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In Vitro Organogenesis and Epiphyllous Secondary Buds Formation in *Kalanchoe tubiflora* (Harvey), Hamet, (Crassulaceae)

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In-vitro organogenesis and epiphyllous secondary buds production in Kalanchoe tubiflora were carried out using MS medium supplemented with appropriate growth regulators. This protocol can be valuable for the mass propagation of this plant species, which is crucial for effective conservation efforts. The highest yield of whitish green massive callus was obtained in MS medium supplemented with 2.0 mg/l of 2,4-dichlorophenoxy acetic acid and 1.0 mg/l of Indole-3-butyric acid. Rapid shoot formation was achieved in MS medium supplemented with 2.0 mg/l of Benzyl amino purine, 1.0 mg/l of kinetin, and 1.0 mg/l of IBA. Effective root induction was observed in MS medium supplemented with 1.5 mg/l of Indole-3-acetic acid, 1.0 mg/l of BA, and 0.5 mg/l of Naphthalene acetic acid. Epiphyllous secondary bud induction was successfully achieved in Murashige and Skoog medium supplemented with 1.0 mg/l of BAP, 0.5 mg/l of kinetin, 0.5 mg/l of IBA, and 0.5 mg/l of Gibberellic acid 3. The plantlets were successfully acclimatized and thrived well in the greenhouse environment. This protocol has significant potential for large-scale production of K. tubiflora and can contribute to its conservation efforts.

Keywords: Callus; Epiphyllous secondary buds; Kalanchoe spp ; Organogenesis.

Kalanchoe tubiflora (Harvey) Hamet (Crassulaceae), commonly known as 'Mother of thousands', is a succulent plant that is native to Madagascar[1]. This plant species has significant economic and medicinal importance due to its ornamental value and traditional medicinal properties. In traditional medicine, it has been used for the treatment of various ailments such as rheumatism, inflammation, immunosuppressive, hypertension, and treatment of kidney stones, liver damage, and anti-tumor activities[2]. The plant has the ability to multiply through epiphyllous secondary buds, but it is necessary to develop a protocol for rapid multiplication through *in-vitro* culture since an effective regeneration system is a prerequisite for all biotechnological tools[3] for conservation programs. *In-vitro* tissue culture is regarded as the most efficient method for plant propagation and is widely used for several succulent plant species[4,5]. Alam, et al.[6] describes the successful in vitro propagation of *K. tubiflora* through leaf explants and optimized the growth regulators in the culture medium and achieved high regeneration rates. Sayari et al.[7] focuses on *in-vitro* propagation of

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K. tubiflora through leaf explants, used different growth regulators and achieved a high regeneration rate using a combination of auxins and cytokinins. Studies on *in-vitro* propagation of Kalanchoe spp. such as *K. daigremontiana*, *K. blossfeldiana*, *K. laciniata*, *K. tomentosa, K. pinnata* was done and research has demonstrated their potential in treating diabetes, inflammation, and cancer due to its antioxidant, antidiabetic, and cytotoxic properties[8–9]. *Kalanchoe daigremontiana* is another succulent species that is indigenous to Madagascar characterized by its ability to produce numerous plantlets along the margins of its leaves, which can be detached and rooted to form new plants.[10-11] It has been traditionally used as a medicinal plant to treat wounds, burns, and skin diseases. However, there is few existing protocols for the *in-vitro* propagation of *K. tubiflora*. The main objective of this study is to develop an effective propagation method and also to induce epiphyllous secondary buds under *in-vitro* conditions. The development of a reliable protocol for *in-vitro* propagation will be essential for mass propagation of this plant species and will significantly contribute to its conservation efforts.[12-13] This study is expected to provide a valuable foundation for further research on the biotechnological application of *K. tubiflora*.

METHODOLOGY

K. tubiflora, also known as *Bryophyllum tubiflorum*, is a fascinating succulent plant that has a unique mode of vegetative propagation.[15-16] Unlike many other plants, it produces epiphyllous buds on the matured leaf margin, which grow tiny rootlets, detach from the mother plant, and develop into individual plants. This process is called vivipary and is a characteristic feature of many plants in the family Crassulaceae, to which *K. tubiflora* belongs. Interestingly, *K. tubiflora* has also been synonymized with the genus Kitchingia by some taxonomists.[17-18] This highlights the challenges in classifying plants and the need for careful examination of morphological, molecular, and ecological features.[19-21] In terms of cultivation, *K. tubiflora* is relatively easy to grow and maintain, making it a popular choice among succulent enthusiasts. It requires well-draining soil, moderate watering, and bright, indirect sunlight. With proper care, it can thrive and produce many new plants through its unique method of vegetative propagation.[22]

Source of plant materials

The plants were collected from the Botanical Garden, Department of Botany, National College, Tiruchirapalli, Tamil Nadu, India.

Plant Tissue Culture

Explants Preparation

To propagate *K. tubiflora* through *in-vitro* culture, the selection of healthy leaf segments and epiphyllous buds is crucial. These explant materials must be thoroughly washed with running tap water and then rinsed with distilled water to remove any external debris or contaminants. Afterward, a surface sterilization process using an aqueous solution of $HgCl_2$ (0.1 % w/v) for 2 minutes and 70% ethanol for 1 minute is carried out to eliminate any microbial contaminants. The explant materials are then washed thrice with sterilized distilled water. Once the explants are sterilized, they are carefully blotted with sterile blotting paper to remove any excess moisture and prevent contamination. The explants are then inoculated into culture tubes containing a modified MS medium under aseptic conditions. This medium is optimized for the growth of *K. tubiflora* and contains all the necessary nutrients and growth regulators required for the *in-vitro* culture of the explants.

Cultural medium and Culture conditions

Cultural medium and culture conditions were optimized for the successful propagation and growth of *K. tubiflora*. A modified MS medium was used, supplemented with 30% w/v sucrose and 8% w/v agar-agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C and 15 lbs pressure for 20 minutes to ensure sterility. To induce callus formation, different growth regulators including 2,4-D, IAA, IBA, BAP, BA, Kin, GA3, and NAA were added to the MS medium at various concentrations and combinations. The *in-vitro* cultures were maintained under controlled environmental conditions to promote optimal growth and development. The cultures were kept in 25*160 mm culture tubes covered with cotton plugs to maintain a relative humidity of 60-80%. The cultures were illuminated for 16 hours with fluorescent light at a photosynthetic photon flux density of 18-24 µmol/m^2/sec followed by an 8-hour dark period. The temperature was

maintained at 24°C ± 2°C throughout the culture period.

Callus induction

To investigate the optimal combination of growth regulators for inducing callus production, healthy leaf segments of explants were carefully selected and inoculated in a sterile MS medium. Various combinations of growth regulators, including 2,4-D, IAA, and IBA, were tested for their efficacy in callus induction. MS+2,4-D+IBA,were added under the following concentrations1.0 + 1.0, 1.5 + 1.5, 2.0 + 1.0, MS+2,4-D+IAA were added using these contrations 1.0 + 1.0, 1.5 + 1.5, 2.0 + 1.0 To ensure the reliability of the results, a total of twenty cultures were raised for each treatment, and all experiments were repeated thrice. By systematically varying the growth regulator concentrations and observing the resulting callus production, we aimed to identify the most effective combination for inducing callus growth in the explants.

Organogenesis and epiphyllous secondary buds induction

For induction of organogenesis and epiphyllous secondary buds in *K. tubiflora*, a series of experiments were conducted using various concentrations and combinations of growth regulators in MS medium[23]. The healthy leaf buds of the explants were used as the starting material, and 20 cultures were established for each treatment, with all experiments repeated three times to ensure accuracy and reproducibility of the results[24]. Several different growth regulators, including BAP, IBA, GA3 were used in various combinations to determine their efficacy in promoting organogenesis and epiphyllous secondary buds induction. The results showed that certain combinations of growth regulators were highly effective in inducing the desired response, while others had little to no effect.

RESULTS

K. tubiflora, an important medicinal herb with potential anticancer properties used to treat pulmonary infections and rheumatoid arthritis. The current study aimed to investigate *in-vitro* organogenesis and epiphyllous secondary bud induction, with the results being presented below.

Callus Induction

Leaf segments were used to induce callus, and the combination of 2,4-D and IBA proved to be highly effective in producing a substantial amount of massive calli as shown in Fig.2. The morphology of the callus was found to be friable and whitish green in color. MS + 2,4-D (2.0 mg/l) + IBA (1.0 mg/l) resulted in the highest number of massive calli, followed by MS + 2,4-D (2.0 mg/l) + IAA (1.0 mg/l) shown in Table.1.

Organogenesis

In the present study, the *in-vitro* organogenesis of *K. tubiflora* was induced using MS medium supplemented with different combinations and concentrations of growth regulators. Shoot and root induction were the two major parameters studied, and the results were analyzed based on the number and morphology of the shoots and roots produced. The maximum number of shoots was obtained when the explants were cultured on MS medium supplemented with BAP (2.0 mg/L), Kin (1.0 mg/L), and IBA (1.0 mg/L). On the other hand, the combination of MS+BAP+NAA+IAA found to shown lesser number of shoot resulted

Table 1. Callus induction from the leaf segments explant of *K. tubiflora* cultured on MS with various combinations of growth regulators. *The values represents means ± SD from 20 replicates

Medium + Growth regulator	Conc	No of explant innoculated	No of explant produced calli	Morphology of calli
MS+2,4-D+IBA	1.0 + 1.0	20	06 + 0.05*	Friable, whitish,
	1.5 + 1.5	20	06 + 0.70*	Green, Massive
	2.0 + 1.0	20	14 + 1.4*	
MS+2,4-D+IAA	1.0 + 1.0	20	04 + 0.10*	Friable, whitish,
	1.5 + 1.5	20	04 + 0.20*	Green, Massive
	2.0 + 1.0	20	08 + 0.40*	

in a lesser number of shoots. Among the other combinations tested, MS+IAA (1.5 mg/L), BA (1.0 mg/L), and NAA (0.5 mg/L) were found to be the best-suited combination of growth regulators for shoot induction which is shown in figure2. This combination was found to be more effective than the MS+IBA+BA+Kin combination. The results were tabulated to compare the effects of different

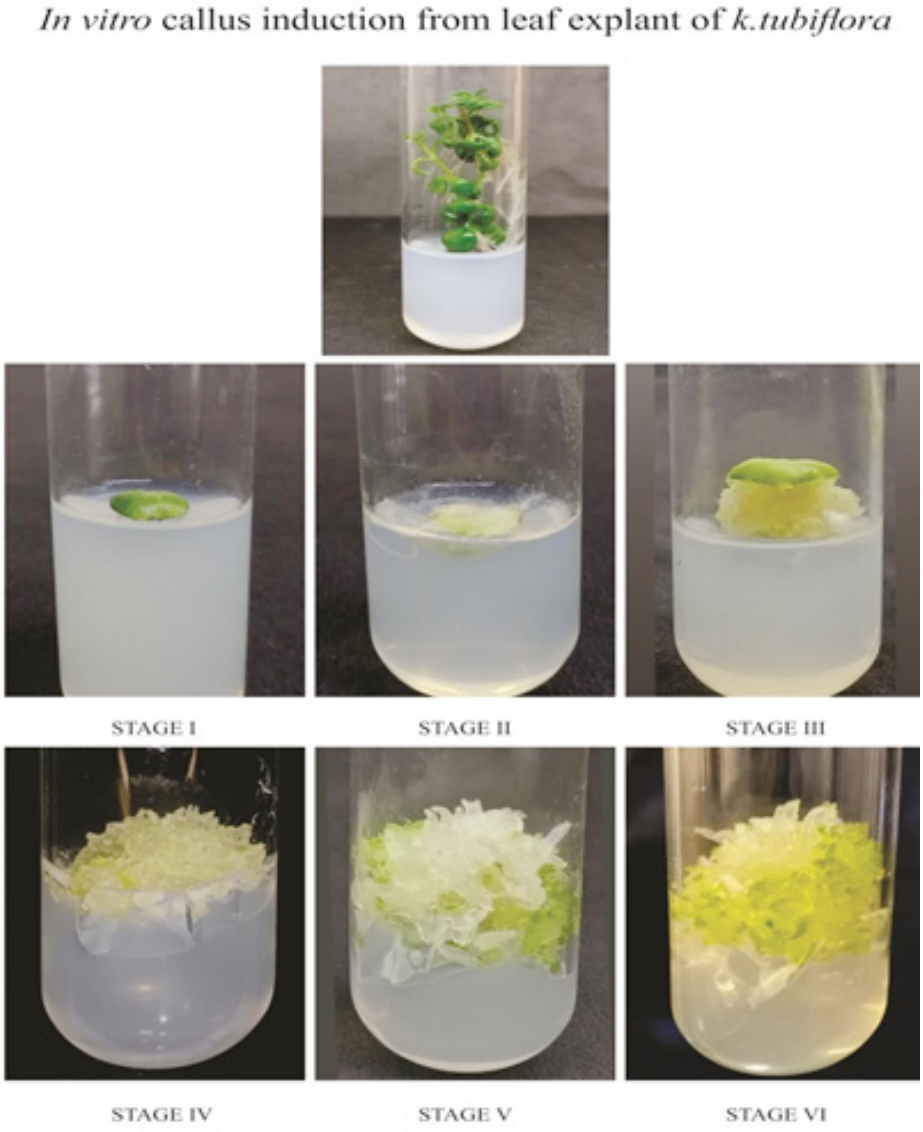

Fig. 1. *In vitro* Callus induction from the leaf explant of *K. tubiflora*

combinations of growth regulators on shoot and root induction responses observed as shown in Table.2.

Epiphyllous secondary buds induction

In-vitro organogenesis of *K. tubiflora* was induced using MS medium supplemented with different combinations and concentrations of growth regulators. The young plantlets developed *in vitro* was subjected to subculture with MS + BAP + Kin + IBA + GA_3 for the induction of secondary buds. The secondary buds developed on the leaf margin in 15 days as well as at the stem basal region of the plant lets which is shown in figure 3. Maximum secondary buds formation was noticed in MS + BAP (1.0 mg/L), Kin (0.5 mg/L), IBA (0.5 mg/L), GA_3 (0.5mg/L) when compared with MS + IAA + kin + GA_3 Combinations and the results were tabulated (Table 3).

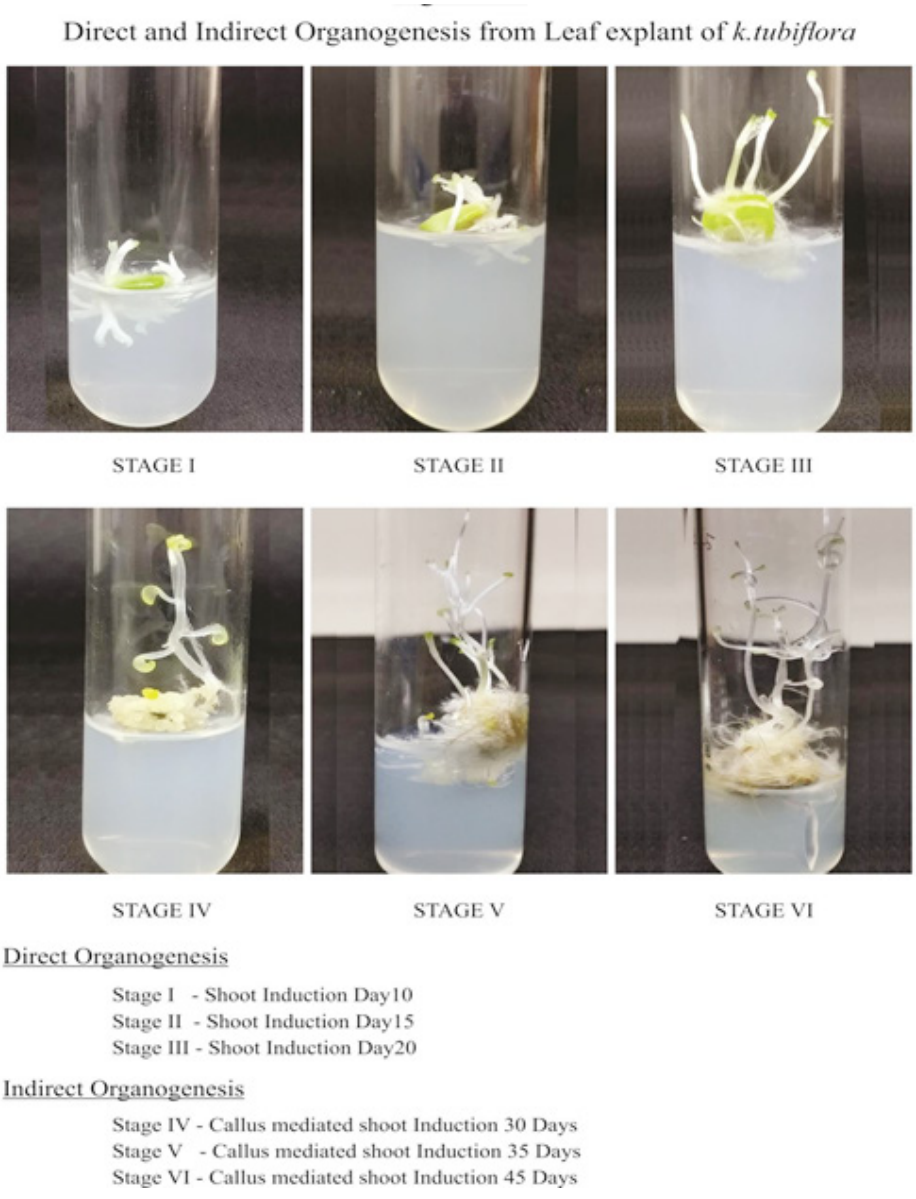

Fig. 2. Direct and Indirect Organogensis from Leaf explant of *K. tubiflora.*

DISCUSSION

India possesses a rich diversity of biotic resources, and among the 34 recognized hotspots, the Eastern Himalayas and the Western Ghats are two major hotspots that harbor high biodiversity.[26] However, the constant expansion of global trade and inadequate cultivation fields have led to the depletion of natural resources and a decline in biodiversity.[27] Therefore, biotechnological methods such as *in vitro* cultures and clonal propagation can help in the conservation of threatened plant species such as *K. tubiflora*.

Callus induction

Callus induction was maximum in MS, +2, 4-D (2.0mg/L) and IBA (1.0 mg/L), the Callus was friable whitish green and massive.[28] Obtained callus using MS + 2, 4 -D (1, 2, 3 mg/L) + IAA, 0.1, 0.5 mg/L in Bryophyllum sp leaf explant, whereas the present study have shown 2,4-D and IBA combinations induced callus. It is due to the specific phytochemicals present in the different species of *Bryophyllum / Kalanchoe* responded differently for callus production.

Organogenesis

Maximum number of shoot formation was found to be on MS + + BAP 2.0 mg /L, kin,

Table 2. Effect of Growth Regulators on Leaf Bud Organogenesis in *K. tubiflora*. The values represent means ± SD from 20 replicates, indicated by the asterisk (*)

Medium + Growth regulation	Concentrations	Number of explants inoculated	Number of explants produced shoots	A number of explants produced roots.
MS+BAP+Kin+IBA	1.0 + 0.5 + 0.5	20	06 ± 0.10*	_
	1.5 + 1.0 + 1.0	20	12 ± 0.40*	_
	2.0 + 1.0 + 1.0	20	18 ± 0.71*	_
MS+BAP+NAA+IAA	1.0 + 0.5 + 0.5	20	04 ± 0.20*	_
	1.5 + 1.0 + 1.0	20	08 ± 0.70*	_
	2.0 +1.0 + 1.0	20	08 ± 0.20*	_
MS+IAA+BA+NAA	0.5 + 0.5 + 0.5	20	_	04 ± 0.25*
	1.0 + 0.5 + 0.5	20	_	08 ± 0.10*
	1.5 + 1.0 + 0.5	20	_	12 ± 0.10*
MS+IBA+BA+Kin	0.5 + 0.5 + 0.5	20	_	02 ± 0.20*
	1.0 + 0.5 + 0.5	20	_	06 ± 0.15*
	1.5 + 1.0 + 0.5	20	_	08 ± 0.30*

Table 3. Induction of secondary buds from the *in-vitro* plantlets of *K. tubiflora* on MS with various combinations of growth regulators. The values represent means ± SD from 20 replicates, indicated by the asterisk (*)

Medium + Growth regulators	Concentrations (mg/l)	No of explant Sub cultured	No of plants developed secondary buds on leaf	No of plants developed secondary buds on stem basal region
MS+BAP+Kin+IBA+GA3	0.5 + 0.5 + 0.5 + 0.5	20	02 ± 0.10*	-
	1.0 + 0.5 + 0.5 + 0.5	20	08 ± 0.03*	04 ± 0.20*
	1.5 + 1.0 + 0.5 + 0.5	20	05 ± 0.05*	02 ± 0.20*
MS+IAA+Kin+GA3	0.5 + 0.5 + 0.5	20	-	-
	1.0 + 0.5 + 0.5	20	02±0.02*	02 ± 0.04*
	1.5 + 1.0 + 0.5	20	02±0.10*	-

l.0 mg/L and IBA, 1.0mg/L and the highest as number of root formation in the combinations of MS+ IAA, 1.5 mg/L, BA, 1.0 mg/L, NAA 0.5mg/L, compared with other combinations of growth regulators[29]. *In vitro* regeneration of *Kalanchoe blossfeldiana* and obtained maximum number of shoots on MS + BAP, 1.0 mg/L from model explant and the best rooting was obtained in MS + BAP, 1.0mg/L used various concentrations of thidiazuron (TDZ) and found that lower concentration of TDZ (2.5pm) + BAP (1, 2 and 3PM) produced multiple shoots from leaf explant in Bryophyllum

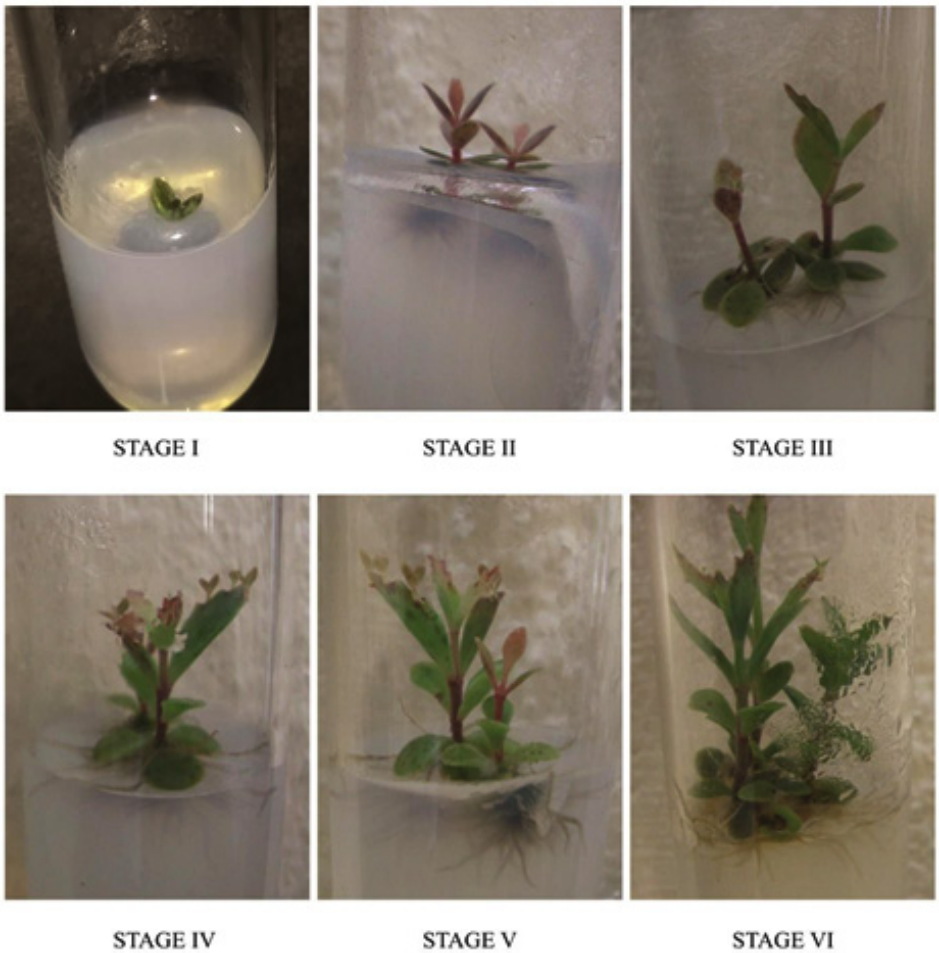

Fig. 3. *In-vitro* shoot and secondary bud induction in *K. tubiflora.*

daigremontiannm. The variations always possible because of different species of *Kalanchoe* respond differently for organogenesis[14].

Epiphyllous Secondary bud Induction

Eepiphyllous secondary bud induction in *K. tubiflora* observed with the use of BAP, IBA, and GA3 in various combinations and concentrations was essential for the induction of secondary buds on the leaves. In this study, the maximum number of secondary buds was observed in MS+BAP,1.0mg/L+ 0.5 mg/l of kinetin IBA,0.5mg/L, and GA3 0.5mg/L combination. It is noteworthy that Kin was found to be crucial for the induction of secondary buds, and the optimum concentration of Kin was 0.5mg/L. Higher concentrations of Kin were found to be less effective, which is in line with the work of[30] on *Helianthus annus*. TDZ, which has cytokinin activity, and ETH, an ethylene compound, in the induction of epiphyllous buds in *K. pinata*. These studies suggest that the induction of epiphyllous buds is species-specific and may require different combinations and concentrations of growth regulators. Furthermore, it is important to note that the induction of epiphyllous buds on the leaves of *K. tubiflora* has significant implications for clonal propagation and conservation of this plant species[31]. The ability to induce secondary buds on the leaves would provide an additional means of propagating the plant and may help to conserve it by reducing the pressure on natural resources.

CONCLUSION

Biotechnological methods such as *in-vitro* cultures and clonal propagation can help in the conservation of threatened plant species such as *K. tubiflora*. The highest production of callus was obtained in MS medium supplemented with 2.0 mg/l of 2,4-D and 1.0 mg/l of IBA, Rapid shoot formation was achieved in MS medium supplemented with 2.0 mg/l of BAP, 1.0 mg/l of kinetin, and 1.0 mg/l of IBA, root induction was observed in MS medium supplemented with 1.5 mg/l of IAA, 1.0 mg/l of BA, and 0.5 mg/l of NAA. Epiphyllous secondary bud induction was successfully achieved in MS medium supplemented with 1.0 mg/l of BAP, 0.5 mg/l of kinetin, 0.5 mg/l of IBA, and 0.5 mg/l of GA3. The use of biotechnological methods such as *in-vitro* cultures and clonal propagation can help in the production of plantlets and also conserve natural resources. This protocol provides a reliable and efficient method for mass propagation of *K. tubiflora*, which can be used for the production of anticancer agents, treatment of pulmonary infections, and rheuma- toid arthritis. Moreover, the conservation of *K. tubiflora* is crucial as it is an important medicinal herb and has the potential to provide various benefits to humans. Therefore, this study has practical implications for the conservation and sustainable use of plant resources.

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Conflict of Interest

The authors declare that they have no conflict of interest on publication of this article.

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