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An influence of coco peat and vermicompost on successful *Ex vitro* rooting of micropropagated plantlets of *Oxystelma esculentum* R. Br

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Abstract

In the present study a simple and efficient *in vitro* propagation system was developed from nodal explants of *Oxystelma esculentum* R. Br by using different plant growth regulators. The nodal explants inoculated on the 6-benzyladenine phosphate and Kinetin individually and with combinations for an induction of shoots. The MS medium supplemented with 1.0mg/L of BAP with 1.0 mg/L of Kinetin combinations inducing (95%) the shoots effectively with the shoot length of 7.7±2.2. The well-developed shoots were cut and transferred to IAA (Indole-3-acetic acid) & IBA (Indole-3-butyric acid) individually and with combinations of half strength MS medium for root induction. The high percentage (95%) of rooting was observed in 0.5 mg/L IBA concentration in the mean number of 15.8±1.4 with 12.10±3.3 root length. Simultaneously the well elongated shoots were treating with rooting hormones with various concentrations for 5 minutes and transferred to hardening with the use of coco peat and vermicompost combined with soil with different ratios. The high mean numbers of roots were observed on the combinations of NAA and IBA (200 mg each) concentrations then the 100% survival rate was observed on soil:cocopeat: vermicompost (2:1:1) ratios after 8 weeks of hardening.

Keywords: *In vitro* propagation, medicinal plant, *oxystelma esculentum*, rooting, secondary metabolites, shoot regeneration

Introduction

Climbers are poor stem plants that need support for upright growth and are characterized by types of growth such as tendrillar, twin, scramblers, gripping, adhesive and woody climbers (lianas) (Ali *et al*. 2016) [4]. In Ayurveda, Sidha and Unani traditional medicinal systems, several climber species are useful as herbal remedies (Caesar and Cech 2019) [9]. These are currently under risk of extinction due to habitat loss from overexploitation, climate change, and deforestation, degradation of the atmosphere and occupation of alien life form (Barik *et al*. 2018) [6]. Among these medicinal herbs, *Oxystelma esculentum* R. Br. is a threatened medicinal climber (Senthilkumar et.al., 2009), and produces milky substances in all parts of the plant, commonly called as 'Jaldudhi', distributed in China, Sri Lanka, South Asian countries, South Africa, lower hills of India. It is a perennial twinning edible herb and belonging to Apocynaceae family (Lansdown 2011; Panda 2019; The Plant List 2021) [24]. *O. esculentum* was listed as a least concern medicinal plant by an IUCN (IUCN 2011; Sarvalingam and Rajendran 2016) [4]. Several publications were stated that this plant has been considered as threatened in India and Pakistan owing to the therapeutic necessity of anthropological degradation, pregnane glycosides and many medicinal uses. (Rawat 2014; Zoq-ul-Arfeen *et al*. 2015; Shah and Wani 2016; Shahzad *et al*. 2016; Panda 2019). Whereas, Jayaprakash *et al*. (2021) recently stated that this species is under categorized as a 'rare'. *O. esculentum* has also been reported to possess good therapeutic action against many ailments (Pandya and Anand, 2001) [29]. It has the ability to cure various diseases such as antiseptic, depurative, diuretic, laxative, aphrodisiac, anthelmintic, lactagogue, indigestible, causes flatulence, it is also useful in leucoderma, bronchitis, antitussive, spermatogenic, inhibits lipid peroxidation and antileprotic agent (Kumar *et al*. 2010). Being a threatened and medicinal significance, the plant has been consuming from the wild. The conventional propagation for instance cutting, layering and grafting are problematic task due to their physiological nature. To neglect this problem, *in vitro* propagation/regeneration of cells or tissues or organs is a possible alternative system and has been used to produce large number of regenerants. Over the last decade, *in vitro* propagation technology has been effectively used in the Apocynaceae/Asclepiadaceae members for germplasm conservation (Phulwaria *et al*. 2013; Shekhawat and Manokari 2016; Deepa and Thomas 2020). Few investigations on *in vitro* regeneration of *O. esculentum* have already made (Dharmendra *et al*. 2010; Maliga and Yogananth, 2016; Senthil *et al*. 2009) [15, 37]. However, *in vitro* rooting of *O. esculentum* was marginally challenging due to their phenolic elucidation. Whereas exvitro rooting is an alternative technique to reduces the production cost, time and resources (Shekhawat *et al*. 2015; Panwar *et al*. 2018) [36, 33]. So the aim of this study is communicates an efficiency of *ex vitro* rooting and acclimatization of *Oxystelma esculentum* influenced by Coco peat and vermicompost.

Materials and Methods

Collection and Surface Sterilization

The medicinal plant *Oxystelma esculentum* R. Br. were collected from the river sides of Tirukkattupalli Village,

Tanjore District. The collected material were identified and authenticated in Rapinat Herbarium, St. Joseph's College, Tiruchirappalli (Fig.1). The healthy stem explants of field grown plants *Oxystelma esculentum* R. Br. were thoroughly washed with tap water for 2-3 times to remove any extraneous materials followed by immersion in teepol solution (Reckit Benckiser Pvt. Ltd., Himachal Pradesh, India) for five minutes then washing with distilled water and then for 3 minutes the explant was washed with 5% Bavestin, which is a fungicide used to remove any fungus present in an explant then washed with distilled water. After washing with distilled water, stem explants were again washed in 70% alcohol for few seconds and rinsed three times with distilled water. The stem explants were brought to the inoculation chamber and surface sterilized with 0.1% $HgCl_2$ for 2-3 min and again washed with double sterile distilled water for 5 times to removing the $HgCl_2$ traces (Dubey, 1999) [16].

Growth hormones used

The BAP, KIN (cytokinins) and IAA, IBA (auxins) were used as growth regulators in the culture medium. These are all dissolved by using 0.1N NaoH (Hi-Media) solution.

Culture Media and Conditions

The Basal MS (Murashige and Skoog, 1962) [27] medium containing sucrose was added to a final concentration of 3% (30 g/L). The pH of the medium was adjusted to 5.7 and the adjustment was done with 0.1N HCl (Spectrum Reagents and Chemicals Pvt Ltd., Cochin, India) 0.1 NaOH (Hi-Media). After the pH adjustment 0.8% (w/v) agar was added and heated at 60º C to dissolving the agar and autoclaved for 15 minutes at 15 psi/121ºC. The cultures were maintained at a temperature of 25±2°C relative humidity in the culture room. A rhythmic cycle of 16 hours light followed by 8 hours darkness was given to the cultures. Light was provided by cool white fluorescent tubes (50µmol $m^{-2}s^{-2}$ Photon Flux Density). The sterilized of MS medium with various concentrations of growth hormones were dispensed in Culture tubes.

Sterilization and Inoculation

Forceps, Scalpel *etc*., autoclaved (Atlantis Applications Engg. Pvt. Ltd., New Delhi, India) at 121ºC in 15 lps for 20 min. The inoculation was carried out in laminar air flow chamber. Before starting inoculation, the surface and two sides of the chamber were swiped with alcohol and then all the required equipment's/materials (sterilized forceps, petriplates, sterile blade, Scalpel, sterile distilled water and spirit lamp) were transferred to laminar air flow chamber then the door was tightly closed. After that the UV light was switched on for 15 min. When inoculation, the equipment's were sterilized by dipping in 95% alcohol followed by flaming and cooling. Before starting the inoculation, hands were cleaned with alcohol and the inoculation was carried out in vicinity of the flame. The sterilized explants were placed on the medium at the center of culture tubes. In each concentrations 20 number of explants were cultured.

***In vitro* Shooting and Rooting**

The nodal explants were inoculated and cultured on MS medium containing 100 mg/L^{-1} activated charcoal with 0.5 to 2.5 mg/L^{-1} BAP and KIN (Hi-Media) individually and 0.5 to 2.5 mg/L^{-1} BAP combined with 1.0 mg/L^{-1} KIN. The shoot numbers per explants and length of the shoots were noted after 4 weeks from culture. For *In vitro* rooting the 5-7 cm length of propagated shoots were transferred to the half-strength MS medium comprising 100 mg/L^{-1} activated charcoal with 0.5 to 2.5 mg/L^{-1} IAA and IBA individually and combination of 0.5 to 2.5 mg/L^{-1} IBA with 0.5 mg/L^{-1} IAA. After one week of shoots transfer to rooting medium, the percentage, mean number of roots per shoot were noted.

***Ex-vitro* rooting**

The cutting tips of shoots with a shoot length of 4-5 cm were carefully extracted from shoot clusters and treated with auxins for ex vitro root induction (IBA, IAA and NAA). For 5 minutes, the shoot bases (4-5 mm) were treated with various concentrations (50-250 mg L-1) of IBA, IAA, and NAA alone or in combination. Auxin-treated shoots were placed in autoclaved coco peat and vermicompost mixed soil containing bottles that had been wet with one-fourth strength MS basal salts. These were placed directly in the greenhouse for roots and hardening of the plantlets.

Hardening/Acclimatization

After the development of shoots and roots the cultured plantlets were acclimatized in pots which contains the mixtures of soil and vermicast (Vermicompost yard, St. Joseph's College, Tiruchirappalli) in 1:1 v/v. For one week quarter strength liquid medium was sprayed on this potted medium for fertilizing the shoots and maintained in green house condition. After 2 weeks the potted plantlets were transferred to the field.

Statistical analysis

The results were recorded after 25 days from inoculation and each experiment was noted as in triplicates. The mean number of shoots and roots with standard deviation was calculated by using Online SD calculator (EasyCalculation.com). Duncan's multiple range test (DMRT) was used to compare the treatment means at the level of 5% probability. (Gomez and Gomez, 1976) [19].

Results and Discussion

Effect of auxins and cytokinins in shoot proliferation and multiplication

The least concern medicinal plant, (Lansdown, 2011; Sarvalingam and Rajendren, 2016) [24] *Oxystelma esculentum* L**.** were inoculated in MS medium supplemented with different concentrations of BAP and KIN individually (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L^{-1}) and with combinations (0.5+1.0, 1.0+1.0, 1.5+1.0, 2.0+1.0, 2.5+1.0 mg/L^{-1}). The shoot formation was initiated within 15 days from inoculation period (Fig.2). Among the various concentrations and combinations of the growth regulators used, the percentage of explants responded in shooting ranged from 55% to 90% and the highest number of explants in shoot induction (18/20) with the percentage of 95% was observed in the combinations of BAP and KIN containing MS medium (1.0+1.0 mg/L) and its highest shoot number is 5±0.7 shoots per explant. Next to this the best number of explants (18/20) with the percentage of 90% were responded in the concentration of 2.0 mg/L^{-1} BAP (4.3±1.3 shoots per explant) followed by 1.5 mg/L (4±1.5 shoots per explant) and 0.5mg/L (3.5±1.5 shoots per explant) (Table -1). Different concentrations of kinetin supplemented MS medium provided the best shoot

proliferation, elongation and the percentage of shooting ranges from 65% to 85% (Table – 1). The next best shooting and more number of explant responses (17/20) were observed in the concentration of 1.0 mg/L^{-1} KIN and the percentage is 85% with the mean number of 4.1±1.6 shoots per explant (Fig.2). Similar to our findings the 90% shoot induction was reported in 2.0 mg/L^{-1} BAP concentration in the explant of *Ceropegia mohanramii* (Avinash *et al*. 2019) [5] and *Caralluma edulis* (Patel *et al*., 2014) [32]. *In vitro* shoot proliferation of *Passiflora caerulea* L., the best shoot induction was noted on 3.0 mg/L^{-1} concentration of BAP and the researchers were also reported this was the optimum concentration for best shooting (Prithviraj et. al., 2015) [40]. The similar results also reported in *Cryptolepis grandiflora* by prema *et al*., 2013 [34]. When compare to senthilkumar et.al 2009, the best response were observed in our study. Whereas, Dharmendra *et al*., 2010 [15] mentioned their findings the highest shoot induction of *Oxystelma secamone* (70%) was obtained at 2.0 mg/L BAP. *In vitro* shoot proliferation of many apocyanaceae members was successfully attained in the medium containing the combinations of Kinetin and IBA (Parabia, 2007) [30]. In many cases, the efficacy of the interactive effect of BAP and NAA on multiple shoot differentiation is also being shown (Karami *et al*., 2009; Murthy *et al*., 2010) [22, 28]. In accordance with above statement Goyal *et al*., 2016 [42] reported that the highest number of explant (4±2.8) was observed in the combination of BAP and NAA (5+1 mg/L^{-1}) However in our investigation the best shoot multiplication was achieved in Kinetin and BAP combination (Table -1).

After the elongation of the shoots, the shoot length was measured and the height of the shoots ranges from 10-22 cm. According to patel *et al* 2014a [32], the highest shoot length of *Leptadenia reticulate* is ranges from 6-8 cm, whereas the best shoot length was reported in our study with the same level of concentration. In Fig.3 represents the highest shoot length (22 cm) in 1.5 mg/L KIN supplemented MS medium.

Table 1: Effect of BAP and KIN (mg/L) on nodal explant of *Oxystelma esculentum* R.Br.

S. No	PGR		No of Explant Inoculated	No of Responded	% of Responded	No of shoot per explant (mean ± SD)	Shoot length in cm (mean± SD)
	BAP	KIN					
1	0.5	0	20	15	75	3.0±1.6^{a}	4.3±0.8^{x}
2	1.0	0	20	14	70	3.1±2^{a}	4.2±0.9^{x}
3	1.5	0	20	16	80	4±1.5^{ab}	11.8±1.8^{y}
4	2.0	0	20	18	90	4.3±1.3^{ab}	15.1±2.5^{xy}
5	2.5	0	20	11	55	3.5±1.5^{b}	7.7±2.2^{z}
1	0	0.5	20	11	55	3.1±1.4^{a}	9.5±1.1^{yy}
2	0	1.0	20	17	85	4.1±1.6^{ab}	11.4±3.1^{y}
3	0	1.5	20	15	75	3.9±0.7^{b}	9.8±2.1^{yy}
4	0	2.0	20	11	55	3.1±0.1^{a}	8.5±1.4^{xz}
5	0	2.5	20	13	65	3.6±0.6^{b}	9.6±2.9^{yy}
1	0.5	1.0	20	16	80	3.7±1.1^{b}	10.4±3.3^{zz}
2	1.0	1.0	20	19	95	5±0.7^{c}	18.2±3.3^{yz}
3	1.5	1.0	20	17	85	4.2±1.0^{ab}	12.7±2.1^{zx}
4	2.0	1.0	20	15	75	3.0±0.7^{a}	10.4±2.4^{zz}
5	2.5	1.0	20	16	80	3.5±0.6^{b}	10.7±2.7^{zz}

Values represents *means* ± *standard deviation*. *Means* followed by the *same letter* within each *column* are not significantly different *(P<0.0001)* using Duncan's Multiple Range Test.

PGR – Plant Growth Regulators, *BAP* – 6-Benzyladenine Phosphate, *KIN* - Kinetin

Effect of auxins in Root induction

After the elongation, the shoots excised from *in vitro* were transferred to half strength MS containing rooting medium. Different concentrations of IAA and IBA were used individually and with combinations for rooting (Table-2). The various concentrations of growth regulators used for rooting, the percentage of rooting has been ranges from 55-95%. Owing to the concentrations of IBA was significantly affected the rooting (Chavan et. al., 2014). Hence, the maximum number of explants (19/20) was responded in low concentration of IBA (0.5 mg/L) with 95% also the same result noted in the concentration of IAA+IBA (0.5+1.0 mg/L) combination (Table -2). The similar results were obtained in *Oxystelma secamone* (Dharmendra et. al., 2010) [15]. Whereas, 1.0 mg/L concentration of IAA were produces significant rooting with 85%. Chavan *et al*., 2014 [11] documents their findings, the root induction of *C. noorjahaniae* in IAA were produced very poor results. However, they showed significant root induction when combined with IBA. The highest number of roots (15±1.4) was formed in half strength MS medium at the concentration of 0.5 mg/L IBA with the root length of 9 cm (Fig.3). Likewise many of the researchers found an efficient root induction is successfully promoted by IBA in *Hemidesmus indicus* (Sreekumar *et al*. 2000) [38], *Holostemma ada-kodien* (Martin 2002) [26], *Ceropegia candelabrum* (Beena *et al*. 2003) [7], *Cunila galioides* (Fracaro and Echeverrigary, 2001) [17], *Aloe polyphylla* (Abrie and Van Staden, 2001) [1] and *Ceropegia attenuata* (Chavan *et al*. 2011a) [12]. In IAA+IBA (0.5+1.0 mg/L) combinations showed more number of roots (12.16±3.7) and with the average length is ranges from 2-7 cm. In contrast with our findings, the highest frequency of rooting was obtained in the combinations of IBA (0.5mg/L) and IAA 0.5mg/L containing MS medium on *Caralluma diffusa* (Karthik prabu *et al*, 2013) [23]. When the combination of hormones (IAA+IBA) concentration level increases then the response of rooting was decreased (Table -2). The auxins containing MS medium is more efficient for rooting in many species and the auxins such as NAA and IBA are the most common plant growth regulators which is widely used for root induction (Bhojwani and Razdan 1992) [8].

Table 2: Effect of IAA and IBA in Root formation from nodal explants of *Oxystelma esculentum* R. Br.

S. No.	Plant Growth Regulators (PGRs)		% of root induction	No. of roots / shoot Mean ± SD	Root length in cm Mean ± SD
	IAA (mg/L)	IBA (mg/L)			
1.	0.5	0.5	85	8.0 ± 2.64^{e}	10.5 ± 3.6^{x}
2.	0.5	1.0	90	12.1 ± 3.7^{de}	11.5 ± 4.3^{y}
3.	0.5	1.5	80	4.4 ± 1.1^{ab}	6.5 ± 3.5^{xy}
4.	0.5	2.0	70	4.2 ± 1.2^{ab}	5.6 ± 2.8^{z}
5.	0.5	2.5	60	5.3 ± 1.1^{c}	4.4 ± 2.3^{yy}
1.	0.5	00	80	6.8 ± 1.3^{d}	5.2 ± 3.1^{z}
2.	1.0	00	85	7.1 ± 1.4^{d}	5.9 ± 4.1^{z}
3.	1.5	00	70	5.7 ± 1.2^{c}	4.2 ± 2.3^{yy}
4.	2.0	00	55	3.5 ± 1.1^{b}	4.3 ± 2.4^{yy}
5.	2.5	00	45	2.6 ± 0.6^{a}	3.4 ± 2.0^{xz}
1.	00	0.5	95	15.8 ± 1.4^{ef}	12.10 ± 3.3^{zz}
2.	00	1.0	90	13.2 ± 1.7^{f}	10.6 ± 2.8^{x}
3.	00	1.5	75	5.7 ± 1.2^{c}	4.0 ± 1.8^{yy}
4.	00	2.0	65	4.2 ± 1.6^{ab}	3.6 ± 1.4^{xz}
5.	00	2.5	55	2.2 ± 0.8^{a}	3.2 ± 1.3^{xz}

Values represents *means* ± *standard error*. *Means* followed by the *same letter* within each *column* are not significantly different *(P<0.0001)* using Duncan's Multiple Range Test.
IAA - Indole-3-Acetic Acid, *IBA* – Indole-3-Butyric Acid

Ex vitro Rooting and Hardening/Acclimatization

For Ex-vitro rooting the shoot bases (4-5 mm) were treated with various concentrations (50-250 mg L-1) of IBA, IAA, and NAA alone or in combination for 5 minutes. The best rooting were reported in table-3. After the development of shoots with well rooted plants were transferred subsequently to the natural field conditions and these plants were grown well and exhibits 92% of survival rate. (Fig.4). Plant conservation and restoration can be achieved by transplanting in vitro raised plantlets into natural habitats. (Chavan *et al*., 2018; Aggarwal et.al., 2012)

Table 3: Effect of various concentrations of auxins (NAA, IAA & IBA) on *ex vitro* rooting of *O. esculentum*

S. No.	Plant Growth Regulators (PGRs)			% of root induction	No. of roots / shoot Mean ± SD	Root length in cm Mean ± SD
	NAA (mg)	IAA (mg)	IBA (mg)			
1.	00	50	00	60	6 ± 1.2^{a}	6.6 ± 1.5^{x}
2.	00	100	00	70	6.7 ± 1.0^{a}	6.5 ± 1.8^{x}
3.	00	150	00	75	7.2 ± 1.4^{b}	6.8 ± 1.5^{x}
4.	00	200	00	80	9.1 ± 0.7^{ab}	7.6 ± 1.1^{y}
5.	00	250	00	60	6.3 ± 1.7^{a}	7.3 ± 0.7^{y}
1.	00	00	50	75	10.1 ± 2.0^{c}	7.4 ± 0.9^{y}
2.	00	00	100	80	12.3 ± 2.4^{ac}	8.2 ± 1.1^{xy}
3.	00	00	150	85	14.1 ± 2.5^{bc}	8.3 ± 1.1^{xy}
4.	00	00	200	90	16.5 ± 2.6^{cc}	12.8 ± 4.3^{z}
5.	00	00	250	80	16.2 ± 2.4^{cc}	13.1 ± 4.5^{z}
1.	50	50	00	65	6.8 ± 1.3^{a}	11.3 ± 4.4^{zy}
2.	100	100	00	80	8.2 ± 1.1^{d}	11.7 ± 3.9^{zy}
3.	150	150	00	85	9.2 ± 1.1^{ab}	12.7 ± 3.8^{z}
4.	200	200	00	80	10 ± 1^{c}	15.2 ± 3.5^{zz}
5.	250	250	00	70	8.8 ± 0.6^{d}	15.1 ± 4.1^{zz}
1.	50	00	50	80	14.6 ± 2.2^{bc}	13.5 ± 3.8^{z}
2.	100	00	100	90	17.4 ± 2.2^{cd}	15.0 ± 2.8^{zz}
3.	150	00	150	95	20.1 ± 3.2^{dd}	16.2 ± 4.0^{yz}
4.	200	00	200	100	25.9 ± 3.5^{e}	17.1 ± 4.1^{xx}
5.	250	00	250	80	18.3 ± 1.6^{de}	15.5 ± 3.4^{zz}

Values represents *means* ± *standard error*. *Means* followed by the *same letter* within each *column* are not significantly different *(P<0.0001)* using Duncan's Multiple Range Test.
NAA - 1-Naphthyl Acetic Acid, *IAA* - Indole-3-Acetic Acid, *IBA* - Indole-3-Butyric Acid

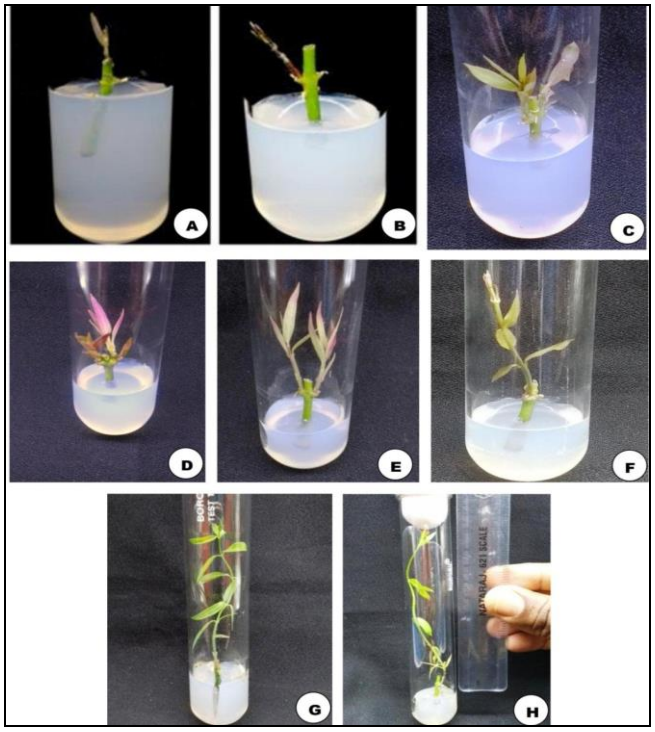

Fig 1: Effect of cytokinins in shoot prolifereation on *Oxystelma escultem* R.Br.

A & B: Shoot initiation on MS medium
C, D & E: Shoot multiplication on 1.0+1.0 mg/L (BAP+KIN) supplemented MS medium
F & G: Elongation of shoots on 1.0 mg/L KIN supplemented MS medium
H: Average length of shoot (10 cm).

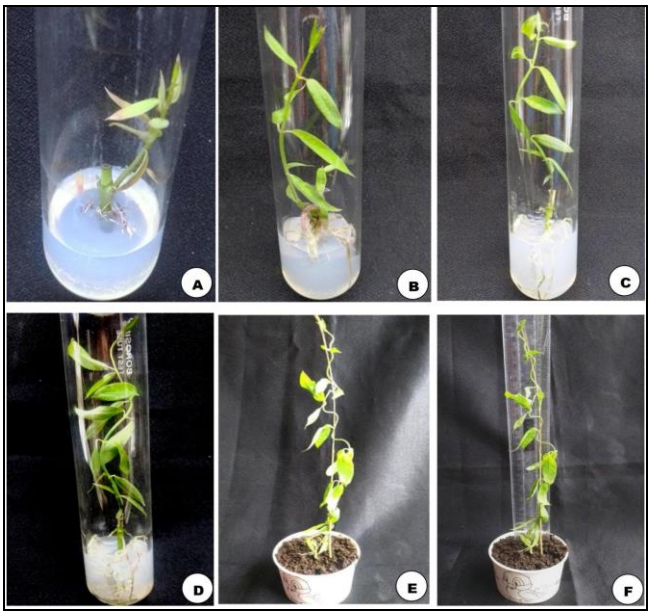

Fig 2: Effect of auxins in root prolifereation on *Oxystelma escultem* R.Br.

A & B: Root initiation on half-stregth MS medium
C & D: Root multiplication on 0.5 mg/L (IBA) supplemented half-strength MS medium
E: Hardened *In vitro* plantlets on soil+vermicast mixture (1:1 v/v)
F: Measurement of the higest shoot length (22cm)

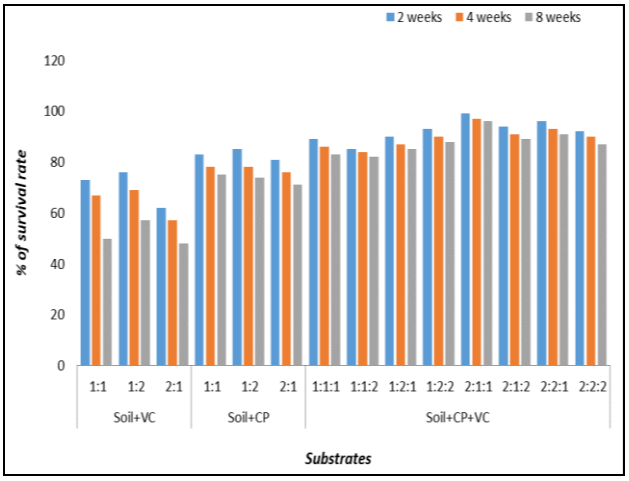

Fig 3: Effect of different substrates on an *ex vitro* rooting & acclimatization of *Oxystelma esculentum* VC – Vermicompost CP – Coco peat

Conclusion

In conclusion, this is an efficient, highly reproducible and rapid micropropagation protocol has been established for large-scale production of *Oxystelma esculentum*. The protocol outlined here provides a potential *in vitro* method for medicinal herb growth, conservation, and efficient mass multiplication. This protocol is highly beneficial due to its improved multiplication rate and cost effectiveness. Additionally, the established protocol can be used to mass multiply and preserve other *Oxystelma* species.

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Authors' Contributions

RS and SB were carried out the experiments, analyzing the data of the experiments and prepared the manuscript. TFX and AFR gave the outline of the experimental design and edited the manuscript.

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