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# ROSMARINIC ACID CONTENT OF GENETICALLY STABLE CLONES OF *Ocimum americanum* L. (LEMON BASIL) REGENERATED THROUGH *IN VITRO* SHOOT BUD MULTIPLICATION AND ROOT CULTURE

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## ABSTRACT

*Ocimum americanum* L. is an annual herb with citrus scent therefore called lemon basil, reflects their high content of phenolic compounds including rosmarinic acid. In the present investigation an attempt has been made to establish *in vitro* shoot bud multiplication and root culture, for the maintenance of uniform progeny of the plant as a source of explant as well as rosmarinic acid production in a short period of time. Murashige and Skoog's basal medium supplemented with 1.0 mg/l kinetin in combination with 0.2 mg/l α-napthaleneacetic acid (NAA) was found to be effective for inducing multiple shoots (27.4±0.534) from the apical bud explants. The shoots were transferred to ½ MS basal medium for rooting. Roots of *in vitro* regenerated plants were used to establish *in vitro* root cultures in liquid MS basal media of one fourth strength, supplemented with 1.0 mg/l NAA and 10.0 mg/l putrescine. Somatic chromosome analysis has indicated genomic stability of the regenerates. The increased level of rosmarinic acid content of the *in vitro* root cultures (2.45±0.06 % of dry weight) as compared to *in vivo* plants was recorded.

**Keywords: Genomic stability, *Ocimum,* Rosmarinic acid**

**Abbreviations: MS - Murashige and Skoog's basal medium, NAA - α -napthaleneacetic acid**

## INTRODUCTION

*Ocimum americanum* L. commonly known as lemon basil, is widely distributed in tropical Africa and Asia [1]. *O. americanum* L. is morphologically intermediate between *O. canum* and *O. basilicum.* It is suggested that *O. americanum* arose as a natural hybrid between the diploid *O. canum* (2n=24) and the tetraploid *O. basilicum* (2n=48) followed by doubling of chromosomes (2n=72) [2]. It has been reported that rosmarinic acid is the predominant phenolic compound found in basil accessions including lemon basil and is supposed to act as a preformed constitutively accumulated defence compound [3]. The biological activity of rosmarinic acid is described as antibacterial, antiviral, and antioxidative [4]. Rosmarinic acid content of basil varies greatly in wild population because of cross pollination, heterozygosity and seasonal variations. In addition, the pharmaceutical companies also make indiscriminate use of wild populations of different medicinal plants for their own interests hence reducing the quality of active principle contents. The conventional method for propagation of *Ocimum americanum* L. is seed germination and this plant cannot be vegetatively propagated. The present study describe establishment of uniform progeny of the plant through *in vitro* shoot bud multiplication and establishment of root culture for production of rosmarinic acid since they show rapid growth and stable metabolic productivity without the influence of seasonal variations.

## MATERIALS AND METHODS

Seeds of *Ocimum americanum* L. (lemon basil) were collected from Sutton and Sons (India) Pvt Ltd, Kolkata having accession number 16 35 58. The plants grown from these seeds were maintained in the experimental garden, Department of Botany, University of Calcutta, for further studies. Voucher specimens of the species are deposited in the herbaria of University of Calcutta, (CUH, Kolkata) bearing accession number - West Bengal, Kolkata, Ballygunge Science College, Experimental Garden, date 22.09.10, *Biswas* 005, Acc. no 200010 (CUH). Apical twigs (2.0 cm) from *in vitro* germinated seedlings (21 days after germination) were used as explants for *in vitro* shoot bud multiplication. Adventitious roots (1.5-2.0 cm in length, 0.1 g fresh weight) excised from *in vitro* plants regenerated through shoot bud multiplication, were used as explants for root culture establishment. Seeds of *O. americanum* L. were first washed in aqueous liquid detergent solution (Tween 20, SRL; two drops in 100 ml) for 12 min at room temperature and rinsed thrice with distilled water and were then treated with

an aqueous 0.1 % (w/v) $HgCl_2$ solution in order to establish maximum contaminant free cultures without affecting percentage of germination. Seeds were germinated *in vitro* on Murashige and Skoog's (MS) [5] modified basal medium supplemented with 3% sucrose, 0.05 mg/l (w/v) ascorbic acid, and 0.1 mg/l (w/v) glutamine and 0.25% (w/v) Gelrite®. The shoot bud multiplication was carried out using modified MS basal medium supplemented with NAA (α-napthaleneacetate, $C_{12}H_{10}O_2$) and kinetin (6-furfurylaminopurine, $C_{10}H_9N_5O_0$). The research is conducted in factorial design based on Completely Random Design (CRD) [6]. The factorial levels of 5 treatments (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) were repeated for three times in five replicates. The concentrations of auxin (NAA) were 0.0 and 0.2 mg/l and tested in case of all the 5 treatments. The regenerated shoots were further cultured in basal media for 4 weeks for further elongation and after attaining a length of more than 3.0 – 4.0 cm, were excised and transferred individually to rooting medium. In this case, first factor was strength of MS, full and half strength respectively and the second factor was NAA (0.0 and 1.0 mg/l). Media used for *in vitro* root culture was same modified MS basal medium as *in vitro* regeneration. The treatments were concentrations of NAA (1.0 and 2.0 mg/l) and putrescine (0.0 and 10.0 mg/l) (2 x 2) and each treatment was repeated for three times in five replicates. Chromosome analysis from root tip cells of *in vitro* regenerates was carried out following propionic orcein staining method to determine the somatic chromosome number of individual clones of each species [7,8].

Dried plant tissues were used for extraction of phenolic compounds. 5.0 mg of dried shoots and roots were pulverized in a mortar and suspended in 2.0 ml of aqueous methanol (80:20 absolute methanol: water, w/v) and absolute methanol (100% methanol, HPLC grade) separately in ambient conditions. Crude extract was prepared for quantitative determination of total phenol and rosmarinic acid following the method of Ellis and Towers (1970) [9] and Didier and Pasquier (1994) [10]. The crude extracts were weighed to calculate the yield (g crude/100 g dried plant material i.e. percentage) [11,12] and stored in a refrigerator (- $4^0$C), until further use for qualitative and quantitative estimation. Separation was achieved by using HPLC (Shimadzu, SPD-10A UV-Vis detector, LC-10AD Liquid chromatograph) with a C18 column, (150 x 4.6 mm, Hypersil) of particle size 5.0 µm. using water: acetonitrile (83:17 v/v) as mobile phase. Ultraviolet detection was set at 330 nm. Rosmarinic acid contents were calculated using a calibration curve (i.e., concentration

vs peak area) and expressed as percentage of dry weight (% of dry wt) [13].

Data were analyzed statistically following one way ANOVA and two way ANOVA [14]. If the sample means differ more than the LSD (Least Significant Difference), it was considered that the two were significantly different ($P \leq 0.05$). Statistical analyses were done using SPSS version 15.0 for Windows (SPSS Inc., USA).

**RESULTS & DISCUSSION**

In the present investigation apical shoot buds from germinated seedling have been found to be suitable explants for *in vitro* shoot bud multiplication. There are various reports of utilizing different shoot bud morphogenesis in different species of *Ocimum* [15, 16, 17, 18, 19, 20, 21, 22]. The earliest (4.0±0.01 days) shoot initiation as well as maximum multiplication fold (27.4±0.534) obtained in the medium containing 0.2 mg/l NAA and 1.0 mg/l kinetin (Fig 1). The number of shoot buds reduced beyond 1.0 mg/l level of kinetin. A further increase in NAA concentration caused callusing and hyper hydricity of regenerated shoots without increasing multiplication fold. All the cultures were maintained up to 16 weeks until the multiplication rate declined and despite the quantitative variation in number of shoot buds induced, the shoots remained healthy with green leaves and without showing signs of hyper hydricity by the end of the third subculture passage. In *O. americanum* L. the half strength of MS basal medium showed the highest numbers of shoots with roots and the strength of medium have significant effect on rooting as revealed from the ANOVA test. The influence of mineral concentration of culture medium on rooting can be attributed to the participation of inorganic ions in processes regulating hormonal balance [23]. The cytological status of the regenerates of these three species was verified using somatic chromosome analysis and the genomic stability was found to be maintained. *In vitro* root culture was successfully established in low nutrient concentration (1/4 MS medium) along with an optimum concentration of an auxin (NAA 1.0 mg/l) which are critical determinants in controlling the growth of adventitious roots. In case of *O. americanum* L., maximum average rosmarinic acid content 2.45±0.06 % of dry wt obtained in liquid ¼ MS medium supplemented with 1.0 mg/l NAA and 10.0 mg/l putrescine. Two way analysis of variance of observed data revealed significant ($P \leq 0.05$) influence of concentration of NAA (1.0 and 2.0 mg/l) on average rosmarinic acid content (Table 2). Presence of putrescine (0.0 and 10.0 mg/l) also showed significant effect in ($P=0.004$) though the interaction of NAA (1.0 and 2.0 mg/l) and putrescine (0.0 and

10.0 mg/l) found to be non significant. The present investigation revealed superiority of *in vitro* root cultures for production of rosmarinic acid in *O. americanum* L. The rosmarinic acid content has been increased by 2.25 times in root culture than those of *in vivo* plants (1.09±0.072). Exogenous incorporation of polyamines and putrescine in particular, has a significant effect on growth and secondary metabolite accumulation in root cultures [24, 25, 26]. The role of polyamines as a source of nitrogen influencing the metabolic process interferes greatly with the secondary metabolite accumulation as reported by other authors [27, 28, 29, 30, 31].

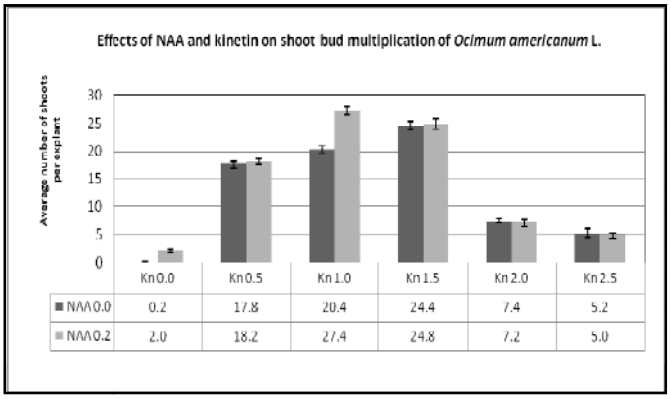


**Fig 1 Graphical representations of effects of different concentrations of NAA and Kinetin on shoot bud multiplication of *Ocimum americanum* L.**

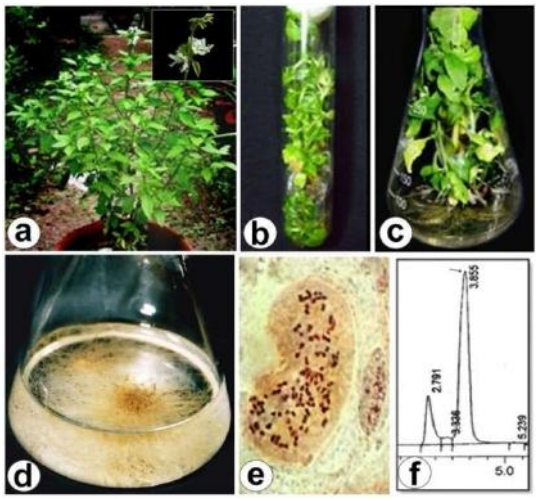


**Fig 2 a. Mature plants of *Ocimum americanum* L. with flowers in inset (c.a. x 0.28); b. Shoot bud multiplications after 8 weeks in culture (NAA 0.2 mg/l and Kinetin 1.0 mg/l) (c.a. x 0.56); c. *In vitro* regenerated plant maintained in half strength of MS modified basal medium (4 weeks) (c.a. x 0.32); d. *In vitro* root cultures in liquid media (1/4 MS, NAA 1.0 mg/l, Putrescine 10.0 mg/l) after 90 days in culture (c.a. x 0.74); e. Somatic chromosome preparation of *in vitro* roots showing 2n=48 chromosomes at metaphase stage (x 2500) f. The graph showing rosmarinic acid peak (Retention Time 3.855 min) of extracted sample from *in vitro* root culture**

**Table 1: Synergistic effect of strength of MS and NAA on rooting of shoots of *Ocimum americanum* L. [à]**

| Media | Number of days taken for root initiation | Average number of shoots rooted/explants |
|---|---|---|
| MS / NAA 0.0mg/l | 14.2±0.200 | 57.4±0.748 |
| ½MS / NAA 0.0mg/l | 7.2±0.200 | 68.4±0.509 |
| MS / NAA 1.0mg/l | 14.0±0.0 | 50.0±1.043 |
| ½ MS / NAA 1.0mg/l | 9.20.200 | 48.5±0.245 |
| F value | 716.576* | 165.98* |

**à Data represented as mean±SE from five replica of an experiment.**

***P<0.0001, (df 3, 16)**

**Table 2: Average rosmarinic acid content of *in vitro* root cultures *Ocimum americanum* L. in different liquid media.[à]**

| Strength of MS + concentration of NAA/Putrescine ( mg/l ) | Average rosmarinic acid content (% of dry wt) |
|---|---|
| 1/4 MS/ 0.0/0.0 | 1.85±0.03[a] |
| 1/4 MS/ 1.0/0.0 | 2.30±0.13[b] |
| 1/4 MS/ 1.0/10.0 | 2.45±0.06[c] |
| 1/4 MS/2.0/0.0 | 2.02±0.30[d] |
| 1/4 MS/ 2.0/10.0 | 2.32±0.30[b] |
| LSD* | 0.11 |

**à Data represented as mean±SE from five replica of an experiment.**

*** Means followed by different superscript letters in the same column present significant** difference (P≤0.05).

## CONCLUSION

In the present investigation combination of *in vitro* micropropagation and root culture, therefore, provides maintenance of uniform progeny of the plant as a source of explant as well as stable rosmarinic acid production in a short culture period. There are reports of micropropagation of *Ocimum americanum* L. [15, 32, 22] but this is the first attempt of utilising *in vitro root* culture of the plant for the production of rosmarinic acid . The genetically stable root culture of the present study can be further utilised for estimating other secondary metabolites including phenolic compounds. With the advancement of plant molecular biology and plant tissue culture techniques, the idea can be conceived that genes from other sources can be integrated into *in vitro* cultures to confer resistance to pathogens and pests that would not only greatly enhance but also stabilize production.

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