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In vitro *Ocimum basilicum* L: Cotyledon Potential in Callus Induction and Evaluation of Prior Leaf-based Studies

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Abstract

Ocimum basilicum L is a valuable medicinal plant with a wide range of healing effects. The current study looked at the potential of the cotyledon of *O. basilicum* to produce callus and compared it to the potential of the leaf to find the best way to get a high biomass and evaluated prior studies on leaf efficiency in callus induction. Nine plant growth regulator (PGR) treatments with the highest callus biomass were selected from previous studies relevant to the current research and used the leaf as an explant. The cotyledons, from 6-day-old, and young leaves, 5mm x 5 mm, were aseptically inoculated in Murashige and Skoog (MS) medium. The concentrations of these nine treatments ranging from 0.3 –5.0 mg/L, were added either alone or in binary combination, which are 2,4-dichloro-phenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), benzyl amino purine (BAP), indole acetic acid (IAA), indole butyric acid (IBA) and kinetin (KIN). There were significant differences between the treatments. All the treatments containing NAA showed high potential in callus induction either using cotyledon or leaf ranged between 15.11 – 18.37 g/L. The leaf explant treated with 0.6 mg/L NAA and 0.3 mg/L BAP formed the most callus, 18.37 g/L. The cotyledon explant treated with 1.2 mg/L NAA and 1.0 mg/L BAP produced the second most callus, 17.58 g/L. Adversely, 2,4-D acted as an inhibitor of callus formation. The study showed that the leaf and cotyledon of *O. basilicum* have different capabilities to produce biomass from callus, which indicates how their physiological performance is different between them.

***Key words*:** *Ocimum basilicum*, Callus initiation, Efficiency, Cotyledon, Leaf, NAA

The medicinal plant *Ocimum basilicum* L (sweet basil, Tulsi, in India named Sabja) belongs to the family of Lamiaceae, which contains about 200 general and 3200 species. It is an annual or perennial fragrant herbal plant. [1] it has more vitality compared to other species under the genus of *Ocimum* [2]. It has become established in tropical Africa, Asia, and America. It is grown throughout North Africa, Europe, and Southwest Asia (Paton and Paton 1991). It is also considered an important species in the home kitchen because of its aromatic properties, which add flavour to food. It has various medicinal properties such as anticancer [2], antioxidant [3], antiseptic [4], cardiac stimulant, anti-ulcer genic [5], anti-inflammatory [6], and antimicrobial [7]. Hence, *O. basilicum* has enticed entrepreneurs to discover its biological components. In the chemical composition of *O. basilicum*, there are numerous pharmaceutical active ingredients such as alkaloids, flavonoids, phenolic and terpenoids. Methyl, 1,8-cineole, Linalool and eugenol are the main biotic active ingredients in *O. basilicum* [8-9]. The dominant pharmaceutical active ingredients in *O. basilicum* have been reported to be rosmarinic acid, chicoric acid, rutin, and isoquercetin in phenolic compounds [10]. Consequently, the components of *O. basilicum* are used to produce medicines to meet the population's needs. To use *O. basilicum* in the pharmaceutical industry, the quality and quantity of the compounds extracted from it must be standardized. In light of that, in vitro callus induction is a better approach to extracting secondary metabolites in terms of quality and quantity. Elicitation has proven to be one of the most effective ways for secondary metabolite enhancement and biotechnological production [11]. Callus cultures of Ocimum have untapped potential as an alternative production source of betulinic acid, which is gaining unmatched attention owing to its unique anti-cancer activity in *O. basilicum*, *O. kilimandscharicum* and *O. sanctum* [12]. Ethanol extract from the leaf and leaf callus of *O. basilicum* had anti-inflammatory effects on LPS-stimulated RAW 264.7 macrophage cells [13].

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[14] enhanced total phenolic content, antioxidant potential, rosmarinic acid, eugenol, flavonoid content, caffeic acid, peonidin, cyanidin and cichoric acid by being treated with leafy cotyledon light emitting diodes (LEDs) properties. The cotyledon is an embryonic leaf that arises from germinated seed and develops into a plantlet. The plantlet needs to get nutrients and energy from the cotyledons to carry out its cell division at a high vitality till roots form. The results of [15] demonstrate that the leafy cotyledon (LEC) genes are essential for inducing somatic embryogenesis in vitro and play a crucial function in regulating several aspects of zygotic embryogenesis in *Arabidopsis*. The best explant based on callus weight was the cotyledon explant of *Trachyspermum ammi* [16]. However, to our knowledge, there have been insufficient attempts in vitro to use the cotyledons of *O. basilicum* to produce callus and improve and uniform its biomass production protocol economically. The present study aimed to explore the potential of the cotyledon in callus induction and production by studying a group of different PGRs treatments which have been applied in previous studies on the leaf of *O. basilicum;* which makes the comparison between leaf and cotyledon as explants intriguing, being cotyledon is closest to the leaf structurally and functionally. Furthermore, the study aimed to evaluate the findings of previous studies regarding the efficiency of the leaf in the induction of callus, as well as to standardize and improve the protocol for its economical production.

MATERIALS AND METHODS

Treatments of the experiment

Based on previous studies [2], [6], [17-20] which are closely related to the present research, nine different PGR treatments were applied in the current study by using leaf and cotyledon as explants of *O. basilicum.* PGRs, which include 2.4-D, IAA, NAA, BAP, and KIN (High Purity Laboratory Chemical, Mumbai, India), were used alone or composite. Uniform conditions were applied to all treatments. In addition, the study included MS medium free of PGRs (MS0) as control and underwent the same conditions.

Table 1 Concentrations of PGRs treatments (mg/L)

Treatments	Type of PGRs				
	2,4-D	IAA	NAA	BAP	KIN
T_1	0.5				
T_2	1				
T_3	2				
T_4		5			
T_5			0.6	0.3	
T_6			1.2	1	
T_7	1				0.25
T_8	1				0.5
T_9			3		1.2

Plant materials and callus induction

Cotyledons 6-day-old and juvenile leaves of *O. basilicum* were collected on 10/06/2022 (First experiment) and 06/09/2022(second experiment) from Himayat Bagh in Rauza Bagh, district of Aurangabad, Maharashtra state, India. Explants were rinsed by running tap water for 60 minutes. After that, they were immersed in 70% alcohol for 20 seconds and then rinsed with sterile distilled water three times until all traces of alcohol was eliminated. Next, explants were disinfected by immersing in 10% sodium hypochlorite for 5 minutes, followed by rinsing with sterile distilled water five times until all traces of sodium hypochlorite was eliminated. Disinfected leaves were cut into 5 mm x 5 mm in size and were inoculated along with disinfected cotyledons in MS medium (Murashige and Skoog 1962) supplemented with 3.0% (w/v) sucrose, 0.7% (w/v) agar (High Purity Laboratory Chemical, Mumbai, India) as well as above treatments of PGRs (Table 1). The pH of the medium was adjusted at 5.8±1 with 1.0 M HCl or 1.0 M NaOH before adding agar, then sterilized by autoclaving for 20 minutes at 121°C under 1.1 kg/cm^2 pressure. All the cultures were kept at 25±1ºC in an incubator with a photoperiod of 16 hours light and 8 hours dark provided by white fluorescent tubes with 20004000 lux intensity. The cultures were incubated for eight weeks, and daily observations were taken to track the day when the first callus appeared and its progress. In the eighth week, calli biomasses were harvested, and weighting was carried out. The calli were again weight after oven dried.

Statistical analysis

The experiment in the present study was conducted in a randomized design twice independently. Each treatment consisted of three replicates. Statistical analysis was carried out using SPSS 26.0. Pearson's correlation coefficient was used to evaluate the relationship between various factors. Tukey's HSD was performed for post hoc analysis to determine whether the mean difference was statistically significant. Results were evaluated as significant at a P 0.05.

RESULTS AND DISCUSSION

All PGR treatments applied in the present research initiated the callus formation in both explants (cotyledons & leaves). The callus formation was initiated on the 6th - 8th day following inoculation in all treatments, including control. The results showed significant differences in the dry weight of induced callus between the control and all treatments in both explants. Using cotyledon explants, a T_6 achieved a high dry weight of induced callus 17.58 g/L, which was incubated on MS medium supplemented with 1.0 mg/L BAP in combination with 1.2 mg/L NAA followed by T_7, then T_5. At T_1, by using cotyledon explant, the lowest concentration from 2.4-D (0.5 mg/L) achieved the highest dry biomass 11.25 g/L of callus among all treatments in which was used 2.4-D either alone or in combination with KIN (Fig 1). There is a negatively correlated reached 0.86 at the level of statistical significance 1% between the concentration of 2.4-D (range 0.5-2.0 mg/L) and dry weight of callus, as the lower the concentration of 2.4-D the higher the biomass (Table 2). The leaf explant achieved the highest callus dry weight (18.37 g/L) among all treatments by T_5, which contained 0.6 mg/L NAA in combination with 0.3 mg/L BAP; followed by T_7, as achieved 17.08 g/L (Table 2). With a comparison between cotyledon and leaf, there are significant differences between them in induced biomass. The largest significant difference in the dry weight of callus between them was seen in T_2, as both explants were treated with 1.0 mg/L of 2.4-D alone, resulting in 14.80 g/L from dry callus derived from leaf explant; however, cotyledon explant produced only 6.43 g/L. Conversely, the cotyledon explant achieved a significant difference in dry weight of callus 17.58 g/L compared with leaf explant 15.1 g/L in T_6, which contained 1.2 mg/L NAA in combination with 1.00 mg/L BAP (Table 2). Various explants of the same species might respond differently to the same hormonal treatment due to the specific biochemical and physiological capabilities of different tissues. [21] have recorded Callus induction of *O. basilicum* during the period from 15-20 days following explant inoculation in all treatments. Notably, in our study, NAA showed a critical role in the callogenesis of *O. basilicum* as the treatments containing NAA, which are T_5, T_6 and T_7, showed the highest potential in callus induction in comparison to other treatments either using

cotyledon or leaf explants (Table 2). Hormone NAA critically influences biomass accumulation when using leaf explants of *O. basilicum* [17]. [3] have tested NAA alone in different concentrations (0.1, 1.0, 2.5, 5.0, 10.0 & 20.0 mg/L) where the concentration 2.5 mg/L of NAA achieved a maximum dry weight of callus biomass by leaf explant. The current study emphasized the outcome of [18] those who demonstrated that the callus developed more frequently in the presence of 0.3 BAP combination with 0.6 NAA mg/L as well as the treatment containing 1.0 BAP combination with 1.2 NAA mg/L. Different explants may respond differently due to the physiological capabilities of different tissues. In this study, we have highlighted the cotyledon of *O. basilisum* as an explant that has promising potential in different applications of tissue culture technology. By cotyledon explant, the highest weight of callus induction of *Trachyspermum ammi* was obtained using 0.25 mg/L BAP with 2 mg/L 2,4-D [16]. The response of leaf explants to 2.4-D was better than that of Cotyledon explants, which may be attributed to the tender tissue of the cotyledon, which was detected in T_1, T_2, and T_3 (Table 2). [2] reported that 1.0 mg/l of 2,4-D in combination with 0.25 mg/l Kin produced high callus biomass using leaf explants of *O. basilicum*. After four weeks of leaves culture as an explant in the light or dark, a large callus formation by using various combinations of KIN and 2,4-D have been obtained in MS medium [6]. Medium with a high concentration of 2,4-D alone (up to 1.0 mg/L) produced a remarkable callus from leaf explant [22]. High callus biomass of *O.basilicum* was obtained using MS medium fortuned with 1.0 mg/L 2,4-D combination with 0.5 KIN using nodal explants [2].

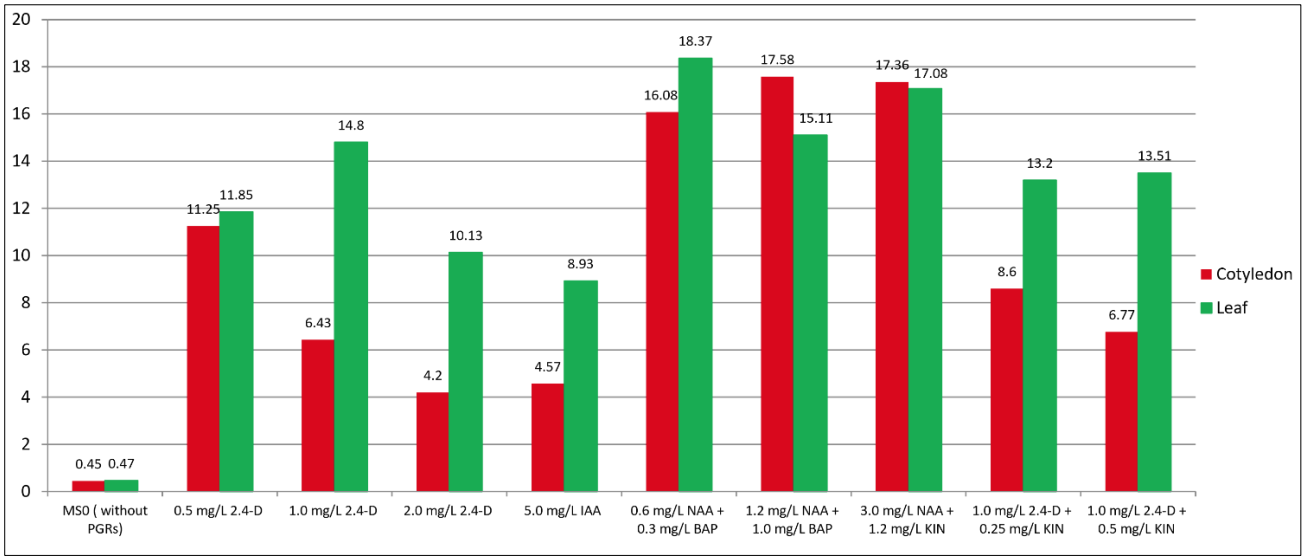

Fig 1 Effect of PGRs on the dry weight of the callus derived from cotyledon and leaf explants of *O. basilicum* (gram/1 litter MS medium)

Table 2 Effect of different PGRs on callus formation derived from cotyledon and leaf explants of *O. basilicum* during eight weeks of culture

Treatments	PGR type and concentration in MS media (mg/L)				Percentage of Callus induced		The dry weight of the callus (gram/1 litter MS medium)		Colour of callus	
					Cotyledon	Leaf	Cotyledon	Leaf	Cotyledon	Leaf
MS0					100	100	ef 0.45 ± 0.08	ef 0.47 ± 0.07	Pale yellow	Pale yellow
T_1	2,4-D	0.5			100	100	bc 11.25 ± 1.75	bc 11.85 ± 1.73	White	Greenish white, brown
T_2	2,4-D	1			100	100	d 6.43 ± 0.36	b 14.80 ± 0.79	White, brown	Greenish white, brown
T_3	2,4-D	2			100	100	de 4.20 ± 0.10	c 10.13 ± 1.99	White, brown	White, brown
T_4	IAA	5			100	100	de 4.57 ± 0.42	cd 8.93 ± 1.27	Dark brown	Dark brown
T_5	NAA	0.6	BAP	0.3	100	100	ab 16.08 ± 0.35	a 18.37 ± 1.03	White, cream, brown	Whitish green, Brown
T_6	NAA	1.2	BAP	1	100	100	a 17.58 ± 1.11	ab 15.11 ± 1.56	Cream	Cream, dark brown
T_7	NAA	3	Kin	1.2	100	100	a 17.36 ± 0.86	a 17.08 ± 0.55	Greenish white, brown	Whitish green, Brown
T_8	2,4-D	1	Kin	0.25	100	100	cd 8.60 ± 0.39	b 13.20 ± 1.91	Cream	White, brown
T_9	2,4-D	1	Kin	0.5	100	100	d 6.77 ± 0.47	b 13.51 ± 0.70	White, brown	Light green, Brown

Values represent mean ± standard error (SE). The results were analyzed through one-way ANOVA. The means with the same letters within each column are not different significantly at ($p < 0.05$) using Tukey's multiple comparison tests. The values were collected in the 8th week. (MS0), i.e., media without PGRs

The morphological characters of the callus reflect the physiological conditions to which it was exposed. Based on that, the morphological variations in callus colour were recorded. After three weeks, the callus derived from cotyledons and leaf of *O. basilicum* were a light colour (green, yellow, white), but in the seventh week, the callus changed from light colours to different degrees of brown colour in almost treatments (Table 2). There were slight significant differences between cotyledon and leaf in callus browning. Generally, the callus derived from the leaf explants was browner than the callus derived from the cotyledon. Noteworthy, the callus in all treatments was compact in density. Still, an irregular white spongy thin layer on the upper surface of the callus in varying degrees was formed in all treatments (Fig 2).

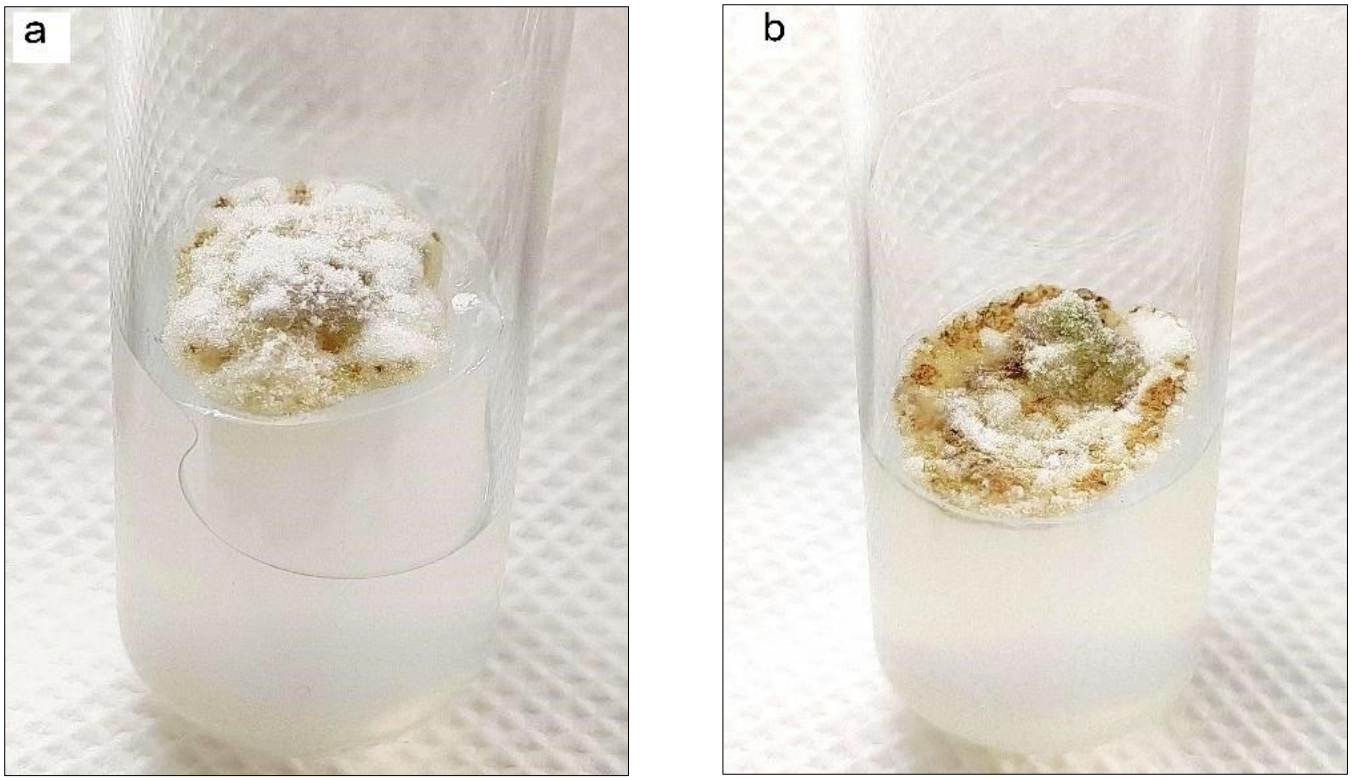

Fig 2 Browning reaction on cotyledon and leaf explants of *O. basilicum* at 7th week from inoculation
a. callus derived from cotyledon explant; **b**. callus derived from leaf explant

CONCLUSION

The present study explored that the cotyledon of *O. basilicum* possesses a strong potential for the formation of callus. It demonstrated that the NAA was an essential factor in achieving the highest significant differences in callus induction of *O. basilicum* either using cotyledon or leaf explant. In contrast hand, 2,4-D acted as an inhibitor of callus formation, particularly if it raised more than 0.5 mg/L. The relationship between the callus's dry weight and the concentration of 2.4-D, which ranges from 0.5-2 mg/L, is inverse

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