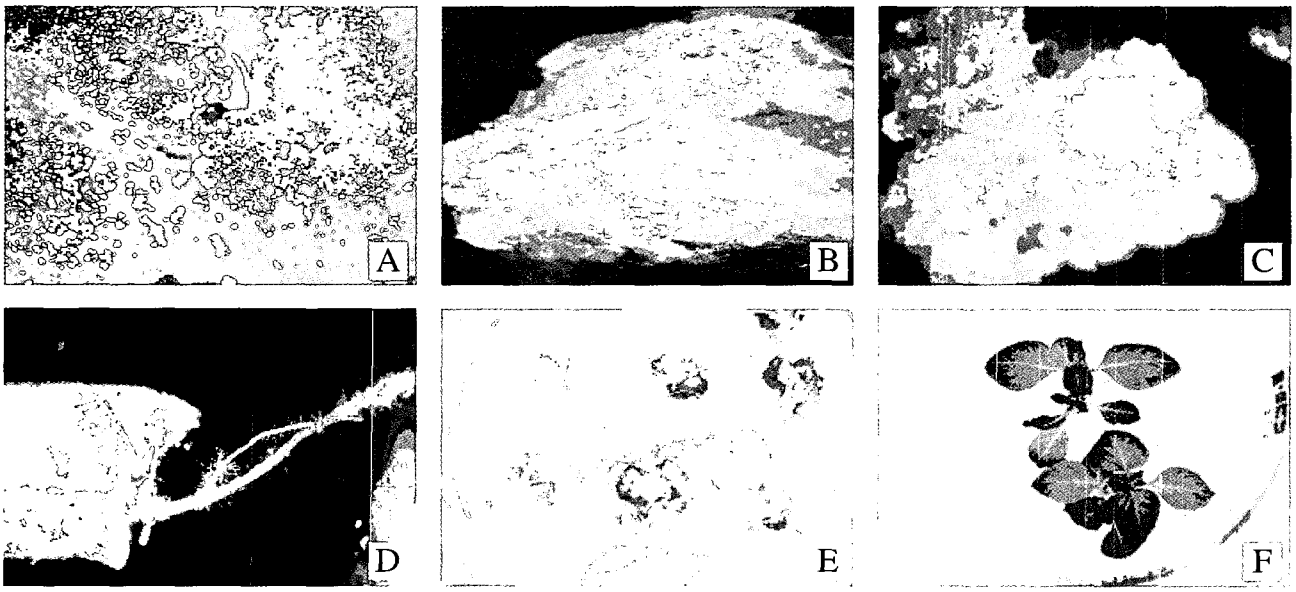

Fig. 3.Various tissue culture response of Basil. A)Anthocynin spot derived from callus in 'Sweet Dani', B)initial shoot originated from pink color callus of 'Sweet Dani', C)Vigorous callus formation in 'Juicy Fruit', D)Hairly root growing in addition to charcoal, 'African Beauty', E)Juvenile shoot from cultured leaf segment of 'Sweet Dani'lemon basil, F)Regenerated plantlets from leaf culture of 'Sweet Dani' lemon basil.

Effect of Activated charcoal

As activated charcoal concentration increased, callus formation rate decreased for all varieties. In spite of 'Juicy fruit', exhibited the most vigorous callus formation, callus production clearly decreased by supplement of 0.1 g/L of activated charcoal and did not respond by the higher concentrations.

No pink spots or shoots were observed on any plates. However, limited root formation was observed especially in 'African beauty', inducing long hairly roots in 23 to 33 % of the explants. The rate of adsorption of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) by activated charcoal from liquid and semi-solid tissue culture media was determined using BAP and 2,4-D(Ebert *et al.*, 1993)

Effect of gelling agents

'Sweet Dani' reached 100% of callus formation in all explants. Both gelling agents investigated a decrease in callus growth with increased concentration with agar and phytagel. Pink color callus and shoot regeneration was observed on media supplemented with 9.5 g/L agar. 'Tree Basil' and 'African Beauty' showed similar responses with callus formation rate and callus growth. Callus induction was reduced by increasing the concentration with gelling agent. 'Methylcinnamate' and 'Genevese' exhibited low callus formation rate and poor callus growth. 'Juicy fruit' grow callus very strongly but did not form any shoots or roots.

Changes in medium pH caused by gelling agents, but not charcoal, could be alleviated by adjusting the pH after their addition but prior to autoclaving(Owen *et al.*, 1991).

In conclusion, anthocyanin callus spots were produced only in four varieties among six tested. 'Sweet Dani' showed the best results on anthocyanin accumulation, while 'African beauty', 'Tree basil' and 'Methylcinnamate' produced only a few spots. Shoot regeneration was only achieved from 'Sweet Dani' explants. As the activated charcoal concentration increased, callus formation rate decreased respectively compare to the controls for all varieties. There was a decrease in callus growth with increasing concentration of agar and phytagel.

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REFERENCES

Darrah, H.H. 1980. The cultivated basils. Buckeye Printing, Independence, MO.

Ebert, C.A., F Taylor and J. Blake 1993. Hanges of 6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. Plant Cell Tissue & Organ Culture 33:(2)157-162.

Morales, M.R., D.J. Charles and J.E. Simon 1993. New aromatic lemon basil germplasm. p. 632-639. In: J. Janick and J.E. Simon (eds.), New crops. Wiley, New York.

Murashige, T. and F.A. Skooge 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology 15:473-497.

Owen, H.R., D. Wengerd and A.R. Miller 1991. Avoidance of precipitation and carbohydrate breakdown in culture medium pH is influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage methods. Plant Cell Reports 10:583-586.

Pattnaik, S. and P.K. Chand 1996. *In vitro* propagation of the medicinal herbs *Ocimum americanum* L syn *canum* Sims (hoary basil) and *Ocimum sanctum* L (holy basil). Plant Cell Reports 15:846-850.

Phippen, W.B. and J.E. Simon 2000. Shoot regeneration

of young leaf explants from basil(*Ocimum basilicum* L.). *In Vitro* Cellular Developmental Biology Plant 36:250-259.

Reuvini, R., N.S. Dudai, E. Putievsky, W.H. Elmer and R.L. Wick 1997. Evaluation and identification of basil germplasm for resistance to *Fusarium oxysporum* f. sp.*basilicum*. Plant Disease 81:1077-1081.

Sahoo, Y., S.K. Pattnaik and P.K. Chand 1997. *In vitro* clonal propagation of an aromatic medicinal herb *Ocimum basilicum* L. (sweet basil) by axillary shoot proliferation. *In Vitro* Cellular & Developmental Biology Plant 33: (4)293-296

Simon, J.E., A.F. Chadwick and L.E. Craker 1984. Herbs: An indexed bibliography 1971-1980; the scientific literature on selected herbs, and aromatic and medicinal plants of the temperate zone. Archon Books, Hamden, CT.

Simon, J.E., J. Quinn and R.G. Murray 1990. Basil: a source of essential oils, p. 484-489. In: J. Janick and J.E. Simon (eds.). Advances in new crops. Timber Press, Portland, OR

Singh, N.K. and C.B. Sehgal 1999. Micropropagation of 'Holy Basil' (*Ocimum sanctum* Linn.) from young inflorescences of mature plants. Plant Growth Regulation 29:(3)161-166.

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