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RAPID MICROPROPAGATION, ANTIOXIDANT AND ANTIBACTERIAL ASSAYS OF *OCIMUM* SPP.

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Abstract

In vitro micropropagation was studied in two species of *Ocimum* and one species was used for antioxidant and antibacterial activity. The maximum number of shoots/explant (10.1 and 9.5) was induced in MS + 1.0 mg/l BAP + 0.5 mg/l Kn and MS + 2.0 mg/l BAP + 0.5 mg/l IAA in *Ocimum tenuiflorum* and *O. americanum,* respectively. GA_3 (0.1 mg/l) was found to be effective for shoot induction and elongation in both the species. Strong and stout root formation was observed on half strength MS medium supplemented with 0.2 mg/l IBA for *O. tenuiflorum* and half strength MS + 0.5 mg/l NAA for *O. americanum.* Methanolic leaf extract of *O. tenuiflorum* showed high inhibition of DPPH activity compared to standard antioxidants like quercetin and the chloroform leaf extracts exhibited wide range of antibacterial activity.

Introduction

The genus *Ocimum* is the most important medicinal plant for its therapeutic potentials belonging to Lamiaceae. Whole plant is used as medicine for treatment of many human ailments (Alia *et al*. 2012). The important *Ocimum* species are *Ocimum tenuiflorum* (Tulsi), *O. gratissium* (Ram Tulsi), *O. canum* (Dulal Tulsi), *O. americanum* (Ban Tulsi). Among them *O. tenuiflorum* (also known as *O. sanctum* L.) is the most important herbal plant used in folk medicine as well as in religious and cultural traditions (Engels and Brinckmann 2013).

The leaves of the plant are highly aromatic. The leaves of *O. tenuiflorum* contain 0.7% volatile oil comprising about 71% eugenol, 20% methyl eugenol (Patil *et al.* 2010). It shows antibacterial property against *Salmonella typhosa* (Faisal *et al.* 2012). It is also used as pharmaceutical agents because of it's anti-asthmatic, antioxidant, anti-microbial, anti-emetic, anti-diabetic, anti-fertility, anti-stress, antifungal, insecticidal, diuretic, expectorant and analgesic properties (Ram *et al*. 2011). Previous studies reported about antioxidant activities of *Ocimum* spp. (Filip *et al*. 2013). *Ocimum* species is conventionally propagated through seeds. But seed viability is very poor and low germination rate restricts its mass scale multiplication. Moreover, progenies derived from seeds are not always true to type due to cross-pollination (Ghani 2003). So conservation of the plants is also badly needed. Micropropagation is an *in vitro* technique that enables rapid, large-scale plant propagation and development of disease free plant in small spaces (Tulika 2015). The present investigation was undertaken with a view to developing an efficient *in vitro* regeneration protocol of two *Ocimum* species, namely *O. tenuiflorum* and *O. americanum* for continuous supply of true to type plants and germplasm conservation. Antioxidant and antibacterial properties of *O. tenuiflorum* were also evaluated because of its great use in pharmaceutical industries in Bangladesh.

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Materials and Methods

Plants of *Ocimum tenuiflorum* and *O. americanum* were collected from BCSIR medicinal plant garden and used as source of explants. The nodal segments were sterilized using soft detergent, Tween 20 (1.0 drops/100 ml distilled water), 70% alcohol (30 sec) and 0.1% mercuric chloride (w/v, for 2.0 -2.5 min) by proper rinsing. Then the sterilized explants were inoculated on basal MS (Murashige and Skoog 1962) medium supplemented with different concentrations and combinations of BAP, Kn, NAA, IAA, GA_3 and $ZnSO_4$ for direct shoot regeneration. The *in vitro* regenerated shoots were sub-cultured to fresh medium at an interval of 20 - 25 days for further multiplication. About 3-4 cm long shoots were separated and transferred to half strength of MS basal medium supplemented with different concentrations of IBA and NAA. The profusely rooted plantlets were transferred to plastic pots containing soil for acclimatization in outside environment.

To evaluate the antioxidant property, leaf extract was extracted from the dried powdered leaf (200 g) of *O. tenuiflorum* in 550, 600 and 650 ml n-hexane, chloroform and methanol, respectively with sequential manner. The leaf extracts were then evaporated, dried and stored in refrigerator till use. The free radical scavenging activity of extracts were analyzed by DPPH (1, 2-diphenyl 1-picryl hydrazyl) developed by Brand-Williams *et al.* (1995). Quercetin was used as standard. Negative control was prepared in the same way as the sample except addition of sample or standard.

Percent scavenging activity was calculated using the formula: Scavenging activity = (A0-A1)/A0 × 100%, where A0 is the absorbance of control, and A1 is the absorbance of sample or standard. The experiment was carried out in triplicate. By using the equation $y = mx + c$ (where c is intercept and m is slope); IC_{50} value of extract was calculated.

Crude leaf extracts (n-hexane, chloroformic and methanolic) of *O. tenuiflorum* were used to evaluate the antibacterial activity following disc diffusion method. Five bacteria such as *Bacillus megaterium* (ATCC14581), *Staphylococcus aureus* (ATCC 9144), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 13311) were used in this test. Gentamycin (10 mcg/disc) and neomycin (30 mcg/disc) were used as standard antibiotic. The sterile filter paper discs were prepared by adding 500 µg extracts per disc. The negative control was ethanol (100%).

Results and Discussion

An efficient protocol was developed for *in vitro* regeneration of *O. tenuiflorum* and *O. americanum.* Maximum adventitious shoots were derived directly from the nodal explants of *O. tenuiflorum* and *O. americanum* when cultured on MS with 1.0 mg/l BAP + 0.5 mg/l Kn and MS with 2.0 mg/l BAP with 0.5 mg/l IAA, respectively (Fig. 1a, e). The regenerated shoots were stunted and growth rate was too poor in these media (Fig. 1b). Rapid elongation was observed when 0.1 mg/l GA_3 was added in both MS + 1.0 mg/l BAP + 0.5 mg/l Kn for *O. tenuiflorum* and MS + 2.0 mg/l + BAP + 0.5 mg/l IAA for *O. americanum* (Table 1, Fig. 1c). MS with BAP and NAA; MS with BAP and IAA also showed good response towards multiple shoot formation that supported by the report of Sunil *et al*. (2012), Tulika (2015), Anjana *et al.* (2016) and Banu *et al.* (2017) in case of medicinal plant *Gynura procumbens.* In all the cases GA_3 (0.1 mg/l) was found to be effective for proper elongation of shoots (Fig. 1d, Table 1).

In tissue culture cytokinins may play a vital role in combinations with gibberellic acids or auxins depending on the individual plant species or genotypes (Gulati *et al*. 2015). In the present study similar observations were recorded. Individual shoots were separated from cluster and sub-cultured on GA_3 supplemented regeneration media and the highest elongation was recorded (Fig.

1f, g). Zinc played a vital role for shoot formation and elongation. Therefore, zinc sulfate (4.3 - 21.5 mg/l) along with 1.0 mg/l IAA was added to the medium. In case of *O. tenuiflorum* the highest mean number of shoots/explant (9.4) and mean length of shoot (6.6 cm) was recorded on MS with 17.2 mg/l $ZnSO_4$ + 1.0 mg/l IAA (Fig. 1h) while highest mean number of shoots (9.2) and length (6.2 cm) was recorded in *O. americanum* on MS with 21.5 mg/l $ZnSO_4$ + 1.0 mg/l IAA (Fig. 1i). Interestingly, no root formation was observed in developing shoots from nodal explants when grown on $ZnSO_4$ and IAA containing MS medium. Sandeep *et al.* (2016) reported that spontanious root formation was ovserved along with multiple shoot formation in $ZnSO_4$ and IAA containing MS medium.

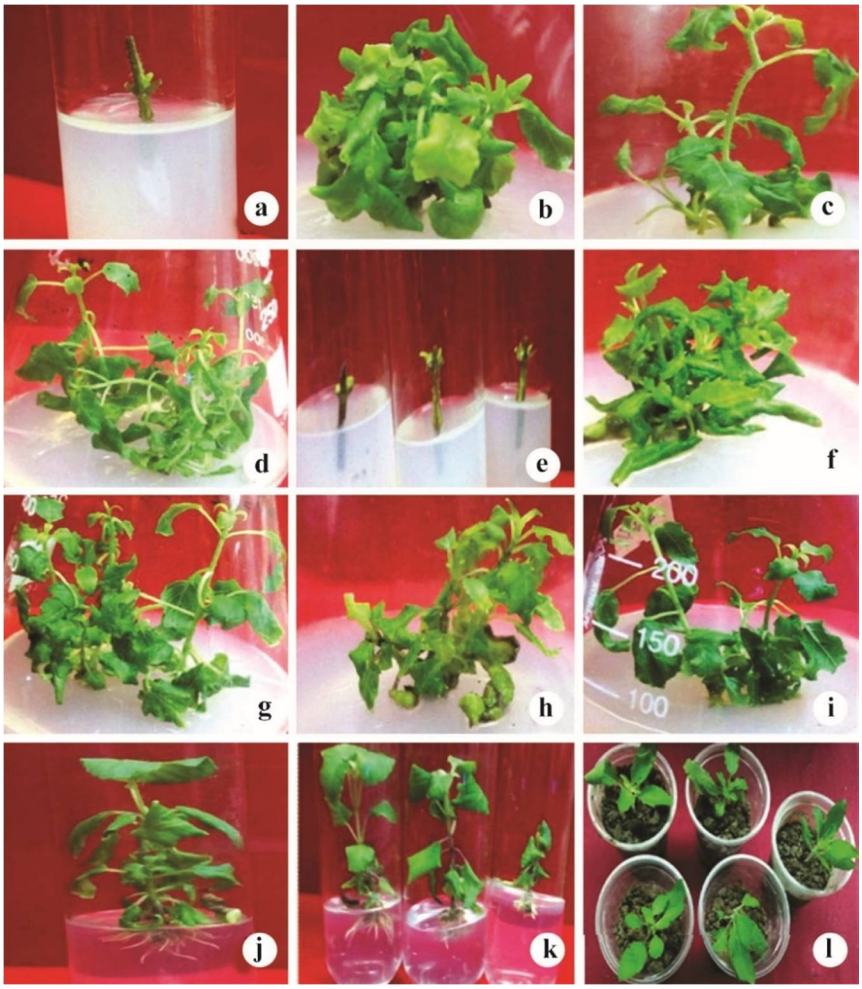

Fig.1. Different stages of regeneration from nodal segments of *O. tenuiflorum* and *O. americanum*: (a) Initiation of shoots within 7 - 10 days in *O. tenuiflorum*; (b) Proliferation of shoots; (c) Elongated shoots; (d) Shoots developed on MS with 1.0 mg/l BAP, 0.5 mg/l Kn and 0.1 mg/l GA_3; (e) Initiation of shoots in *O. americanum*; (f) Compact multiple shoots formation; (g) Elongated shoots; (h) Shoots on MS with 17.2 mg/l $ZnSO_4$ and 1.0 mg/l IAA within 110 days in *O. tenuiflorum*; (i) Shoots developed on MS with 21.5 mg/l $ZnSO_4$ and 1.0 mg/l IAA in *O. americanum*; (j and K) Induction of stout roots in *O. tenuiflorum* and *O. americanum*, respectively, (l) Plantlets transplanted in small pots in outside environment.

Table 1. Effect of MS medium supplemented with different concentrations of hormones on shoot regeneration from nodal explants of *O. tenuiflorum* and *O. americanum*.

	Hormonal supplements (mg/l)							Days required for shoot initiation	% of explants regenerated	Mean no. of shoots/ explants after 90 days	Mean length of shoots (cm) after 90 days
	For micropropagation					For shoot elongation					
	BAP	NAA	IAA	Kn	GA_3	GA_3	$ZnSO_4$				
Ocimum tenuiflorum	2.0	0.4	-	-	-	0.1	-	10-15	89.00	9.5	5.0
	2.0	-	0.2	-	-	0.1	-	7-10	90.00	9.2	6.5
	1.0	-	-	**0.5**	-	0.1	-	**7-10**	**99.00**	**10.1**	**6.5**
	1.0	-	-	1.0	-		-	10-12	79.00	6.1	5.5
	1.0	-	-	0.5	0.1		-	10-12	70	6.4	5.2
	-	-	1.0	-	-		17.2	10-15	83.33	9.4	6.6
O. americanum	2.0	0.4	-	-	-	0.1	-	7-10	99.00	8.5	4.5
	2.0	-	**0.5**	-	-	0.1	-	**10-12**	86.66	**9.5**	**4.8**
	1.0	-	-	0.5	-	0.1	-	10-12	73.33	8.4	4.0
	1.0	-	-	0.5	0.05		-	8-11	80	7.3	4.0
	1.0	-	-	1.0	0.05		-	8-10	90	7.2	5.0
	-	-	1.0	-	-		21.5	10-12	80.33	9.2	6.2

Half and full strength MS medium supplemented with various concentrations of IBA and NAA were used for root induction. In case of *O. tenuiflorum* half strength MS medium with 0.2 mg/l IBA was the best for induction of stout root system while half strength MS medium with 0.5 mg/l NAA was the best for *O. americanum* (Fig. 1j, k, Fig. 2). Venugopal *et al.* (2015) and Tulika (2015) reported that MS with IBA was optimal for root formation of *O. tenuiflorum.* The rooted plantlets were transplanted in soil with 80% survivability.

Antioxidant activity of *O. tenuiflorum* leaf extracts were examined by DPPH free radical scavenging assay and methanolic leaf extracts exhibited the potent antioxidant activity. On the other hand, chloroformic extract showed moderate and n-hexane leaf extracts showed the lowest antioxidant activity by comparing the IC_{50} value (Fig. 3). The IC_{50} value of methanolic leaf extract of *O. tenuiflorum* was 7.63 µg/ml that obtained from the linear regression equation which was very close to the IC_{50} value of the standard (7.65 µg/ml, Fig. 4). This finding is supported by Bandita *et al.* (2013) and Deepak *et al.* (2015) who reported the highest antioxidant activity in ethanolic leaf extracts of *Ocimum* species.

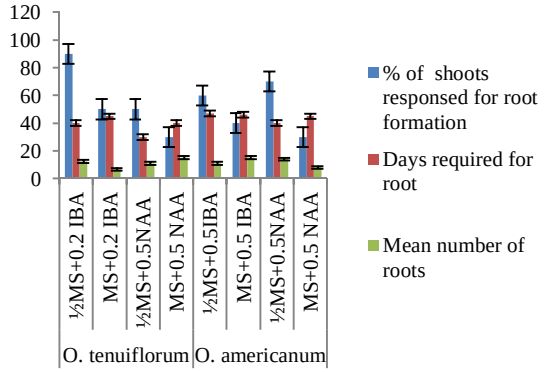

Fig. 2. Effects of different concentrations of IBA and NAA on root formation of two species of *Ocimum.*

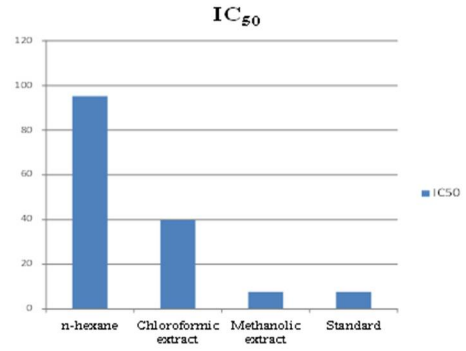

Fig. 3. Comparison of IC_{50} values of different plant extracts of *O. tenuiflorum*

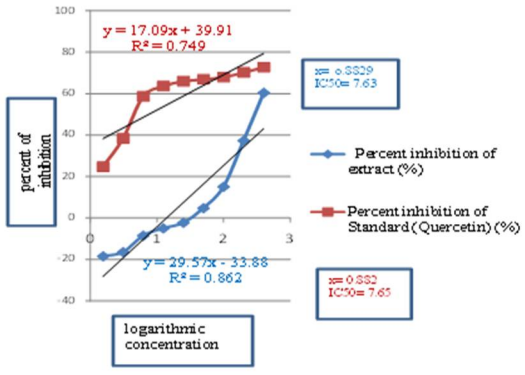

Fig. 4. DPPH scavenging assay of *O. tenuiflorum.* Methanolic leaf extracts (% inhibition vs logarithomic concentrations).

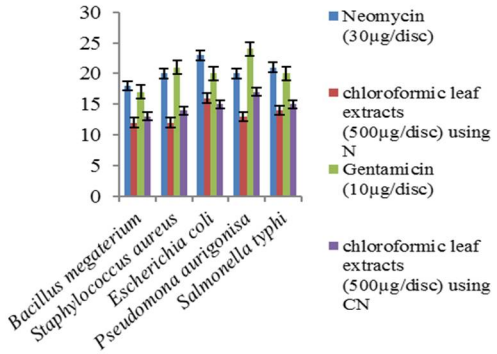

Fig. 5. Evaluation of antibacterial activity using chloroformic leaf extracts of *O. tenuiflorum* and positive control of neomycin (N) and CN antibiotic disc (microorganism vs zone of inhibition).

In case of antibacterial screening, all the leaf extracts (n-hexane, chloroformic and methanolic) of *O. tenuiflorum* showed potent to moderate antibacterial activity against tested bacteria (*Bacillus megaterium*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*). The chloroformic leaf extract showed notable zone of inhibition against all tested bacteria and maximum zone of inhibition was 17.0 mm as compared to standard gentamicin (CN: 24.0 mm inhibition) antibiotic against *P. aeruginosa* which is a pathogenic bacteria of pulmonary infection (Figs 5, 6). Methanolic leaf extract produced moderate zone of inhibition (14 - 15 mm) (Fig. 6c). On the other hand, n-hexane leaf extract produced lower zone of inhibition against the tested microorganisms (Fig. 6a). Anjana *et al.* (2016) reported that chloroform extracts exhibited wide range of antibacterial activity than methanolic extract. Venugopal *et al.* (2015) reported that methanolic extracts produce maximum inhibition zone against *B. subtilis* and *S. aureus* and Yamani *et al.* (2016) reported that essential oil extracted from *O. tenuiflorum* has less antimicrobial activity against *P. aeruginosa.* All these reports supported the present findings.

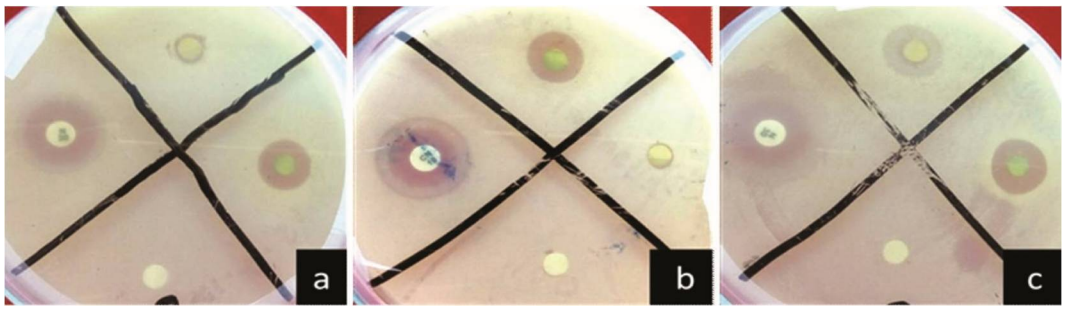

Fig. 6(a-c). Different inhibition zone using *n*-hexane, chloroformic and methanolic leaf extracts. (a) Inhibition zone (9.5 mm) using *n*-hexane leaf extracts against *S. typhi* bacteria. (b) Inhibition zone (17 mm) using chloroformic leaf extracts against *P. aeruginosa*. (c) Inhibition zone (15 mm) using methanolic leaf extracts against *P. aeruginosa* bacteria.

An efficient micropropagation protocol for two species of *Ocimum* from nodal explants has been developed that will ensure large scale propagation, as well as *ex situ* conservation of this important aromatic and medicinal herb. Encouraging antioxidant and antibacterial activity were determined which will facilitate in designing and discovery of effective drug for combating challenging diseases.

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