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The effect of thidiazuron (TDZ) enhances shoot organogenesis, *in-vitro* flowering, and secondary metabolism accumulation of *Oldenlandia umbellata* L. leaf explants

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Abstract

Oldenlandia umbellata L. is widely used for its medicinal properties, particularly in pharmaceutical industries and conservation endeavors. Our study aimed to enhance *in vitro* plant regeneration techniques by supplementing thidiazuron (TDZ) and cytokinins (CKs) (KI; kinetin, BAP; 6-benzylaminopurine), bolstering conservation efforts and therapeutic applications. The results revealed concentration-dependent responses, with KI (6.96 µM) and BAP (4.44 µM) significantly promoting callus formation, while TDZ (12-18 days) notably accelerated shoot induction. Root induction demonstrated variability based on indole-3-acetic acid (IAA) concentrations, whereas 1-naphthaleneacetic acid (NAA) influenced root formation. Particularly noteworthy were the substantial shifts in growth parameters induced by CK supplementations, including enhanced shoot length, biomass, and modifications in leaf-root ratios. BAP (6.66 µM) notably augmented leaf growth, whereas TDZ (4.54 µM) facilitated root elongation. Additionally, CK supplementation exhibited a stimulating effect on secondary metabolites, thereby enhancing shoot biochemistry. These results shed light on the intricate regulatory mechanisms underlying *O. umbellata* tissue culture, providing valuable insights for tailored conservation strategies and pharmaceutical innovations. Notably, identifying optimal TDZ concentrations underscores its potential for stress-free growth promotion. These findings propel the refinement of tissue culture methodologies, unlocking the therapeutic potential of *O. umbellata* while safeguarding its genetic diversity. Furthermore, the elucidation of such mechanisms serves as a cornerstone for future research endeavors aimed at harnessing the full medicinal potential of this invaluable plant species.

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Introduction

Oldenlandia umbellata L. is a perennial herb in the Rubiaceae family that is characterized by small, delicate white flowers that grow at the tips of its slender stems. Native to the lush terrain of the Indian subcontinent, it flourishes in the warm embrace of tropical and subtropical climates, where it has been assimilated for centuries into the fabric of regional customs. This herb has many therapeutic benefits ingrained in age-old Ayurvedic knowledge. Its roots have long been valued for their therapeutic qualities as a source of compounds such as pigments called rubicholric acid and alizarin. These compounds help extract a vivid red dye, but they are also potent medicines that can be used to treat various illnesses (Siva, 2007).

Siva *et al.* (2009) reported that *O. umbellata* extract effectively treats severe respiratory diseases like asthma and tuberculosis, providing a glimmer of hope to those suffering from these disabling illnesses. The plant's in treating various health diseases is further demonstrated by its leaf infusion into folk medicine to lessen the symptoms of venomous bites (Behera *et al.*, 2018). Even with its great therapeutic potential, *O. umbellata* propagation is still a closely guarded procedure that depends only on seed cultivation. With great care and attention to detail, this plant is harvested before it produces seeds, guaranteeing its lineage's survival and preserving its medicinal heritage (Jayabal *et al.*, 2019).

Plant tissue culture (PTC) techniques, an alternative approach have gained prominence in light of the significance of conserving species like *O. umbellata*, especially given its medicinal importance. By guaranteeing the preservation of this plant species for future generations, these techniques present a promising avenue for their *in vitro* conservation. PTC is the process of growing plant cells, tissues, or organs in a controlled environment to enable precise modification and the inheritance of desired characteristics. In the induction of shoots from explants, a small portion of plant material is used as starting material for tissue culture.

For the *O. umbellata*, shoot induction is crucial to the plant's ability to reproduce and produce genetically identical plants. This process guarantees the preservation of genetic diversity within cultivated populations while facilitating the quick multiplication of desired plant traits.

In PTC, CKs, a class of plant growth regulators, are essential for the induction and development of shoots. Kinetin (KI) and 6-benzylaminopurine (BAP) are two of these CKs that have become important hormones in a variety of plant species for promoting shoot regeneration and flowering induction (Skoog and Miller, 1957; Premkumar *et al.*, 2011). These CKs' ability to promote shoot induction has been thoroughly investigated and proven.

Skoog and Miller (1957) laid the groundwork for the current understanding of CKs' function in PTC by showcasing their capacity to stimulate cell division and encourage the formation of shoots. Our previous research also further clarified the precise mechanisms behind CK-mediated shoot induction and offered insights into how to optimize tissue culture protocols for improved plant regeneration (Premkumar *et al.*, 2011).

Thidiazuron (TDZ) as a cytokinin has become a game-changer in PTC, providing a robust and efficient artificial plant growth regulator for various uses. Dewir *et al.* (2018) reported that TDZ is remarkably effective at the development of somatic embryogenesis, shoot proliferation, and *in vitro* flowering. The response of TDZ in culture medium has been extensively studied over time, with notable progress made in understanding its function in phenol production and accumulation. A more sophisticated manipulation of TDZ to achieve desired results in tissue culture systems has been made possible by this nuanced understanding. However, in addition to its remarkable effectiveness, previous research has highlighted some drawbacks of TDZ; supplementation of this hormone can cause abnormal bud development, which emphasizes the need for careful optimization and dosage control (Wang *et al.*,

1991; Xiang *et al.*, 2012). Notwithstanding these difficulties, TDZ can be used in plant breeding. The idea of *in vitro* flowering, made possible by TDZ, establishes the foundation for cutting-edge techniques, which present viable means of quickening the breeding process (Zulkarnain, 2016). Kshirsagar *et al.* (2017) report that TDZ application can be strategically used to induce multiple shoots with synchronized flowering and maturity, offering valuable tools for improving plant breeding techniques.

The present study aimed to develop an efficient *in vitro* plant regeneration protocol with *in vitro* flowering of *O. umbellata* using TDZ with other plant hormones. This research would enable biodiversity conservation by preserving this dye-producing medicinal herb.

Materials and methods

Plant materials

O. umbellate young leaves were obtained from healthy and field-grown plants from The Botanical Garden, V.H.N. Senthikumara Nadar College, Virudhunagar, Tamil Nadu, India, and used as explants for the initiation of shoot cultures. The explants were carefully selected based on their vigor and absence of visible diseases or abnormalities.

Sterilization

The explants were washed in running water for 30 min and then thoroughly rinsed in a detergent solution (Tween 20) for 5 min. They were then surface sterilized with $HgCl_2$ (0.1%) and 70% ethanol for 2 and 3 min, respectively, followed by thorough washing with sterile distilled water.

Inoculation

Surface sterilized explants were inoculated on Murashige and Skoog's medium (MS medium) (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.8% (w/v) agar Type1 (Hi Media, India) supplemented with various concentrations of CKs including BAP, KI, and TDZ ranged from 2.22 µM to 9.26 µM. The MS medium was adjusted to pH 5.7. The 20 ml of sterile medium was dispensed in a 100 ml sterile glass conical flask. *In vitro* originated shoots from the explants were sub-cultured after 15 days. Shoots taller than 3 cm were transferred to ½ MS medium supplemented with different auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA). All cultures were incubated at 25 ± 2°C with a 16 hr photoperiod under fluorescent light with a photon flux of 52 µmol $m^{-2}s^{-1}$ (Moher *et al.*, 2021). All experiments were repeated thrice with nine explants per conical flask. The cultures were continuously observed for any response. The days required for direct shoot initiation, average number of shoots per explant, percentage of explants producing shoots, average shoot and root length, average number of roots, and rate of rooted plantlets were evaluated in all experiments.

Determination of secondary metabolites

After 6 weeks of culture, total phenol and flavonoids were determined in the produced shoots. The shoot samples were collected, dried at 30 ± 2 °C to a constant dry weight, and ground into powder. Five hundred milligrams of powder were mixed with 10 ml of 100% methanol and then extract was allowed to dry up to 5 ml and then centrifuged at 10,000 rpm. The supernatant was collected and used to determine total phenol and flavonoids.

Total phenol estimation

The total phenol content was determined according to the method of Singleton *et al.* (1999). In brief, 100 µl of extracts were mixed with 100 µl of Folin-Ciocalteu reagent, and after 6 min, 150 µl of a 20% sodium carbonate solution was added to the mixture. After 30 min at room temperature incubation, the total phenol content was estimated and was expressed as mg of pyrogallol per g of DW.

Estimation of total flavonoids

The total flavonoid content was analyzed using a method by Chang *et al.* (2002). In brief, 1.5 ml of methanolic extract and 4.5 ml of distilled water were into a 25 ml flask and mixed with 1 ml of 5% sodium

nitrate solution. After incubation for 6 min, 1 ml of 10% aluminum chloride solution was added to the mixture. The mixture was kept for 6 min before adding 10 ml of 4% sodium hydroxide solutions and fixed to 25 ml with 60% ethanol aqueous. Finally, the mixture was incubated for 15 min, and the absorbance of the mixture solution was measured with a spectrophotometer at 510 nm against a blank containing 5 ml of extraction solvent. The total flavonoid content was expressed as mg quercetin equivalent per g DW.

Quantification of oleanolic acid

The oleanolic acid quantification was done using a modified method by Liang *et al.* (2009). Oleanolic acid was determined in the produced shoots after 6 weeks of culture cultivation. The samples were ground into powder and passed through a 10-mesh (2 mm) sieve. One gram of sample was transferred into a 50 ml centrifuge tube and extracted with 25 ml of acetone by sonication for 60 min at room temperature. Supernatants were collected after centrifugation at 3000 rpm for 10 min. The resultant solid residues were dissolved in methanol and made up to 25 ml volume with methanol. Ten microliters of samples were injected manually into a Shimadzu CBM-20A HPLC system (Kyoto, Japan) equipped with a C18reverse-phase column (5 µm, 4.6 × 250 mm) (Alltech Associates, Waters, MA, USA). The mobile phase was a methanol-water mixture (83:17 containing 0.2% NH_4OAc). The mobile phase was pumped at 1 ml min^{-1}. Absorbance was read at 230 nm by Shimadzu SPD-20A UV-Vis wavelength detector, and data were processed using the software.

Statistical analysis

The results represent the mean (± SD) of the triplicate. The results were analyzed statistically by one-way ANOVA and compared using Duncan's multiple-range tests (DMRT) using the R program (Duncan, 1955).

Results

Effects of CKs on direct shoot induction and flowering

The results showed that different KI concentrations influenced shoot induction and flowering in *O. umbellata*, ranging from 2.22 to 9.26 µM over 21-25 days (Table 1). An optimal concentration of 6.96 µM KI + 4.44 µM BAP can enhance callus development. Shoots per explant peaked at 1.89 ± 0.33 (20.99%) with 9.26 µM KI, but no flowering was induced despite robust shoot growth.

Table 1. *In vitro* response to different concentrations of cytokinins on direct shoot induction and flowering of young leaf explants in *O. umbellate*

Kinetin	Concentration (µM)	Direct shoot induction(days)	Response	Degree of callusing	Shoots per explant (No.)	Explants producing shoots (%)	Flowering (%)
KI	2.32	22	C+S	+	1.22 ± 0.44^{c}	13.58^{gh}	0
	4.65	21	C+S	++	1.78 ± 0.44^{bc}	19.75^{de}	0
	6.96	23	C+S	+++	1.67 ± 0.50^{bc}	18.52^{ef}	0
	9.26	25	C+S	++	1.89 ± 0.33^{bc}	20.99^{d}	0
BAP	2.22	16	C+S	+	1.78 ± 0.44^{bc}	19.75^{de}	0
	4.44	18	C+S	+++	1.11 ± 0.60^{c}	12.35^{h}	0
	6.66	20	C+S	++	1.33 ± 0.50^{bc}	14.81^{g}	0
	8.88	22	C+S	++	1.11 ± 0.33^{c}	12.35^{h}	0
TDZ	**2.27**	15	S+F	–	5.03 ± 0.50^{a}	55.86^{a}	41.67^{c} ± 0.55
	4.54	**12**	S+F	–	3.00 ± 0.70^{b}	33.33^{b}	80.56^{a} ± 0.41
	6.81	16	S+F	–	2.44 ± 0.53^{bc}	27.16^{c}	47.22^{b} ± 0.41
	9.08	18	C+S+F	++	1.56 ± 0.73^{bc}	17.28^{f}	11.11^{d} ± 0.52

Young leaves explants were cultured on an MS medium supplemented with various KI, BAP, and TDZ concentrations. Triplicates were used per treatment. S = shoot, C = callus, and F = flowering + prolonged callus growth, + + slow callus growth, and +++ fast callus growth. Variability around the mean was represented as ± SD. Duncan's multiple comparison tests did not significantly differ in data with the same letter in a column (P<0.05).

BAP concentrations ranging from 2.22 to 8.88 µM exhibit similar concentration-dependent trends as KI. BAP induces shoot formation within 16 to 22 days, a shorter period than KI. Higher BAP concentrations increase callus formation, with the highest shoot production (1.78 ± 0.44; 19.75%) at 2.22 µM BAP. Like KI, no flowering is observed with any BAP treatment (Table 1). The rapid callus growth is also evident at 4.44 µM KI. TDZ concentrations ranging from 2.27 to 9.08 µM elicit unique responses, with shoot induction taking 12 to 18 days, a shorter period than KI and BAP. Notably, TDZ induces flowering, peaking at 80.56% for 4.54 µM TDZ, reducing the average shoots per explant to 3.00 ± 0.70 (33.33%). However, at 2.27 µM TDZ, the average shoots per explant peak at 5.03 ± 0.50 (55.86%) (Table 1).

Table 2. Effect of different concentrations of auxin on root induction of *O. umbellate*

Auxin	Concentration (µM)	Average no. of roots	Rooted plantlets (%)
IAA	2.85	0.83 ± 0.41^{c}	13.89^{f}
	5.71	2.00 ± 0.63^{bc}	33.33^{d}
	8.56	0.67 ± 0.52^{c}	11.11^{g}
IBA	2.46	2.33 ± 0.82^{bc}	38.89^{c}
	4.92	2.67 ± 0.52^{b}	44.44^{b}
	7.38	0.83 ± 0.41^{c}	13.89^{f}
NAA	2.68	4.67 ± 0.82^{a}	77.78^{a}
	5.37	2.00 ± 0.00^{bc}	33.33^{d}
	8.05	1.00 ± 0.63^{bc}	16.67^{e}

Triplicates were used per treatment; variability around the mean was represented as ± SD. Duncan's multiple comparison tests did not significantly differ in data with the same letter in a column ($P<0.05$).

Effects of different concentrations of auxins on root induction

The varying concentrations of IAA influenced root induction in *O. umbellata*, with concentrations ranging from 2.85 to 8.56 µM showing notable trends (Table 2). At 2.85 µM, a lower concentration, moderate root initiation was observed (0.83 ± 0.41 roots per plantlet, 13.89% rooted). Increasing IAA to 5.71 µM led to a significant rise in root induction (2.00 ± 0.63 roots per plantlet, 33.33% rooted). However, at 8.56 µM IAA, root numbers declined (0.67 ± 0.52 roots per plantlet, 11.11% rooted). The response to IBA concentrations followed a similar trend to IAA. At 2.46 µM IBA, root induction increased significantly to 2.33 ± 0.82 roots per plantlet, with a higher percentage of rooted plantlets at 38.89%. This trend continued at 4.92 µM IBA, with the average roots per plantlet rising to 2.67 ± 0.52 and the percentage of rooted plantlets increasing to 44.44%. However, at 7.38 µM IBA, both the average roots per plantlet (0.83 ± 0.41) and the percentage of rooted plantlets (13.89%) decreased. NAA concentrations (2.68 to 8.05 µM) showed distinct patterns in root induction compared to IAA and IBA, with a concentration-dependent effect on *O. umbellata*. At 2.68 µM NAA, substantial root initiation was observed at 4.67 ± 0.82 roots per plantlet, with most plantlets (77.78%) developing roots. However, at 5.37 µM NAA, the average roots per plantlet decreased to 2.00 ± 0.00, and the percentage of rooted plantlets dropped to 33.33%. Interestingly, at 8.05 µM NAA, both the average roots per plantlet (1.00 ± 0.63) and the percentage of rooted plantlets (16.67%) decreased further (Table 2).

Effect of various concentrations of CKs on morphometric parameters in O. umbellata

The CK supplementation on *O. umbellata* regenerated plants from leaf explants showed significant variations in growth parameters. Shoot length decreased with higher KI concentrations, from 2.23 ± 0.35 cm at 9.26 µM KI to 2.80 ± 0.10 cm at 4.65 µM KI, with 2.32 µM KI having a relatively higher shoot length of 2.70 ± 0.10 cm (Table 3). Fresh weights decreased with increasing KI, showed 1.12 ± 0.54 g at 9.26 µM KI *vs.* 1.48 ± 0.15 g at 4.65 µM KI. Dry weights mirrored this trend, declining from 0.35 ± 0.02 g to 0.67 ± 0.02 g over the same KI range. Higher KI concentrations resulted in fewer leaves, with 6.96 µM showing approximately 6.67 ± 1.15 leaves compared to 3.67 ± 0.58 leaves at 9.26 µM KI. Root lengths varied, with lower concentrations (2.32 µM) yielding longer roots (2.17 ± 0.15 cm) than higher concentrations 9.26 µM KI, which led to shorter roots (2.00 ± 0.10 cm). Fresh and dry weights of roots decreased with higher KI concentrations, the root fresh weights were 1.23 ± 0.02 g and 1.79 ± 0.09 g, at 9.26 µM and 4.65 µM KI, respectively (Table 3).

Table 3. Morphometric analysis of regenerated plants from young leaves explants of *O. umbellata*

Treatments	Regenerated shoots				Regenerated roots		
	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	No. of leaves	Root length (cm)	Fresh weight (g)	Dry weight (g)
KI (µM)							
2.32	2.70 ± 0.10	1.35 ± 0.16	0.53 ± 0.02	5.67 ± 1.53	2.17 ± 0.15	1.58 ± 0.03	0.66 ± 0.02
4.65	2.80 ± 0.10	1.48 ± 0.15	0.67 ± 0.02	6.00 ± 2.00	2.63 ± 0.15	1.79 ± 0.09	0.85 ± 0.04
6.96	2.53 ± 0.15	1.16 ± 0.29	0.45 ± 0.02	6.67 ± 1.15	2.80 ± 0.10	1.25 ± 0.09	0.59 ± 0.01
9.26	2.23 ± 0.35	1.12 ± 0.54	0.35 ± 0.02	3.67 ± 0.58	2.00 ± 0.10	1.23 ± 0.02	0.62 ± 0.02
BAP (µM)							
2.22	3.50 ± 0.20	1.99 ± 0.37	0.97 ± 0.01	4.00 ± 0.00	1.90 ± 0.10	1.35 ± 0.03	0.45 ± 0.04
4.44	3.86 ± 0.10	2.19 ± 0.55	1.24 ± 0.05	4.03 ± 0.15	2.03 ± 0.12	1.21 ± 0.07	0.24 ± 0.01
6.66	3.10 ± 0.26	2.45 ± 0.34	1.53 ± 0.01	5.00 ± 0.10	2.70 ± 0.10	1.15 ± 0.02	0.34 ± 0.05
8.88	3.30 ± 0.10	2.13 ± 0.46	1.57 ± 0.01	4.00 ± 0.10	2.20 ± 0.30	1.09 ± 0.02	0.43 ± 0.02
TDZ (µM)							
2.27	3.73 ± 0.10	2.97 ± 0.21	1.95 ± 0.03	6.00 ± 2.00	2.27 ± 0.21	2.15 ± 0.02	1.16 ± 0.04
4.54	2.50 ± 0.26	2.67 ± 0.17	1.73 ± 0.01	7.03 ± 0.06	2.47 ± 0.06	2.54 ± 0.02	1.71 ± 0.05
6.81	1.50 ± 0.20	2.55 ± 0.09	1.52 ± 0.01	4.00 ± 0.10	1.73 ± 0.21	2.36 ± 0.21	1.62 ± 0.10
9.08	1.60 ± 0.17	2.21 ± 0.33	1.50 ± 0.01	3.93 ± 0.15	1.67 ± 0.25	1.76 ± 0.10	0.83 ± 0.35

Triplicates were used per treatment; variability around the mean was represented as ± SD.

BAP supplementation on *O. umbellata* regenerated plants showed significant variations in growth parameters. Shoot lengths ranged from 3.10 ± 0.26 cm to 3.86 ± 0.10 cm, with the highest observed at 4.44 µM BAP. Fresh weights varied from 1.99 ± 0.37 g to 2.45 ± 0.34 g, peaking at 6.66 µM BAP. Dry weights ranged from 0.97 ± 0.01 g to 1.57 ± 0.01 g, with the highest observed at 8.88 µM BAP. Leaf counts ranged from 4.00 ± 0.00 to 5.00 ± 0.10, the highest number of leaves are noticed with 6.66 µM BAP. Root lengths varied from 1.90 ± 0.10 cm to 2.70 ± 0.10 cm, the longest at 6.66 µM BAP. Fresh weights of roots ranged from 1.09 ± 0.02 g to 1.35 ± 0.03 g, a significant high was observed at 2.22 µM BAP, and dry weights ranged from 0.24 ± 0.01 g to 0.45 ± 0.04 g, also highest at 2.22 µM BAP (Table 3).

Shoot lengths varied widely, from 1.50 ± 0.20 cm to 3.73 ± 0.10 cm across various TDZ concentrations, 2.27 to 9.08 µM. The highest shoot length of 3.73 ± 0.10 cm was noted at 2.27 µM TDZ. Fresh weights ranged from 2.21 ± 0.33 g to 2.97 ± 0.21 g, peaking at 2.27 µM TDZ with 2.97 ± 0.21 g. Dry weights ranged from 1.50 ± 0.01 g to 1.95 ± 0.03 g, with the highest at 2.27 µM TDZ. Leaf counts varied from 3.93 ± 0.15 to 7.03 ± 0.06 leaves, with the highest count at 4.54 µM TDZ. Root lengths ranged widely from 1.67 ± 0.25 cm to 2.47 ± 0.06 cm across TDZ concentrations. The longest roots, 2.47 ± 0.06 cm, were seen at 4.54 µM TDZ. Fresh weights varied from 1.76 ± 0.10 g to 2.54 ± 0.02 g, peaking at 4.54 µM TDZ with 2.54 ± 0.02 g. Similarly, dry weights ranged from 0.83 ± 0.35 g to 1.71 ± 0.05 g, the highest was seen at 4.54 µM TDZ (Table 3).

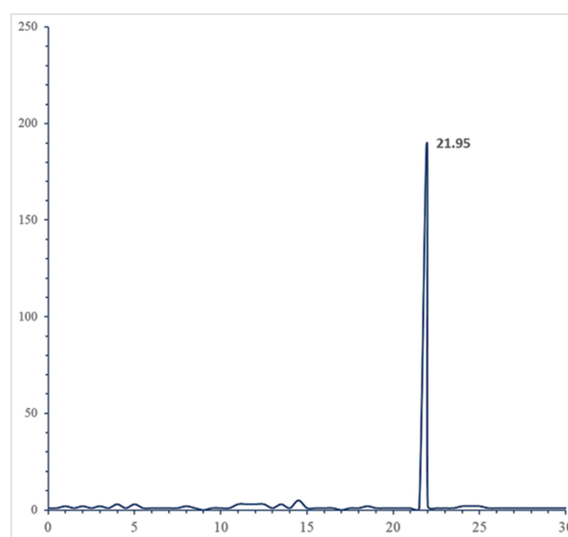

Fig. 1. HPLC chromatogram of standard oleanolic acid

Effects of CKs in secondary metabolites of O. umbellata

Fig. 1 shows the HPLC standard graph for oleanolic acid, highlighting HPLC's efficacy in compound identification. Total flavonoid content varied with KI concentration, peaking at 6.96 µM KI (90.75 ± 2.38 mg/g) and decreasing at 9.26 µM KI (75.20 ±

2.84 mg/g). Similarly, total phenols and oleanolic acid peaked at 2.32 µM KI (3.43 ± 0.59 and 1.83 ± 0.06 mg/g, respectively), declining notably at 9.26 µM KI (0.83 ± 0.29 and 1.65 ± 0.09 mg/g, respectively) (Table 4).

In BAP supplementation, total flavonoids increased at 2.22 µM BAP (91.67 ± 2.08 mg/g) compared to the wild plant (54.33 ± 2.08 mg/g). However, at higher BAP concentrations, flavonoid content gradually decreased. Total phenols peaked at 2.22 µM BAP (4.33 ± 0.42 mg/g) and decreased at 8.88 µM BAP (72.20 ± 2.31 mg/g). Oleanolic acid content varied slightly across BAP concentrations, highest at 6.66 µM BAP (1.26 ± 0.20 mg/g). In TDZ supplementation, total flavonoids peaked at 6.81 µM TDZ (141.53 ± 3.27 mg/g). Total phenols and oleanolic acid also peaked at 2.27 µM TDZ before decreasing at higher concentrations. Compound content fluctuated across concentrations but remained notably higher than the wild plant (Table 4).

Table 4. Various KI, BAP, and TDZ concentrations affect total flavonoid, phenolic, and oleanolic acid content in the produced shoots

Treatments	Total flavonoids (mg/g)	Total phenols (mg/g)	Oleanolic acid (mg/g)
KI (µm)			
2.32	81.73 ± 2.19	3.43 ± 0.59	1.83 ± 0.06
4.65	84.17 ± 2.22	2.30 ± 0.26	1.75 ± 0.05
6.96	90.75 ± 2.38	2.33 ± 0.31	1.77 ± 0.15
9.26	75.20 ± 2.84	0.83 ± 0.29	1.65 ± 0.09
BAP (µm)			
2.22	91.67 ± 2.08	4.33 ± 0.42	1.29 ± 0.10
4.44	82.03 ± 1.95	4.33 ± 0.15	1.23 ± 0.12
6.66	85.40 ± 2.52	3.27 ± 0.31	1.64 ± 0.12
8.88	72.20 ± 2.31	1.33 ± 0.15	1.26 ± 0.20
TDZ (µm)			
2.27	105.07 ± 4.90	7.13 ± 0.71	2.63 ± 0.15
4.54	112.17 ± 3.80	6.13 ± 0.32	1.85 ± 0.13
6.81	141.53 ± 3.27	4.13 ± 0.32	1.77 ± 0.13
9.08	121.67 ± 2.57	3.90 ± 0.10	1.54 ± 0.08
Wild plant	54.33 ± 2.08	2.33 ± 0.42	1.55 ± 0.11

Triplicates were used per treatment; variability around the mean was represented as ± SD.

Fig. 2 illustrates how different TDZ levels influenced callus induction and subsequent shoot growth. After 18 days, successful callus initiation occurred at a low TDZ concentration of 2.27 µM (Fig. 2a), stimulating cell dedifferentiation and callus formation. The experiment revealed a dose-dependent response to TDZ; higher concentrations led to growth inhibition, notably at 4.54 µM (Fig. 2b), intensifying at 6.81 µM (Fig. 2c) and 9.08 µM (Fig. 2d), resulting in stunted growth. Surprisingly, 2.27 µM TDZ facilitated elongated shoot induction and robust plantlet development of *O. umbellata* (Fig. 2e).

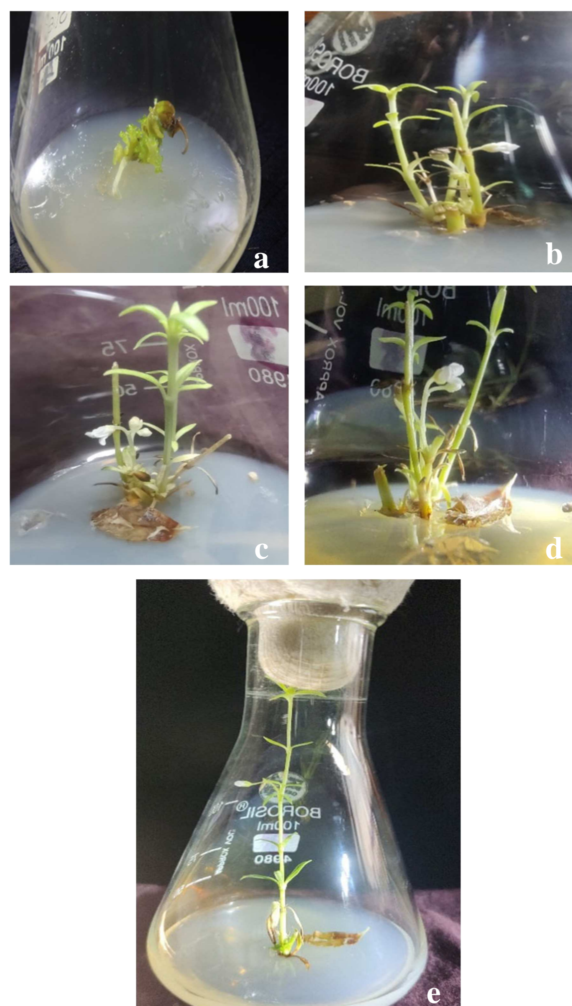

Fig. 2 (a-e). Callus induction and shoot growth in various TDZ concentrations

Discussion

Plant growth regulators, including CKs, including, KI, BAP, and TDZ, and auxins such as IAA, IBA, and NAA, play pivotal roles in plant tissue culture. They manipulate processes like cell division, shoot and root formation, and overall growth, essential for tissue culture propagation and genetic transformation (Small *et al.*, 2018). The balance between these hormones is crucial for growth and development and

is critical in achieving induction of shoot proliferation, root formation, and plant regeneration. In our study, KI influences shoot induction and callus formation, with an optimal concentration of 6.96 µM triggering specific gene expression patterns linked to cell proliferation and differentiation (Patel, 2021). This leads to notable callus formation and increased shoot numbers per explant, although it may not activate pathways essential for floral development. BAP promotes cell division and shoot initiation as a CK, exhibiting a dose-responsive effect on shoot induction and callus growth. The study's integration of efficiency assessment using CKs, auxins, and associated secondary metabolism is significant for conserving this medicinal plant, *O. umbellata* (Varghese *et al.*, 2022).

In the current study, the trade-off between shoot number and flowering induction at 4.54 µM TDZ reflects intricate hormonal interactions and regulatory mechanisms affecting plant development. Variability in the days needed for shoot induction with different hormones indicates varying potency and efficiency in stimulating growth processes. Moreover, concentration-dependent trends in callus formation and shoot induction underscore the crucial role of hormonal balance and precise regulation for achieving desired outcomes (Magioli *et al.*, 1998). Similar research has been done previously on using varying concentrations of CKs and auxins in conjunction with optimal growth regulators like TDZ. Siva *et al.* (2009) reported that coconut milk has been found to enhance callus induction, shoot development, and rooting in *O. umbellata*. Furthermore, a liquid medium supplemented with BAP, and other growth hormones has been validated for enhancing shoot amplification, improving flowering, and facilitating *ex vitro* rooting in *O. umbellata* (Jayabal *et al.*, 2019).

In the current study, the effects observed with varying concentrations of IAA, IBA, and NAA on root induction in *O. umbellata* are influenced by their specific mechanisms of action and concentration-dependent responses. Lower concentrations, like 2.85 µM IAA and 2.46 µM IBA, led to moderate root initiation, indicating a threshold for induction without compromising plant growth. As auxin concentrations increased (5.71 µM IAA, 4.92 µM IBA), root induction significantly improved, showing a dose-dependent relationship. Higher auxin levels may activate genes crucial for root initiation and growth, resulting in more roots per plantlet and increased rootedness. Conversely, higher concentrations (8.56 µM IAA, 7.38 µM IBA, 8.05 µM NAA) decreased root induction, possibly due to excessive levels of auxin inhibiting root development pathways. These differential responses across auxins stem from their varying receptor affinities and downstream signaling pathway activations. In tissue culture, optimizing auxin concentrations is crucial for desired root induction and plant growth. Similar findings were noted using quercetin to enhance shoot production, elucidating apical dominance (Saranya Krishnan *et al.*, 2017).

The inhibitory effect on shoot elongation in *O. umbellata* as KI concentration increases likely stems from its impact on cell division and differentiation pathways. In the present study, higher KI concentrations may disrupt these processes, reducing shoot length and biomass production. This is evident in the range of shoot lengths observed, from 2.23 ± 0.35 cm at 9.26 µM KI to 2.80 ± 0.10 cm at 4.65 µM KI, highlighting a dose-dependent trend and emphasizing the importance of precise concentration adjustments in tissue culture media. The variability in shoot length underscores *O. umbellata*'s nuanced response to different KI concentrations, further stressing the need for careful concentration optimization in tissue culture experiments to achieve desired growth outcomes.

In addition, the concentration-dependent effects observed in BAP supplementation highlight its stimulatory impact on shoot elongation and leaf development, evident from the variability in shoot length peaking at 4.44 µM BAP. This optimal condition promotes shoot growth and enhances leaf development, possibly influencing meristem activity

and leaf primordia initiation. These responses stress the need for selecting appropriate BAP concentrations to maximize shoot and leaf formation in tissue culture experiments. Conversely, the wide variation in shoot lengths and root parameters seen in TDZ supplementations reflects the diverse effects on plant growth. Its concentration-dependent impact on shoot elongation indicates a role in modulating shoot development pathways. Moreover, significant variations in root lengths and biomass accumulation across different TDZ concentrations suggest its potential to influence root growth and biomass production. These responses underline the importance of carefully optimizing TDZ concentrations for desired growth outcomes in tissue culture systems.

The fluctuations in the contents of total flavonoids, total phenols, and oleanolic acid in shoots under varying concentrations of KI, BAP, and TDZ can be attributed to their intricate effects on biochemical pathways. KI exhibited distinct changes in shoot composition, with the peak in total flavonoids, phenols, and oleanolic acid content observed at 2.32 µM KI, indicating an optimal range for stimulating synthesis. However, higher KI concentrations disrupted these metabolites, possibly interfering with their biosynthesis pathways. Similarly, BAP stimulated flavonoids and phenol content at lower concentrations, notably 2.22 µM BAP, likely enhancing synthesis enzyme activity. However, higher BAP concentrations decreased these compounds, indicating a regulatory role that may become inhibitory or induce metabolic shifts.

On the other hand, TDZ demonstrated a concentration-dependent surge in flavonoids, phenols, and oleanolic acid content, peaking at 6.81 µM TDZ. This concentration optimizes enzyme activity for compound synthesis. However, at higher TDZ concentrations, the content declined, hinting at a regulatory mechanism where excessive TDZ might inhibit enzymes or redirect metabolic pathways (Magioli *et al.*, 1998). This intricate balance underscores the interplay between PGRs and biochemical pathways in *O. umbellata* shoots, emphasizing the need for precise concentration optimization in plant tissue culture for targeted metabolite production.

The observed accumulation of secondary metabolites aligns with previous findings in both *in vitro* regeneration and field trials involving *O. umbellata* (Jayabal *et al.*, 2019). Studies on mass multiplication using leaf explants of *O. umbellata* and varying concentrations of IAA, IBA, NAA, or 2,4-D for callus induction indicated that 10 µM NAA resulted in better acclimatization (Saranya *et al.*, 2019). Additionally, experiments using coconut milk and NAA led to high callus induction, contributing to enhance *in vitro* flowering of *O. umbellata* (Behera *et al.*, 2018).

The varying effects observed with different TDZ concentrations on *O. umbellata* plant tissues can be attributed to the intricate mechanisms by which TDZ interacts with plant cells, influencing and regulating growth processes. The successful initiation of callus formation at a relatively low TDZ concentration of 2.27 µM indicates TDZ's critical role in stimulating the dedifferentiation process within plant cells. This process involves transforming specialized cells into undifferentiated cells, leading to the formation of callus tissue. Utilizing elicitors could potentially enhance the industrial applications of this medicinal herb. For instance, the synthesis of anthraquinones has facilitated large-scale production using *O. umbellata* (Krishnan and Siril, 2018).

TDZ activates specific genes or signaling pathways that initiate the dedifferentiation process, transforming cells into a pluripotent state capable of forming calluses. As TDZ concentration increases, diverse responses occur due to its modulatory effects on cellular processes. For instance, .54 µM TDZ shows initial growth inhibition in shoots, potentially impacting cell division and elongation via CK signaling pathways (Magioli *et al.*, 1998). More pronounced growth inhibition at higher TDZ concentrations, such as 6.81 µM and 9.08 µM, indicates a saturation of TDZ's regulatory effects.

These concentrations may disrupt normal cellular processes, leading to stunted growth and reduced shoot development. Higher TDZ concentrations might also disturb hormonal balances or trigger stress responses, hindering proper growth and differentiation. The morphometric analyses corroborate these observations from our study, highlighting the effective lab-to-field transition and *in vitro* regeneration of *O. umbellata*, contributing to biodiversity conservation (Revathi *et al.*, 2018).

The 2.27 μM TDZ notably facilitated elongated shoot induction and robust plantlet development. This optimal concentration likely fine-tuned hormonal balances and signaling pathways, promoting desirable growth responses without inducing excessive growth inhibition or stress effects. This optimal TDZ concentration balances cell proliferation and differentiation, resulting in successful shoot elongation and plantlet development.

Conclusion

Various concentrations of KI, BAP, TDZ, IAA, IBA, and NAA were tested on *O. umbellata* shoot induction, *in vitro* flowering, and plant development. The concentration-dependent responses show that plant growth regulators must be balanced to get the expected outcomes. Because KI supplementations inhibited shoot elongation and leaf production concentration independently, concentration optimization is essential to avoid growth inhibition. BAP increased shoot length and leaf development at optimal concentrations, emphasizing the importance of concentration. We must optimize tissue culture systems to achieve desired growth outcomes, as TDZ supplementations had different effects on shoot elongation and root growth. Our study showed total flavonoids, phenols, and oleanolic acid variations at KI, BAP, and TDZ concentrations. This illustrates how plant growth regulators affect biochemical pathways and how precise concentration control produces targeted biochemicals. This study shows how complex plant growth regulation is and how concentration optimization in tissue culture is crucial. Understanding hormone concentration-dependent responses is necessary for tissue culture propagation, genetic transformation, and secondary metabolite production in *O. umbellata*. Our study supports CK and auxin *in vitro* experiments to preserve this dye-yielding medicinal herb.

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