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Meta-topolin-mediated micropropagation and micromorphological adaptations of Schweinfurthia papilionacea, a xerophytic medicinal herb of the Thar Desert

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Abstract

Schweinfurthia papilionacea is a xerophytic medicinal plant having very low natural regeneration and propagation potential. We have developed an efficient in vitro regeneration protocol using meta-topolin (mT), and micromorphological changes were studied in the leaves and stems during acclimatization. The highest seed germination rate was obtained with 200 mg L^{-1} GA_3 treatment. The highest shoot induction frequency (88.09 ± 4.12%) with the longest shoots (3.19 ± 0.78 cm) were obtained with MS medium containing 0.5 mg L^{-1} meta-topolin (mT), while 1.0 mg L^{-1} mT and 0.10 mg L^{-1} indole-3-acetic acid (IAA) regenerated maximum number of shoots/explant (20.67 ± 2.06). The highest in vitro root induction (63.63 ± 13.88%) was observed at 1.0 mg L^{-1} of indole-3-butyric acid (IBA). The regenerated plantlets exhibited 68% survival rate and established successfully under greenhouse conditions. The comparative micro-morpho-anatomical studies at different development stages showed highest stomatal density (46.61 ± 2.01 mm^{-2}) in 4-week-old in vitro regenerated leaves with anomocytic stomata, as compared to leaves from acclimatized or mother plants having anisocytic stomata. The number of vein islets increased from in vitro to acclimatized plantlet, with maximum number in mother plant leaves (58.33 ± 4.50 mm^{-2}). Leaf and stem anatomy showed progressive development, with a thicker epidermis and secondary xylem with considerable lignification in the acclimatized plantlets. Our results confirm a highly efficient in vitro regeneration of *S. papilionacea* using mT, making this protocol a successful conservation method for this rare and medicinal arid plant species.

Key message

The study establishes a reliable micropropagation protocol for to overcome poor natural seed germination, ensuring proper acclimatization, hardening, and population reinforcement in the Thar Desert.

Introduction

The Indian Thar Desert, which is the driest area in South Asia, climatic conditions are extremely harsh, with variable rainfall, high temperatures and extensive soil salinity, resulting in severe selective pressures on vegetation (Bhandari, 1978). These conditions have created a unique patchwork of xerophytic shrubs and herbs that are little known, yet still persisting in very stressful environments. Therefore, documentation and conservation of this flora on a systematic basis is important to prevent further decline and ensure long-term survival of species with restricted distribution (Joshi et al., 2014). *S. papilionacea* (L.) Boiss. is a small, glabrous, annual xerophyte (Plantaginaceae) that has the ability to thrive on alkaline and infertile soils, with few herbaceous species surviving. It is locally known as Nepal-Nimboo, Sanipat or Glaucous Dog-Flower and is among the relatively under-studied plants of the Thar Desert. It grows in arid and semi-arid regions of Rajasthan and Gujarat in India and is also recorded from Afghanistan, Pakistan, Baluchistan and Sindh (Bhandari, 1978). It was one of a few plants recorded despite repeated attempts over the years to record rare and threatened plants in the Kachchh region (Joshi et al., 2014). According to the World Conservation Monitoring Centre List (WCMC, 1994), *S.*

papilionacea is a rare or uncommon species in India. The species is increasingly under threat owing to changes in environment and grazing pressure in its rocky and alkaline habitat (Meena & Yadav, 2015).

Limited-range population species are especially sensitive to habitat disturbance and other environmental factors. A breach of habitat or intensive grazing, even for short periods, can decrease already low population levels and affect natural regeneration (Gopal et al., 2008). Such taxa losses can also lead to the permanent loss of unique genetic and phytochemical resources. Recruitment is highly dependent on environmental variation, limited habitat availability and low seed viability in the case of rare desert herbs, which becomes very limiting for conventional propagation. Environmental variability, limited habitat availability and low seed germination percentage are factors that could affect the recruitment of rare desert herbs in a very restrictive way in conventional propagation (Nath & Joshi, 2018). We also had some preliminary information on *S. papilionacea*, which showed poor germination under standard propagation conditions, a significant hurdle to large-scale multiplication and reintroduction.

S. papilionacea is a phytochemically valuable species, emphasizing need for its conservation. Traditionally it has been used for the treatment of diabetes, typhoid, epistaxis and malarial fever (Bhandari 1978). Although its chemical constituents are not completely characterized, preliminary metabolite profiling showed the presence of iridoid glycosides and phenolic compounds that corroborate some of its reported medicinal uses (Khan et al., 2023). Its limited distribution, conservation concern, and medicinal value underscore the importance of reliable propagation methods and ex situ conservation strategies before its natural population declines.

An alternative method is micropropagation, which relies on the ability to produce plantlets in a controlled environment and is less limited by seasonal factors that impact traditional propagation (Thorpe & Stasolla, 2021). Plants can also be produced in tissue culture for field establishment, as a permanent source of plants for various pharmacological and biochemical studies and can be used for ex situ collection for conservation. Proper selection of basal nutrient medium and plant growth regulators is an important part of efficient in vitro plant regeneration (Khanam et al., 2019; Manokari et al., 2021; Mishra et al. 2013). Cytokinins are also strongly connected to the activation of axillary buds, their initiation and developmental responses; thus, the selection of the cytokinin is a crucial factor in determining the efficiency of regeneration (Elayaraja et al., 2019). A number of cytokinins have been used for shoot multiplication, but the ability of meta-topolin (mT) to increase shoot proliferation of *S. papilionacea* has not been studied. The mT is a naturally occurring aromatic cytokinin that differs from benzyl aminopurine (BAP) in its metabolization, notably its ability to form and release O-glucoside conjugates, which can lead to prolonged cytokinin activity within cultured tissues (Werbrouck et al., 1996). Therefore, mT is reported to be a helpful alternative to BAP as it resulted in better shoot proliferation and elongation, decreased hyperhydricity, foliar senescence and tissue necrosis, and increased shoot quality, rooting, and acclimatization (Ahmad & Anis, 2019; Khanam et al., 2020). Furthermore, there is a lack of information on whether the characteristics of leaves and stems of the micropropagated plants of *S. papilionacea* are comparable during acclimatization with those of naturally established plants. For xerophytic species, these comparisons are especially significant since stomatal characteristics, venation

patterns and tissue organization are directly involved in adaptation to desert conditions. Hence, the present study was designed to develop an efficient micropropagation protocol for *S. papilionacea*, optimization of shoot induction, multiplication, rooting, and study of micromorphological and anatomical modifications during the process of acclimatization. This integrated approach formed a reliable basis for *ex situ* conservation, establishment and future use of this important rare and medicinal xerophyte.

Materials and Methods

Plant material collection and authentication

The seeds and plant material of *S. papilionacea* were sampled from the Dabhala village of Jaisalmer district, Rajasthan, India (26.867499° N, 70.985005° E) during the winter season. Voucher specimens were prepared as herbarium sheets according to Rao & Sharma (1990) and were deposited at the Arid Zone Regional Centre (AZRC), Botanical Survey of India (BSI), Jodhpur, where they were taxonomically authenticated and identified, with the Accession no. BSJo54854 (Fig. 1).

Optimization of seed germination

Surface sterilization was accomplished by dipping seeds in 70% (v/v) EtOH for 30 sec, followed by five to six washings in autoclaved distilled water, 2 min apart, and then a 30 min soak in different concentrations of GA_3 (0, 100, 200, 300, 400, and 500 ppm). After that, 30 seeds were inoculated in Petri dishes (90 mm diameter) containing half-strength MS medium supplemented with 3% (w/v) sucrose and agar (0.8% w/v) for in vitro seed germination (Murashige and Skoog, 1962). Ex vitro seed germination was carried out on double-layered blotting paper moistened with autoclaved distilled water. Petri dishes were covered with Parafilm M (Bemis Company, Inc., USA) and kept in the dark for 4 days. After that, seeds were exposed to a 16 h photoperiod, 45–50 µmol m^{-2} s^{-1} (cool white fluorescent tubes) at 25 ± 2°C, and 50–60% relative humidity. Germination was observed every other day from day 2 to day 16. Germination of a seed was deemed to have occurred when the radicle broke through the seed coat. For ex vitro seed germination in pots under net-house conditions, seeds were surface sterilized as described above and were directly sown in plastic pots (200 mL capacity) filled with garden soil. The substrate was pre-wetted with distilled water and kept at a field capacity of about 70%. Three replicate pots (30 seeds per pot) were used for each treatment. Seeds were soaked in the same concentrations of GA_3 (0, 100, 200, 300, 400 and 500 ppm) or distilled water (control) at 2-day intervals for consistency in moisture. In the monsoon season (July – August), the natural photoperiod and temperature were maintained in pots in the net house, the average temperature ranged between 28°C to 35°C and relative humidity ranged from 40% to 65%, typical of natural habitat. The net house provided approximately 50% shade to minimize heat stress while maintaining natural daylight. Germination was observed every other day from day 2 to day 16. This method was adopted to assess germination in conditions close to natural establishment, which is pertinent for conservation-oriented propagation research (Hussain et al., 2022; Bhatt et al., 2018).

Explant sterilization, shoot induction and multiplication

Nodal explants collected from nursery-grown plants were surface sterilized under control conditions by sequential treatment for 8–10 min with Bavistin (carbendazim fungicide 0.3% w/v) followed immediately by 0.1% (w/v) mercuric chloride ($HgCl_2$, Hi-media Laboratories), and rinsed five to six times with sterile distilled water, ensuring a minimum contact time of 2 min per rinse. The explants were then removed and cut with sterile scissors at the two ends of each explant and were immediately put on the culture medium under strictly aseptic conditions (Bhojwani & Razdan, 1996; George et al., 2008). MS basal medium (Hi-LR™) was used with growth additives and antioxidants, such as ascorbic acid (50 mg L^{-1}), L-arginine (25 mg L^{-1}), adenine sulphate (25 mg L^{-1}) and citric acid (25 mg L^{-1}), supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The medium was further supplemented with different concentrations of plant growth regulators (PGRs): mT (0.0, 0.1, 0.5, 1.0, 1.5, and 2.0 mg L^{-1}), BAP (0.0, 0.5, 1.0, 1.5, 2.0, and 3.0 mg L^{-1}), and Kn (0.0, 0.5, 1.0, 1.5, 2.0, and 3.0 mg L^{-1}). Hi-LR™ MS medium without growth regulators was used as a control treatment. The pH of the medium was adjusted to 5.8 ± 0.02 before autoclaving. After inoculation culture were maintained at 25 ± 2°C, under a 16 h photoperiod with a cool white fluorescent light source about 30 cm above culture vessels, and 50–60% relative humidity. Axillary shoots, which started within 3 weeks after inoculation, were cut using sterile scissors, the apex was cut off and used as secondary explants for further shoot multiplication. For shoot proliferation, mT (0.1, 0.5, 1.0, 1.5, and 2.0 mg L^{-1}), BAP (0.5, 1.0, 1.5, 2.0, and 3.0 mg L^{-1}), and Kn (0.5, 1.0, 1.5, 2.0, and 3.0 mg L^{-1}) alone, and in combination with IAA (0.1 and 0.25 mg L^{-1}) and NAA (0.10 and 0.25 mg L^{-1}) were tested. To evaluate the effect of vessel size on the response of growth, cultures were maintained in 150 mL flat-bottom Borosil flasks filled with about 50 mL of the culture medium, under the same photoperiod and temperature (25 ± 2°C, 16 h photoperiod, 45–50 µmol m^{-2} s^{-1}). Further subculturing on fresh medium of similar composition was made every 20–25 days and repeated 2–3 times to maintain active growth and perpetual proliferation without any loss of the capacity for regeneration (Aremu et al., 2012; Badhepuri et al., 2023; Reshi et al., 2025).

Induction of in vitro rooting and acclimatization

The in vitro propagated shoots (2 cm in length, with 3–4 visible leaves) were aseptically cut and inoculated on half-strength MS. Various rooting media were used supplemented with 100.0 mg L^{-1} activated charcoal, 3.0% (w/v) sucrose, 0.8% (w/v) agar and one of three auxins, IBA (0, 0.5, 1.0, 2.0, and 3.0 mg L^{-1}), IAA (0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}), and NAA (0, 0.5, 1.0, 2.0, and 3.0 mg L^{-1}). The cultures were kept at 25 ± 2°C on a 16 h photoperiod with a light intensity of 45–50 µmol m^{-2} s^{-1} for 4 weeks. After rooting (3–4 well developed roots ≥ 1cm length), plantlets of *S. papilionacea* were gently removed from the culture vessels, and roots were thoroughly rinsed in sterile distilled water (2–3 times) to remove any agar medium. Rooted plantlets were planted in sterilized glass bottles (10 cm in diameter and 12 cm in height) with autoclaved Soilrite (1:1 w/w; Kel perlite, Bangalore, India). To meet the mineral nutrient requirements during the critical transition phase, the substrate was pre-moistened with ¼ strength MS basal salt solution. During the first week, containers were covered with polycarbonate lids (mini-

propagation chamber) and placed in a greenhouse with 85–90% relative humidity (RH) and 25 ± 2°C. Following week one, the polycarbonate lids were stepped up gradually (approx. 2–3 cm gap) for 3–4 days, before being stepped up further for another 3–4 days and finally removed completely after week 2. Acclimatization took place by gradually exposing plantlets to the warmer and drier environment, the humidity was reduced step-wise from 85–90% to 60–70% (Weeks 2–3) & 50–60% (Weeks 3–4), and the temperature was incrementally increased to 32 ± 2°C by moving them from the cooler humid environment to the warmer environment near the ventilation fans. In weeks 2 and 3, irrigation was reduced from daily to alternate-day with ¼ MS solution and in the fourth weeks, irrigation was reduced to once every 3 days with ¼ MS solution. Plantlets were then transplanted into pots (22 cm diameter × 30 cm height) containing a 1:1 (w/w) mix of Soilrite and garden soil and grown under nursery conditions for further growth. Long-term survival was evaluated by recording survival percentage after four weeks of pot transfer.

Comparative study of foliar micromorphological characters

The leaves from *S. papilionacea* plants grown under three different conditions were used for evaluation of foliar micromorphological traits: (i) in vitro shoots cultured on the MS medium with added mT + IAA for 4 weeks in multiplication stage (4-week-old in vitro plantlets), (ii) ex vitro hardened plants established in the green house for 6 weeks after transfer (6-week-old acclimatized plantlets) and (iii) mother plants from the nursery (10-week-old). To minimize ontogenetic variation, leaves were taken from the 3rd to 5th position from the apex, which are in the middle of their development (Chouhan et al., 2025). The abaxial epidermis, stomatal apparatus, epidermal vein density, epidermal vein islets and distribution were compared. Fresh leaves were immediately fixed in 5% (w/v) NaOH (Hi-Media Laboratories, Mumbai, India) for 24 h at room temperature to soften the tissues for removal of the pigment. This material was then immersed in 70% (v/v) EtOH for 24–48 h to obtain complete discoloration of the material (Ilham et al., 2025). Epidermal peels from the lower surfaces of the leaves were obtained with a sharp blade or separated after softening of the tissues by distilled water (3–4 times), stained with 1% (w/v) safranin in aqueous solution for 1 min and mounted in glycerol (Loba Chemie, Mumbai, India) for microscopic analysis using the Olympus CH20i binocular microscope (Olympus Corporation, Tokyo, Japan). All the images were taken using a Canon PowerShot A2500 digital camera (Canon Inc., Japan) at 4X, 10X and 40X magnification with the same illumination to make them comparable. High-magnification (40X) photomicrographs were also recorded for qualitative data on stomatal morphology. The stomatal density (number of stomata per mm^2 of leaf surface), stomatal type, and stomatal index were calculated as follows:

“Stomatal index (%) = [number of stomata ÷ (number of stomata + number of epidermal cells)] × 100”

Venation analyses were carried out on whole leaves of the same age as those used for stomatal study, and leaves were treated according to the standard diaphanization technique (Cutler et al., 2008). The fresh leaves were treated with 5% (w/v) NaOH solution (Hi-Media Laboratories) for 24–48 h at room

temperature to remove the chlorophyll and soften the tissue, and then it was dehydrated with an EtOH 70% (v/v) for 30 min. Cleared leaves were stained using 1% (w/v) aqueous safranin for 1–2 min and mounted in glycerol and examined under the microscope. Standard methods were used for measuring venation pattern, vein-islet density (number of islets per mm² of leaf lamina) and vein-termination number (number of vein-islet endings per mm²) between in vitro grown leaves and ex vitro hardened leaves (Manokari & Shekhawat, 2016; Manokari & Shekhawat, 2017). Vein density was standardized by taking counts of veins in standardized areas of the field at 40X magnification.

Comparative study of leaf and stem anatomy

The leaves and stem sections of *S. papilionacea* plants of the same age group were used as for the foliar micromorphological study. Transverse sections were cut freehand from the mid region of the stem segments (both internode and node) and leaves with sharp razor blade, washed in distilled water (3–4 times) to remove cell debris and stained with 1% (w/v) aqueous safranin for 3–5 min followed by 0.5% (w/v) fast green (Hi-Media Laboratories) for 30 sec. Sections were rinsed in distilled water to remove excess stain, mounted in glycerin on glass slides and examined under the Olympus CH20i microscope using light microscopy. All the photomicrographs were taken at the same magnification (4X, 10X, and 40X) under the same illumination and camera settings (Canon PowerShot A2500) and thus were comparable. The anatomical characteristics studied were: thickness of the epidermis, organization of the cortex and differentiation of the cell types, vascular bundles, stage of development of the xylem and the phloem, extent of secondary thickening, and pith structure. The anatomical terminologies were used according to the standardized terms (Manokari et al., 2021; Hazzazi et al., 2025).

Experimental design and statistical analysis

The experiments were repeated using a completely randomized design (CRD) with 11 replicates per treatment, with one explant constituting one replicate. Three separate experiments were conducted for all experiments. Morphological traits such as shoot number, shoot height, root number and root length were recorded after 4 weeks. Micromorphological parameters such as stomatal density, stomatal index, stomatal type, vein-islets and veinlet terminations and anatomy of stem and leaf were studied from three replicates at each developmental stage. Data are presented as mean ± SD, and analyzed using IBM SPSS Statistics version 27 with one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) for significant differences between the means of the treatments at $p \leq 0.05$ (Duncan, 1955).

Results

The effect of GA_3 on seed germination

The in vitro seed germination on half-strength MS medium was always low at any concentration of GA_3; maximum seed germination was 17.16 ± 0.56% at 200 mg L^{-1} of GA_3. The same trends were observed

under ex vitro conditions in Petri dishes, where the highest germination percentage was 19.13 ± 0.95% at 200 mg L^{-1} of GA_3. The highest germination rate (22.66 ± 1.33%) at 200 mg L^{-1} of GA_3 was recorded with seeds that were sown directly in pots under net-house conditions during the monsoon period (Table 1).

Table 1
Effect of GA_3 concentrations on the seed germination of *S. papilionacea* under in vitro, ex vitro, and net-house (monsoon) conditions.

S.N.	Concentration of GA_3 in ppm (*in vitro*, Half-strength MS)	Concentration of GA_3 in ppm (*ex vitro*, Petri Dishes)	*ex vitro* (Pots, Net-House; Monsoon season)	Seed germination (%) Mean ± SD
1	0	-	-	12.83 ± 0.56^{de}
2	100	-	-	13.57 ± 1.18^{e}
3	200	-	-	17.16 ± 0.56^{f}
4	300	-	-	8.33 ± 0.95^{ab}
5	400	-	-	11.29 ± 1.60^{bc}
6	500	-	-	10.12 ± 0.87^{bc}
7	-	0	-	10.18 ± 0.87^{c}
8	-	100	-	7.88 ± 1.11^{a}
9	-	200	-	19.13 ± 0.95^{f}
10	-	300	-	12.65 ± 2.20^{de}
11	-	400	-	9.01 ± 1.13^{abc}
12	-	500	-	10.92 ± 1.39^{cd}
13	-	-	0	11.22 ± 0.77^{cd}
14	-	-	100	13.48 ± 1.12^{ef}
15	-	-	200	22.66 ± 1.33^{h}
16	-	-	300	17.66 ± 2.33^{g}
17	-	-	400	10.08 ± 0.87^{bc}
18	-	-	500	9.14 ± 0.25^{abc}

Data are expressed as mean ± SD (%) from 3 independent experiments, with three replicate Petri dishes and pots containing 30 seeds each (n = 3). Means followed by different superscript letters are significantly different according to Duncan's multiple range test (DMRT) at $p \leq 0.05$.

The effect of plant cytokinins on shoot induction

No shoot induction was observed on the PGR-free MS medium, which is evident of the critical requirement of exogenous cytokinins. The mT was found to be the most efficient cytokinin in terms of regeneration efficiency out of the three cytokinins. Maximum shoot induction frequency (88.09 ± 4.12%) and number of shoots per explant (2.92 ± 0.16) were achieved at 0.5 mg L^{-1} mT. Higher concentrations reduced regeneration; 1.0 mg L^{-1} mT produced 73.81 ± 4.15% induction with 2.26 ± 0.28 shoots, while 1.5 and 2.0 mg L^{-1} reduced response to 66.67 ± 3.96% and 35.71 ± 7.14%, respectively. BAP proved inferior, giving the best results when applied at 1.0 mg L^{-1} (optimum response: 71.43 ± 7.14% induction, 2.77 ± 0.29 shoots per explant). Kinetin (Kn) was the least effective, with the highest induction of 47.62 ± 8.24% at 1.5 mg/L. The induction frequency and proliferation of shoots were always higher for mT compared to the two alternatives (Fig. 2A, Table 2).

Table 2

Effect of different concentrations of mT, BAP, and Kn on in vitro shoot induction from nodal explants of *S. papilionacea* cultured on MS medium after 3 weeks of culture.

PGRs (mg L^{-1})			Shoot induction % (mean ± SD)	Number of shoots per explant (mean ± SD)
mT	**BAP**	**Kn**		
0.0	0.0	0.0	00.00 ± 0.00^{a}	0.0 0 ± 0.0^{a}
0.1	-	-	50.00 ± 7.14^{ef}	2.15 ± 0.17^{cdef}
0.5	-	-	**88.09 ± 4.12^{j}**	**2.92 ± 0.16^{g}**
1.0	-	-	73.81 ± 4.15^{i}	2.26 ± 0.28^{ef}
1.5	-	-	66.67 ± 3.96^{ghi}	1.74 ± 0.23^{bcd}
2.0	-	-	35.71 ± 7.14^{cd}	1.41 ± 0.23^{b}
-	0.5	-	61.90 ± 4.12^{gh}	1.52 ± 0.23^{b}
-	1.0	-	**71.43 ± 7.14^{hi}**	**2.77 ± 0.29^{g}**
-	1.5	-	59.52 ± 4.12^{fg}	2.22 ± 0.23^{def}
-	2.0	-	47.62 ± 8.24^{e}	1.66 ± 0.22^{bc}
-	3.0	-	28.57 ± 7.14^{bc}	1.26 ± 0.17^{b}
-	-	0.5	23.81 ± 4.12^{d}	1.52 ± 0.34^{b}
-	-	1.0	35.71 ± 7.14^{cd}	2.44 ± 0.40^{fg}
-	-	1.5	**47.62 ± 8.24^{e}**	**1.77 ± 0.22^{bcde}**
-	-	2.0	45.24 ± 4.12^{de}	1.70 ± 0.39^{bc}
-	-	3.0	28.57 ± 7.14^{bc}	1.54 ± 0.48^{b}

The values represent mean ± SD from 3 separate experiments. The shoot induction frequency and the number of shoots were assessed on 11 replications per experiment. Small letters in the superscript in each column show significant differences among the treatments by DMRT at $p \leq 0.05$.

Effect of combination of cytokinins and auxin on shoot multiplication

The mT alone at 0.5 mg L^{-1} produced 7.48 ± 0.67 shoots per explant with maximum shoot length (3.19 ± 0.78 cm). BAP alone at optimum concentration (1.0 mg L^{-1}) produced 5.55 ± 0.44 shoots per explant

(2.19 ± 0.31 cm length). Multiplication was greatly improved when mT was supplemented with IAA. The highest multiplication rate was obtained with 1.0 mg L^{-1} mT and 0.10 mg L^{-1} IAA (20.67 ± 2.06 shoots per explant), which was almost three times higher than with mT alone. The same effect was also achieved by the combination of 0.5 mg L^{-1} mT + 0.10 mg L^{-1} IAA, which yielded a high multiplication rate (17.81 ± 1.74 shoots per explant). A significant decrease in shoot proliferation with an increase in IAA concentration up to 0.25 mg L^{-1} was observed. The combinations of mT with NAA were much less effective, with a maximum response of 8.63 ± 1.08 shoots per explant at 1.0 mg L^{-1} mT + 0.10 mg L^{-1} NAA. When comparing the multiplication of BAP and IAA combinations, 2.0 mg L^{-1} BAP + 0.10 mg L^{-1} IAA resulted in 18.89 ± 3.49 shoots per explant, while the 1.0 mg L^{-1} BAP + 0.10 mg L^{-1} IAA combination resulted in 14.41 ± 1.56 shoots per explant. The combinations BAP and NAA resulted in fewer shoots compared to their corresponding IAA treatments; the least number of shoots was obtained with BAP + NAA at 3.0 mg L^{-1} (4.78 ± 1.94 shoots, 0.45 ± 0.16 cm length). However, elongation of the shoot was not associated with the proliferation patterns; the treatments that yielded the highest shoot numbers led to comparatively shorter shoots (1.99–2.17 cm), indicating a negative correlation between the multiplication rate and the elongation of each shoot (Fig. 2B, Table 3).

Table 3
Effect of different PGR concentrations and combinations of mT, BAP, IAA, and NAA on in vitro shoot multiplication of *S. papilionacea* cultured on MS medium after 3 weeks of culture.

PGRs (mg L^{-1})				Number of Shoots (Mean ± SD)	Length of Shoots (cm) (Mean ± SD)
mT	**BAP**	**IAA**	**NAA**		
0.0	0.0	0.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0.1	-	-	-	3.18 ± 0.42^{b}	1.57 ± 0.33^{ij}
0.5	-	-	-	**7.48 ± 0.67^{efghij}**	**3.19 ± 0.78^{n}**
1.0	-	-	-	5.41 ± 0.83^{bcdef}	2.64 ± 0.75^{m}
1.5	-	-	-	4.48 ± 0.68^{bcd}	1.58 ± 0.31^{ij}
2.0	-	-	-	4.11 ± 0.40^{bcd}	1.43 ± 0.31^{fghij}
-	0.5	-	-	3.00 ± 0.29^{b}	1.67 ± 0.28^{ij}
-	1.0	-	-	**5.55 ± 0.44^{bcdefg}**	**2.19 ± 0.31^{klm}**
-	1.5	-	-	4.29 ± 0.23^{bcd}	1.93 ± 0.13^{jkl}
-	2.0	-	-	4.15 ± 0.25^{bcd}	1.76 ± 0.23^{ijk}
-	3.0	-	-	4.04 ± 0.46^{bcd}	0.98 ± 0.21^{bcdefg}
0.5	-	0.10	-	17.81 ± 1.74^{o}	2.17 ± 0.16^{lm}
0.5	-	0.25	-	13.15 ± 1.77^{mn}	1.84 ± 0.10^{jk}
0.5	-	-	0.10	8.26 ± 0.89^{ghij}	1.20 ± 0.13^{hij}
0.5	-	-	0.25	6.52 ± 1.95^{cdefgh}	1.37 ± 0.21^{hij}
1.0	-	0.10	-	**20.67 ± 2.06^{p}**	**1.99 ± 0.22^{hij}**
1.0	-	0.25	-	13.63 ± 2.33^{mn}	1.65 ± 0.25^{defghi}
1.0	-	-	0.10	8.63 ± 1.08^{hij}	1.09 ± 0.16^{bcdefg}
1.0	-	-	0.25	7.66 ± 0.58^{fghij}	0.99 ± 0.02^{bcde}
2.0	-	0.10	-	6.82 ± 1.17^{defgh}	1.01 ± 0.10^{bcdef}

Values are mean ± SD of 3 independent experiments, with 11 replicates per experiment. Means followed by different lowercase superscript letters within a column are significantly different according to DMRT at $p \leq 0.05$.

PGRs (mg L^{-1})				Number of Shoots (Mean ± SD)	Length of Shoots (cm) (Mean ± SD)
mT	BAP	IAA	NAA		
2.0	-	0.25	-	5.48 ± 0.79^{bcdefg}	1.16 ± 0.04^{bcde}
2.0	-	-	0.10	5.66 ± 0.80^{bcdefg}	0.88 ± 0.10^{bcde}
2.0	-	-	0.25	3.77 ± 0.69^{bc}	0.97 ± 0.05^{b}
-	1.0	0.10	-	14.41 ± 1.56^{n}	2.37 ± 0.33^{hij}
-	1.0	0.25	-	11.37 ± 2.00^{klm}	1.86 ± 0.16^{efghi}
-	1.0	-	0.10	9.92 ± 1.29^{ijk}	1.54 ± 0.25^{bcde}
-	1.0	-	0.25	8.26 ± 2.59^{ghij}	1.53 ± 0.38^{cdefgh}
-	2.0	0.10	-	**18.89 ± 3.49^{op}**	**1.54 ± 0.33^{ghij}**
-	2.0	0.25	-	12.48 ± 1.06^{lmn}	1.28 ± 0.24^{defghi}
-	2.0	-	0.10	10.11 ± 1.11^{jkl}	0.95 ± 0.10^{bcde}
-	2.0	-	0.25	7.91 ± 1.48^{fghij}	0.90 ± 0.16^{bc}
-	3.0	0.10	-	7.29 ± 2.12^{efghi}	0.92 ± 0.13^{bcd}
-	3.0	0.25	-	5.67 ± 1.19^{bcdefg}	0.93 ± 0.13^{bcde}
-	3.0	-	0.10	6.18 ± 1.58^{cdefgh}	0.79 ± 0.10^{bc}
-	3.0	-	0.25	4.78 ± 1.94^{bcde}	0.45 ± 0.16^{bc}

Values are mean ± SD of 3 independent experiments, with 11 replicates per experiment. Means followed by different lowercase superscript letters within a column are significantly different according to DMRT at $p \leq 0.05$.

Effect of auxins on in vitro rooting, hardening and acclimatization of rooted plantlets

No root induction was observed on an auxin-free medium. IBA was found to be the best auxin for rooting. Maximum rooting frequency (63.63 ± 13.88%), root number per shoot (3.36 ± 0.25), and root length (3.29 ± 0.27 cm) were achieved at 1.0 mg L^{-1} IBA. At 2.0 mg L^{-1} IBA, rooting decreased to half the fold with reduced root number and length, while 3.0 mg L^{-1} IBA produced around 4-fold less compared to 1.0 mg L^{-1} IBA. IAA supported rooting but was less effective, with maximum frequency (57.57 ± 13.88%) at 1.0 mg L^{-1}, producing 1.85 ± 0.38 roots per shoot with 2.01 ± 0.56 cm length. NAA was the least effective, achieving 45.45 ± 5.25% rooting at 1.0 mg L^{-1} with only 0.90 ± 0.16 roots per shoot with 1.47 ±

0.36 cm in length (Fig. 2C, Table 4). Rooted shoots grew excellently from culture to greenhouse in Soilrite substrate. The establishment of plantlets was good and maintained normal morphology in nursery conditions, with a 68% survival rate.

Table 4
Effect of different auxins: IBA, IAA, and NAA on *in vitro* root induction of regenerated shoots of *S. papilionacea* cultured on MS medium after 3 weeks of culture.

PGRs (mg L^{-1})			Root Induction % (Mean ± SD)	Number of Roots per Explant (Mean ± SD)	Length of Roots (cm) (Mean ± SD)
IBA	IAA	NAA			
0.0	0.0	0.0	00.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0.5	-	-	33.33 ± 9.09^{b}	1.70 ± 0.23^{fg}	1.69 ± 0.23^{de}
1.0	**-**	**-**	**63.63 ± 13.88^{d}**	**3.36 ± 0.25^{i}**	**3.29 ± 0.27^{g}**
2.0	-	-	45.45 ± 9.09^{bc}	2.61 ± 0.19^{h}	2.57 ± 0.19^{f}
3.0	-	-	15.15 ± 5.25^{a}	1.25 ± 0.13^{de}	1.21 ± 0.14^{bcd}
-	0.5	-	36.36 ± 9.09^{b}	1.06 ± 0.14^{cd}	1.48 ± 0.46^{cde}
-	1.0	-	57.57 ± 13.88^{cd}	1.85 ± 0.38^{g}	2.01 ± 0.56^{e}
-	2.0	-	39.39 ± 13.88^{b}	1.39 ± 0.14^{ef}	1.39 ± 0.27^{bcd}
-	3.0	-	12.12 ± 5.25^{a}	0.97 ± 0.19^{cd}	0.96 ± 0.19^{bc}
-		0.5	15.15 ± 9.09^{b}	0.87 ± 0.05^{bcd}	1.36 ± 0.35^{bcd}
-		1.0	45.45 ± 5.25^{cd}	0.90 ± 0.16^{bcd}	1.47 ± 0.36^{cde}
-		2.0	27.27 ± 9.09^{b}	0.75 ± 0.10^{bc}	1.15 ± 0.20^{bcd}
-		3.0	12.12 ± 5.24^{a}	0.60 ± 0.05^{b}	0.89 ± 0.21^{b}

Values represent the mean ± SD of 3 independent experiments, each having 11 replications per treatment. Means with different small letters in the same column indicate statistically significant differences at $p \leq 0.05$ based on DMRT.

Analysis of foliar micromorphology and anatomy of leaf and stem

Micromorphological study was performed at three different developmental stages. The 4-week-old in vitro plantlets showed high stomatal density and stomatal index 46.61 ± 2.01 and 28.19 ± 1.05%, respectively, stomata were fully opened and predominantly anomocytic and anisocytic. Acclimatized

leaves (6-week-old) had an intermediate stomatal density & stomatal index (29.90 + 2.74, 24.54 ± 0.18%, respectively), partially closed & anisocytic stomata, and better organization. The lowest stomatal density (21.10 ± 3.49) and stomatal index (20.96 ± 3.04%), and near-completely closed stomata occurred in mother plant leaves, which had only anisocytic stomata, perfectly distributed at uniform intervals and fully functional (Fig. 3A, B, C; Table 5). The in vitro grown leaves had open, simple reticulate venation, low vein-islet number (20.96 ± 3.04 per mm²) and few veinlet terminations (7.33 ± 1.52 per mm²). The intermediate vascular development (34.66 ± 2.51 vein-islets per mm², 14.66 ± 2.08 veinlet terminations per mm²) was observed in the acclimatized leaves. The mother plant leaves were well organized, with closed reticulate venation and significantly higher vein-islet number (58.33 ± 4.50 mm²) and veinlet terminations (23.66 ± 3.51 mm^2) (Fig. 3D, E, F; Table 5). Similarly, the leaf anatomy showed the progressive adaptation of different developmental stages. Leaves of four-week-old in vitro plantlets had thin cuticles, single-layered epidermis and loosely organized mesophyll tissues. The 6-week-old acclimatized leaves showed slightly thickened cuticles, distinct epidermal layers and partially differentiated palisade and spongy mesophyll with higher intercellular spaces. The protective cuticles of mother plant leaves were thick, and the stomatal apparatus was well developed and functional. The epidermis was multi-layered in the mother plant leaves (2–3 layers), the palisade mesophyll was dense, and the spongy mesophyll was well developed with well-differentiated vascular bundles (Fig. 4A, B, C).

Table 5
Stomatal density, stomatal index, number of vein-islets, and number of veinlet terminations in *S. papilionacea* at different development stage.

S.N.	Development stage	Stomatal density	Stomatal index (%)	Number of vein-islets per mm^2	Number of veinlet terminations per mm^2
1	4-week-old (*in vitro*)	46.61 ± 2.01^{c}	28.19 ± 1.05^{b}	20.96 ± 3.04^{a}	7.33 ± 1.52^{a}
2	6-week-old (acclimatised)	29.90 ± 2.74^{b}	24.54 ± 0.18^{ab}	34.66 ± 2.51^{b}	14.66 ± 2.08^{b}
3	Mother plant	21.10 ± 3.49^{a}	20.96 ± 3.04^{a}	58.33 ± 4.50^{c}	23.66 ± 3.51^{c}

Values are presented as mean ± SD of 3 independent experiments, with 3 replicates per experiment. Means followed by different lowercase letters within a column are significantly different according to DMRT at $p \leq 0.05$.

A significant adaptive developmental pattern was observed in internode and node of stem anatomy. Four-week-old in vitro grown plantlets' internode and node had a thin epidermis, narrow cortex comprising loosely packed parenchyma, small vascular bundles with little secondary thickening and a large pith. Acclimatized internode and node of 6-week-old plantlets had thickened epidermis and cortex with differentiated cells, early development of secondary xylem in vascular bundles and an organized pith. Mother plant internode and node had thick epidermis, well-developed cortex with sclerified cells, and vascular bundles with extensive secondary xylem and significant lignification, while the pith was

reduced. Secondary xylem vessel diameter increased, providing the plant with better water and nutrient transport ability (Fig. 5A, B, C). Nodal anatomy was a function of developmental complexity. Nodes of four-week-old in vitro plantlets were characterized by the emergence of the first traces (3–4 bundles), the absence of vascular differentiation and poorly organized parenchymatous tissues. In the nodes of 6-week-old acclimatized plants, the leaf traces (5–6) were somewhat differentiated with the beginnings of vascular connections, and the tissue organization was moderately differentiated. The leaf traces (8–10 bundles) found at the mother plant nodes were well developed, with well discernible vascular bundles, well-defined vascular connections to lateral buds and closely organized anatomical structure (Fig. 5D, E, F).

Discussion

Micropropagation is particularly useful for narrow-range taxa lacking in natural recruitment, as it can be used to supplement or even replace population reinforcement through seeds when germination is limited or unpredictable. In *S. papilionacea*, plant seed germination percentage is very poor, and they grow only in restricted arid regions of India, and suffer from habitat destruction due to construction. The present study demonstrates an efficient in vitro propagation protocol for *S. papilionacea* and importantly illustrates that propagation success must be viewed not only in terms of shoot multiplication and rooting, but in terms of the acquisition of functional foliar and stem anatomy necessary for ex vitro survival. The slight improvement of seed germination under GA_3, especially at the 200 ppm, suggests that there may be some physiological dormancy, but GA_3 is not enough to completely break seed dormancy (He et al., 2020). In many species, moderate levels of GA_3 stimulate embryo growth and the mobilization of reserve food, whereas high levels of GA_3 may have little or no effect due to hormonal imbalance. The slight advantage gained by sowing under a net house during the monsoon further indicates that there is a need for some natural environmental cues such as fluctuating temperatures, moisture pulses and improved substrate aeration to break the partial dormancy (Müller et al., 2019). All of these results suggest the significant application of clonal propagation for this species, especially for the conservation & multiplication.

Effect of cytokinin on shoot induction and combination with auxin on shoot multiplication

The cytokinin specificity is crucial, as the selection of the growth regulators affects both the frequency of regeneration and the anatomical quality and acclimatization ability of the regenerated plantlets. One important finding of the present study is that mT is more superior to BAP and kinetin for shoot induction. A complete lack of shoot formation on the hormone-free MS medium further confirms that endogenous cytokinin is not enough to activate the axillary meristems in *S. papilionacea* and requires the exogenous application of cytokinin for the occurrence of organogenic response. Out of the three cytokinins tested, mT at 0.5 mg L^{-1}, induced the highest number of shoots and had the best regeneration efficiency. This supports that aromatic cytokinins, particularly mT, are capable of inducing shoot proliferation with less

physiological abnormality and healthier plant growth than adenine-type cytokinins (Abdouli et al., 2022). In *Vanilla planifolia*, for instance, mT significantly improved the efficiency of micropropagation and the quality of plants (Manokari et al., 2021). Regeneration success has also been reported for threatened and medicinal plants where selection of cytokinin proved to be critical for the responsiveness of the explants. Based on less number induction percentage at mT concentrations above optimum, *S. papilionacea* appears to have a narrow optimum for cytokinin concentrations. These supra-optimal cytokinin effects are well documented and commonly linked to disturbances in the apical–axillary balance, abnormalities in the organization of meristems and/or lowered quality of shoots (Ptak et al., 2023; Abdalla & Dobránszki, 2024; Chouhan et al., 2025). BAP was moderately responsive, while the least responsive was kinetin, showing a clear genotype-specific preference for mT. It was found that cytokinin and auxin showed synergistic effect when used in combination for multiplication. The combination of low concentration of IAA with mT significantly enhanced multiplication of shoots, especially with mT at 1.0 mg L^{-1} with 0.10 mg L^{-1} IAA. The nearly 3-fold more number of shoots proliferated as compared to mT alone shows a strong synergistic effect between cytokinin and auxin (Novitasari et al., 2025; Masih & Kataria, 2026). Current knowledge of plant regeneration highlights the importance of the balance between PGRs, not the ability of an individual PGR. Low auxin concentrations promote meristematic competence, cell division and bud outgrowth, whereas high auxin concentrations inhibit shoot proliferation or cause callusing. This is the reason why the multiplication rate was decreased with higher IAA concentrations (above 0.10 mg L^{-1}) in the present study. The poor performance of NAA compared with IAA is also indicative of its greater persistence and often poor ability to support organized shoot development in some species, also often associated with compact callus formation. BAP + IAA combinations also improved multiplication, but their response was lower than that of the mT-based combinations, once again demonstrating the superior response of mT for this species. Interestingly, treatments that resulted in the greatest number of shoots also resulted in relatively shorter shoots, suggesting an inverse relationship between shoot proliferation intensity and shoot elongation (Šmeringai et al., 2023).

Effect of auxin on root induction and acclimatization of plantlets

Rooting was completely exogenous auxin-dependent, and IBA seemed to be superior than IAA and NAA, with 1.0 mg L^{-1} being optimum. This finding is similar to the well-established role of IBA in inducing adventitious rooting, as it is relatively stable, easily taken up and remains physiologically active during the initiation and elongation of the roots (Pendli et al., 2019). IAA also stimulated root formation but not as well as NAA, presumably due to its fast metabolism and short lifespan in cultured tissues. NAA was the most inefficient and was even inhibitory at higher concentrations, a phenomenon often explained by basal tissue swelling, high concentrations of auxin or reduced root elongation. The decrease in rooting when using higher IBA concentrations further emphasizes the dose-dependent effect of IBA on root induction in *S. papilionacea*. The in vitro grown plants are transferred into a greenhouse, as the plant parts developed in high humidity, low irradiance and sugar-supported conditions are structurally and

physiologically incompatible with the ambient conditions (Pendli et al., 2019; Masih & Kataria, 2026). Thus, the chances of the survival of the post-rooting plants rely in great measure on the possibility of adapting and remodeling of the tissue culture-derived organs. In our study, the *in-vitro* grown plants were successfully transferred to Soilrite, and the plantlets survived, which indicates the feasibility of the protocol, with an optimized acclimatization step.

Adaptive mechanisms during different development stages at foliar micromorphological and anatomical levels

One of the most valuable findings of the present study is that foliar micromorphology closely reflects developmental adaptation from in vitro to field conditions. High stomatal density, stomatal index, and abnormal stomatal structures like contiguous stomata and persistently open stomata were found in in vitro grown plantlets' leaves (Manokari & Shekhawat, 2017). These are typical characteristics of leaves grown in tissue culture, and are typically observed to be associated with high transpiration rates, low stomatal control and limited ex vitro drought resistance. Acclimatized leaves, on the other hand, had a reduced stomatal density, stomatal index, and stomata were better organized and partially functional, while mother plant leaves had the lowest stomatal density & stomatal index, only anisocytic stomata and stomatal organization was well established. The reduction of stomatal index from in vitro to field-grown plants is a very strong indication of progressive epidermal specialization towards controlling water use. In a desert species this change is significant to limit transpirational loss while ensuring gas exchange that is critical for survival. The stomatal development is known to be regulated by the environment and several in vitro grown plants under high humidity conditions tend to develop many but weak stomata. Similar acclimatization-related changes in foliar micromorphology have been described in several plants, and stomatal normalization has been reported in *Morinda citrifolia*, *Couroupita guianensis*, and in *Hedyotis*/*Oldenlandia* species, correlated with the hardening success (Shekhawat & Manokari, 2017; Revathi et al., 2018; Behera et al., 2018). Based on the results of the present study, the stomatal traits can be used as a functional tool for predicting the readiness of the plantlets to be transplanted. The presence of a gradually more complex venation of in vitro grown leaves to acclimatized and mother plant leaves implies that the maturation of the vascular system is also an important factor in establishing a successful plant. Leaves of in vitro grown plants were simple, open, with few veinlets and veinlet terminations, and low vein-islet frequency, implying limited hydraulic and assimilate transport capacity. These values were much higher during acclimatization and peaked in plants grown in the field, where the leaves had dense, closed reticulate venation and well-developed areolation. An elaborate network of veins provides greater mechanical support, water transport and assimilate export and thus enhances leaf function in nature (Chouhan et al., 2025; Bhusan et al., 2026). This is a venation pattern that matches the overall anatomical development that occurs during hardening. Dense venation could be particularly beneficial in *S. papilionacea* when water is lacking, due to its adaptation to dry habitats, in terms of maintaining hydration status of the plant and efficient water transport.

The leaf transverse sections offer direct proof of significant structural reprogramming during acclimatization of *S. papilionacea*. The in vitro grown plantlets' leaves were characterized by their thin

cuticle, single-layered epidermis and loosely arranged mesophyll, indicating their juvenile and weakly protected state typical of culture-grown tissues. As the plants acclimatized, the cuticle thickened, and epidermal differentiation became better, with more distinct palisade and spongy mesophyll. In mother plant leaves, anatomy showed the well-organized tissue structure with a well-differentiated vascular system. These changes can directly affect the ability of a plant to avoid drought, mechanical protection, internal diffusion and photosynthetic efficiency. Slightly comparable anatomical changes were observed in *Ilex paraguariensis*, where the organization of leaves in vitro was significantly different from that of ex vitro leaves, showing that the culture environment is affecting both mesophyll differentiation and epidermal development (Luna et al., 2017; Suárez et al., 2019). A similar adaptive pattern was observed in the stem anatomy, with the in vitro grown plantlets' stem anatomy showing less tissue differentiation and being more fragile compared to the mother plant, it being the mechanically competent. Limited support and low conductive capacity were found in in vitro internodes due to thinner epidermis, narrower cortex, and smaller vascular bundles, along with the lack of significant secondary thickening and the presence of a large pith. The early development of secondary xylem, good cortical differentiation, and better organization of the pith were observed in the acclimatized stems, while the mother plant internodes exhibited well-developed vascular bundles. All of these observations collectively suggest an increase in mechanical and conductive tissues in the acclimatized plants. Mechanical and temperature stresses, combined with the high evaporative demand, are especially challenging to desert plants, and thus structural reinforcement is particularly important. Shoots produced in vitro may not be well developed in terms of xylem due to the decrease in the necessity of water transport in the culture environment. But during acclimatization, a re-establishment of transpiration demand is followed by a differentiation of additional functional vascular tissues (Suárez et al., 2019; Bhusan et al., 2026). It is therefore suggested that the higher xylem vessel diameter as seen in the older stems of *S. papilionacea* is due to increased hydraulic competence and would be beneficial in field conditions in order to transport water and nutrients. The nodes of in vitro grown plants showed poor tissue organization and only a few rudimentary leaf traces, indicating a limited capacity for branches. The traces were more differentiated and developed vascular continuity in nodes of the acclimatized plantlets, while numerous well-defined leaf traces, clear vascular continuity with lateral buds and compact organization were seen in mother plant nodes. The ecological competitiveness and canopy development depend on nodes because node maturation is the result of the integration of leaf supply, axillary bud activation and branch initiation. The number of shoots and roots is not the only criterion used to assess successful micropropagation. Establishment of the whole plant involves restoration of the competence of the architecture and internal vascular integration, particularly for species that need to quickly stand up after transfer due to natural stress. Similar interpretations have been made in some other micropropagated plants in which anatomical normalization was associated with better field performance (Gautam et al., 2024). In *S. papilionacea*, progressive development of nodes and an increased number of lateral buds, which are connected to an increase in branching and architectural complexity, are required for more competitive in-field growth.

Conservation significance and conclusion

Overall, our results suggest that *S. papilionacea* is a recalcitrant species to propagate from seed, yet an excellent species for conservation-based micropropagation. An optimum regeneration system consisted of mT for shoot induction, mT + IAA for multiplication, and IBA for rooting before a structured hardening in Soilrite. Most importantly, the anatomical and micromorphological data showed that acclimatization is a biological transformation process, where the tissue culture-derived plantlets develop epidermal, mesophyll, vascular and nodal characteristics that enable them to grow and develop independently in the field. The present findings are consistent with those of previous studies which demonstrated that survival and restoration potential of rare plants can be successfully predicted by a combination of gradual acclimatization and foliar and stem anatomical analysis for micropropagated plants. This is particularly useful for rare desert species like *S. papilionacea*, where the ability to combine the regeneration efficiencies with the quality assessment of structure is particularly beneficial. The protocol demonstrated here thus has more than propagation applications, as it sets a standard for the selection of physiologically competent plantlets for nursery hardening, field transfer and eventual population reinforcement. This research concluded that *S. papilionacea* species has limited seed germination in nature; thus, this study aimed to establish an efficient mT-based micropropagation protocol that might be applicable to this species. These integrated anatomical development changes are a result of successful physiological adaptation for field establishment. The present protocol offers a sustainable and reliable method of propagation and ex situ conservation of *S. papilionacea* and could serve as an alternative to the unreliable natural propagation in small and scattered populations, and for possible reintroduction in the Thar Desert.

Future perspectives

Future studies are recommended to optimize field acclimatization protocols to enhance the survival rate above the present 68%, including studying the effect of the use of PGR combinations. Overall, the established propagation protocol should be tested at a larger scale to evaluate the cost-effectiveness and scalability of propagation and conservation initiatives for a commercial application. DNA fingerprinting and metabolite profiling of regenerated plants would provide molecular confirmation of genetic fidelity and pharmacological equivalence to the mother plants, which is necessary for quality control of conservation-propagated plants. Biochemical studies of the observed micromorphological adaptations, such as switch from anomocytic to anisocytic stomata and improved venation development, would offer insight into mechanisms of physiological hardening in acclimatization. The implementation of this micropropagation method in the ex situ conservation programs and Thar Desert habitat restoration projects may be useful for population recovery of *S. papilionacea*. The principles developed here may be potentially applied to other endangered xerophytic medicinal plants with similar propagation problems and may be used in further comparative research on other related species of arid-adapted plants.

Declarations

All the figures are original and drawn by the authors.

Conflict of Interests

The authors declare no financial or commercial conflict of interest.

Ethics Declaration

Not Applicable, as the research does not involve humans and/or animals.

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Declaration:

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Author Contribution

SJ and SKC conceptualized and designed the experiments. DC performed all the experiments, analyzed and interpreted the data and wrote the entire manuscript. All authors critically reviewed and approved the manuscript.

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Data Availability

The datasets generated and analyzed during the current study are available on reasonable request.

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Figures

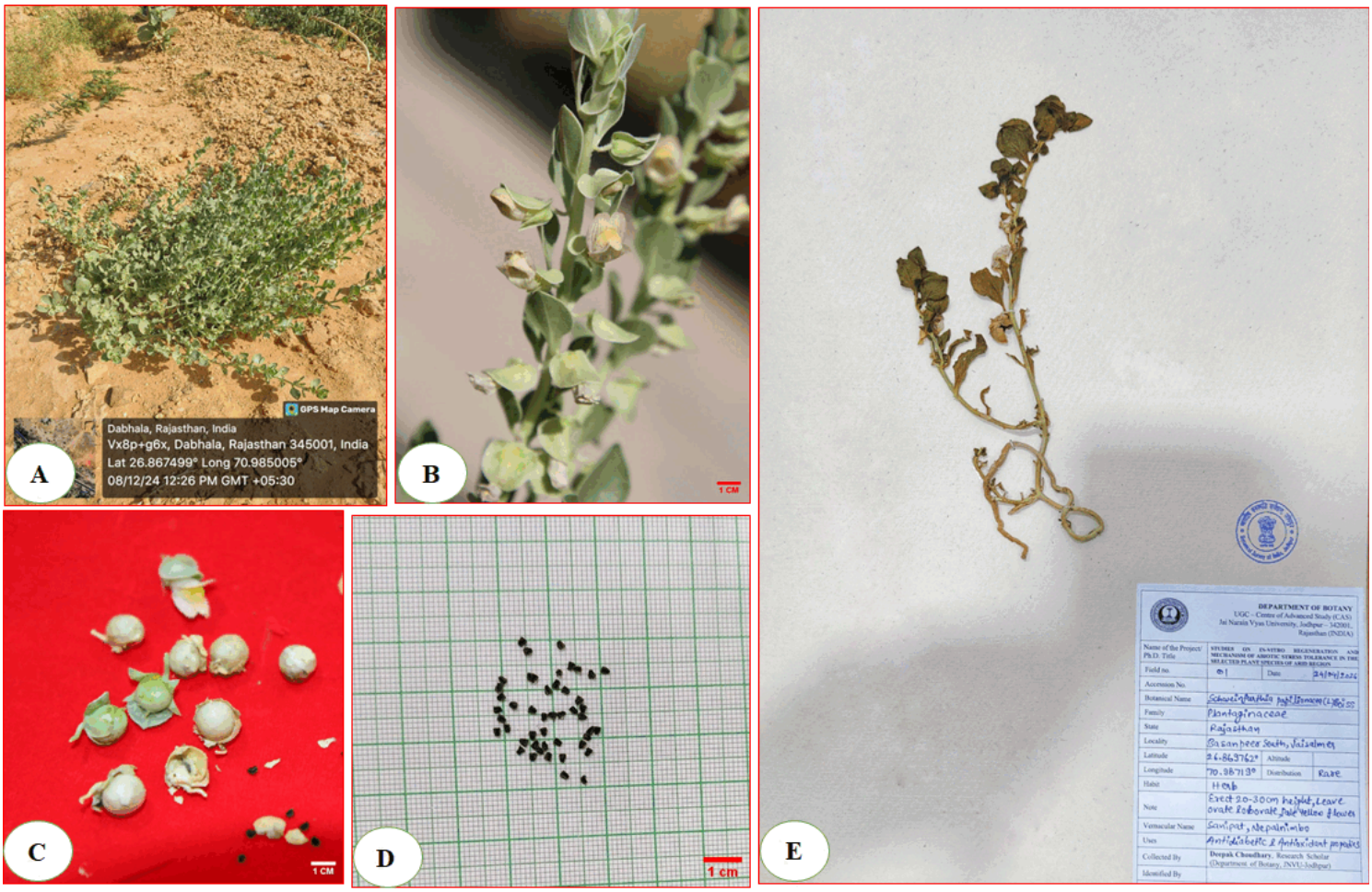


Figure 1

***Schweinfurthia papilionacea*; habitat, morphology, fruits, seeds, and herbarium specimen:** (A) Natural habitat (near Jaisalmer, Rajasthan, India (26.867499° N, 70.985005° E)), showing characteristic succulent rosette growth. (B) Flowering shoots with pale yellow flowers, (C) Mature berry fruits, (D) Mature seeds, (E) Herbarium sheet; authenticated (BSI, Jodhpur) whole plant specimen with root system, stem branching, and reproductive structures, with taxonomic and collection details.



Figure 2

In vitro micropropagation and ex vitro acclimatization of *S. papilionacea*: (A) Shoot induction from nodal explants on MS medium supplemented with mT, showing early shoot and shoot primordium development (B) Multiple shoot proliferation stage on multiplication medium, displaying well-developed green shoots with expanded leaves in MS medium containing mT + IAA. (C) In vitro rooting of regenerated shoots on half-strength MS medium with IBA, showing profuse adventitious root development. (D) Rooted plantlets transferred to Soilrite substrate for initial hardening under controlled humidity and light conditions, demonstrating successful root establishment and early *ex vitro*adaptation. (E) Fully acclimatized, hardened plant in a pot showing vigorous growth and expanded foliage.

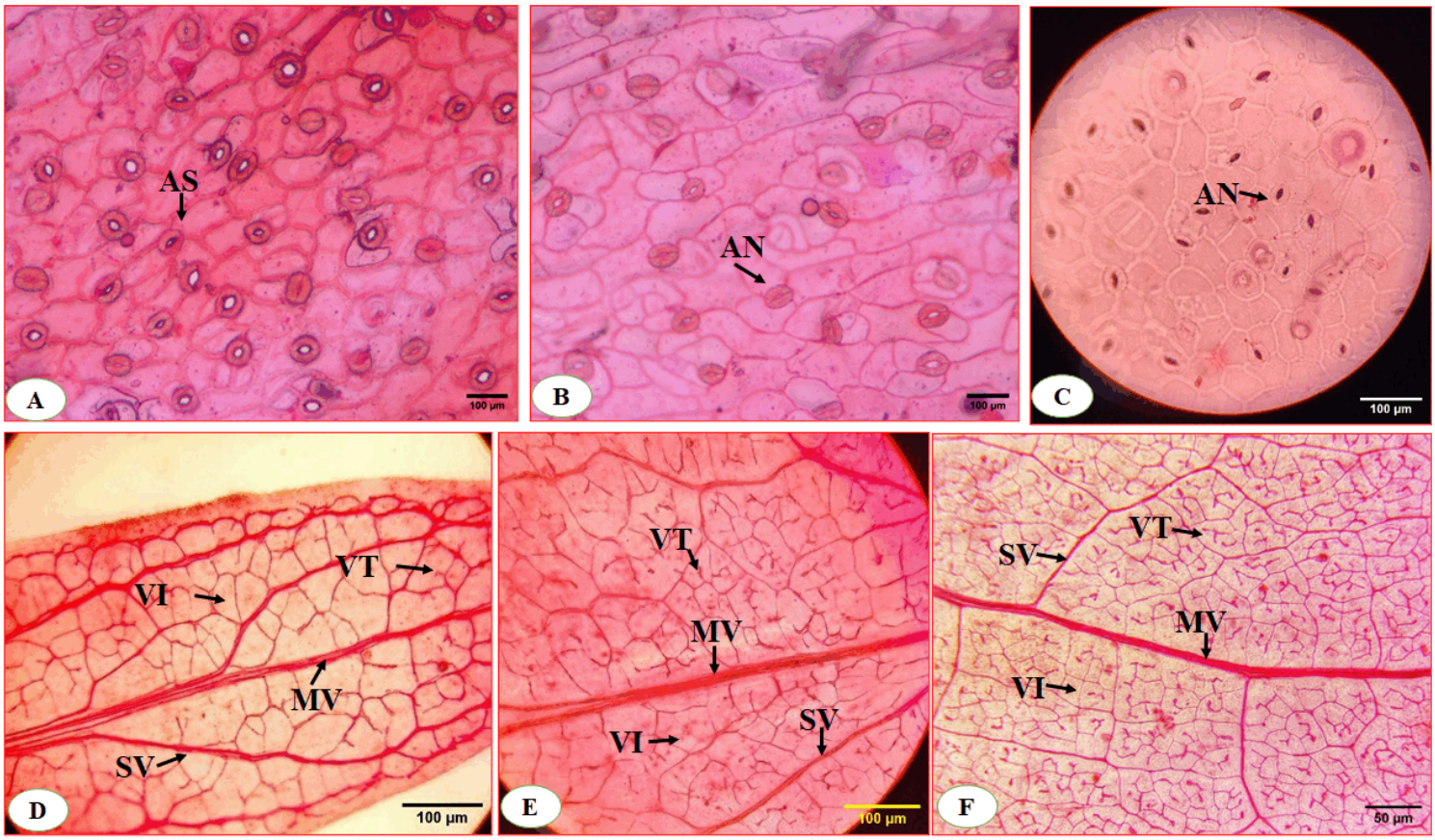


Figure 3

Comparative foliar micromorphology of *S. papilionacea* (stomatal morphology and venation patterns): Stomatal studies (A to C): (A) Abaxial epidermis of a 4-week-old in vitro plantlet showing open and high stomatal density with prominent guard cells, and anomocytic (AS) type stomata present (B) Abaxial epidermis of a 6-week-old acclimatized leaf displaying anisocytic (AN) type stomata with partially opened, reduced stomatal density, (C) Abaxial epidermis of mother plant (field-grown) with low, regularly distributed stomata, AN type stomata and completely closed stomata (scale bar: A-C = 100 µm). Venation studies (D to F): (D) Venation pattern of a 4-week-old in vitro plantlet showing primary(MV; mid vein) and secondary veins (SV), fine tertiary vein differentiated into vein termination (VT), and closed patches of vein denoted by VI; vein islet (E) Venation of a 6-week-old acclimatized leaf with increased vein-islet frequency and refined VT patterns, (F) Mature venation of mother plant displaying dense vein-islet network with highly differentiated tertiary veins (scale bar: D-E = 100 µm; F = 50 µm).

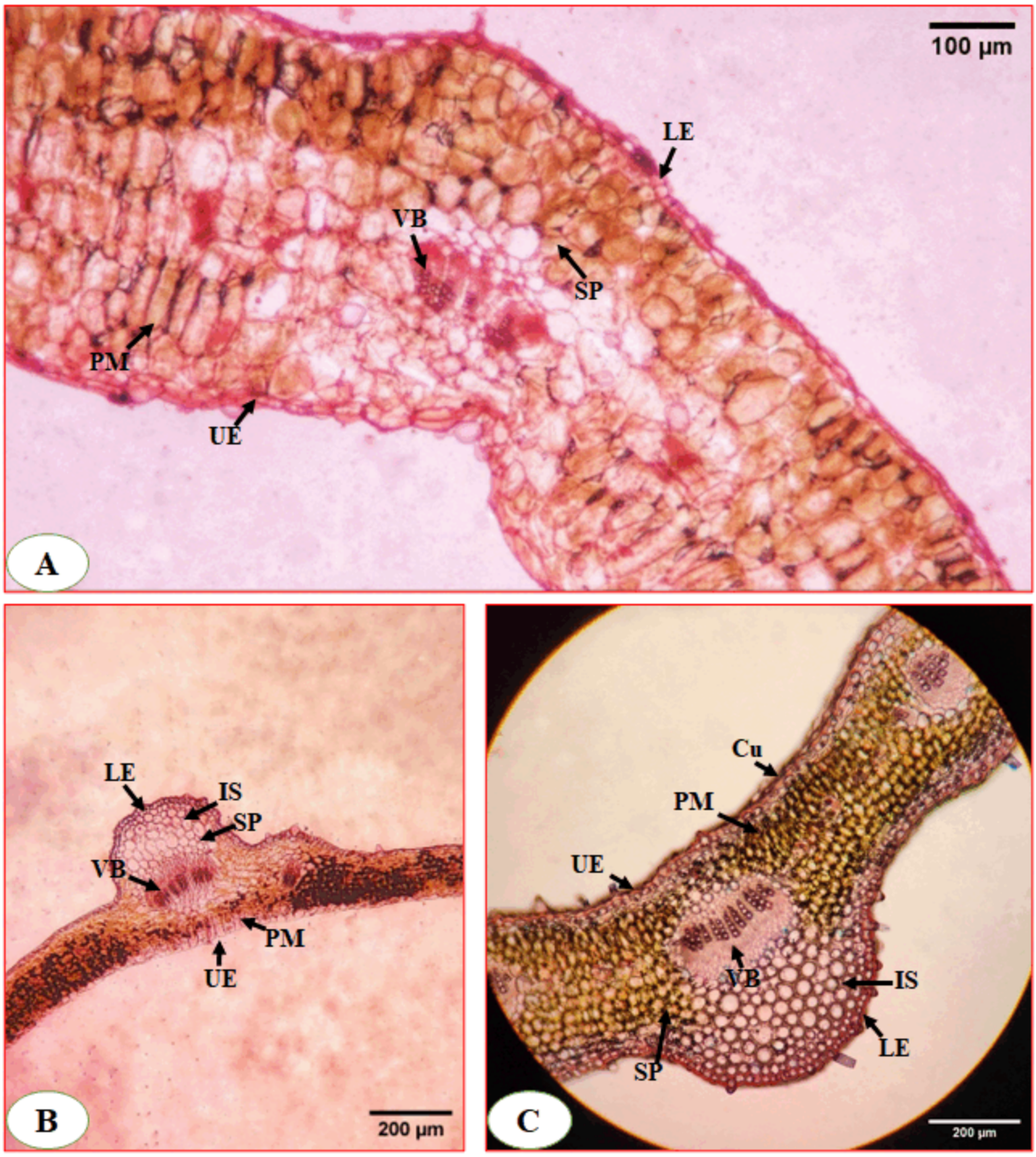


Figure 4

Leaf Transverse Section of *S. papilionacea* Showing Developmental Differences:

Transverse section of a 4-week-old in vitro plantlet leaf (A) displaying a thin cuticle (Cu), single-layered upper epidermis (UE), poorly differentiated palisade mesophyll cells (PM), spongy palisade cells (SP) with loosely arranged parenchyma cells, thin lower epidermis (LE), and reduced stomatal development (scale bar 100 µm). (B) Transverse section of a 6-week-old leaf showing upper epidermis, partially differentiated palisade mesophyll (PM), and developing spongy mesophyll (SM) with increased intercellular spaces (IS) (scale bar 200 µm). (C) Transverse section of mother plant field-grown leaf

exhibiting a well-developed thick cuticle (Cu), distinct upper epidermis (UE), densely packed palisade mesophyll (PM), spongy mesophyll (SP), and differentiated lower epidermis with frequent stomata (scale bar 200 µm).

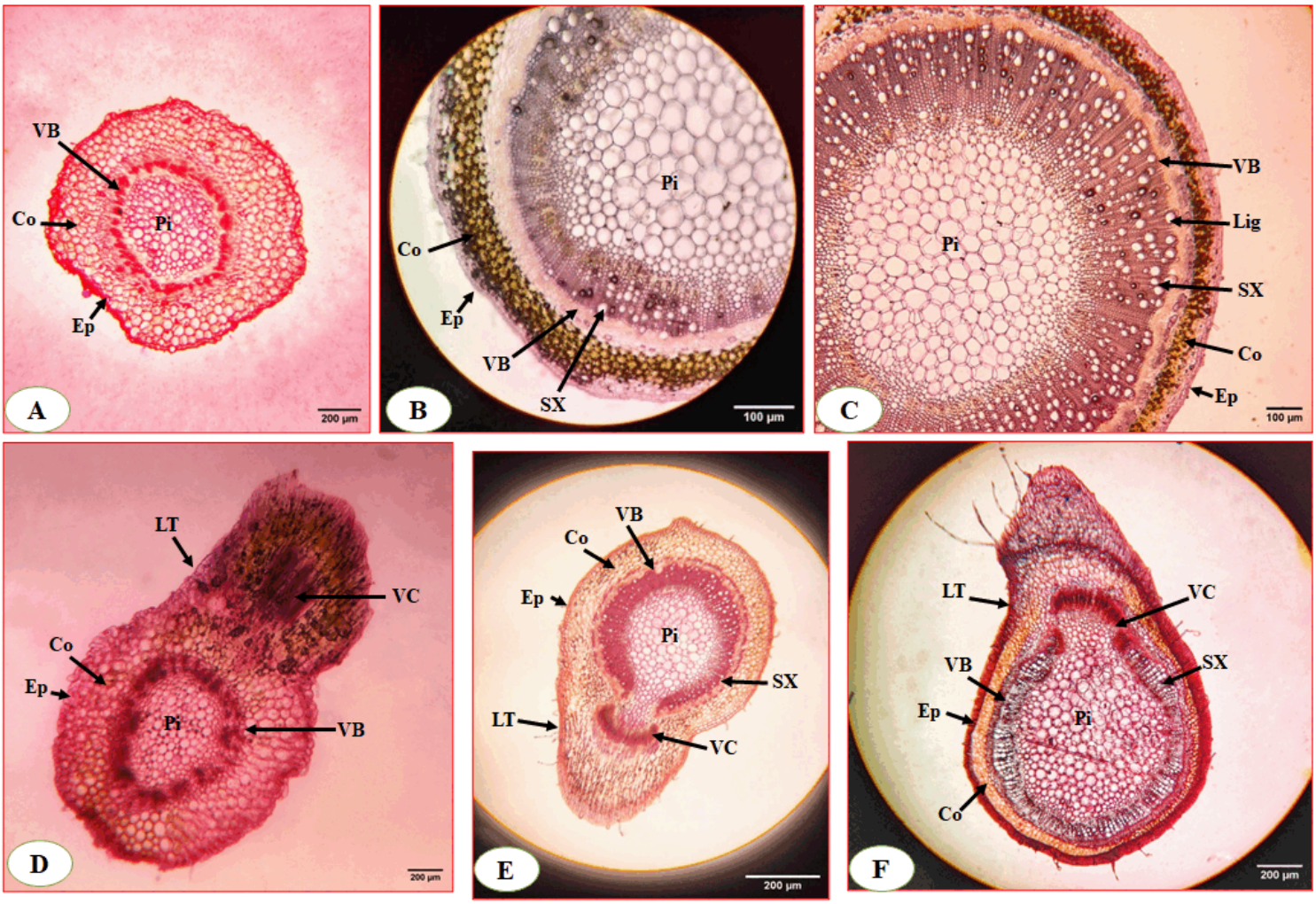


Figure 5

Transverse Section of *S. papilionacea* Stem Showing Developmental Progression: Internode anatomy (A–C): Transverse section of a 4-week-old in vitro plantlet internode (A) displaying thin epidermis (Ep), narrow cortex (Co), small vascular bundles (VB), and large pith (Pi) (scale bar: 200 µm). Transverse section of 6-week-old stem internode (B) showing thickened epidermis, expanded cortex, moderately developed vascular bundles with early secondary xylem (SX), and organized pith (scale bar: 100 µm). Transverse section of mother plant internode (C) exhibiting thick epidermis, well-developed cortex with multiple cell layers, robust vascular bundles with extensive lignification (Lig), and reduced pith (scale bar: 100 µm). Nodal anatomy (D–F): Transverse section of a 4-week-old in vitro plantlet node (D) showing rudimentary leaf traces (LT) and loosely arranged tissues with minimal vascular differentiation (scale bar: 200 µm). Transverse section of a 6-week-old acclimatized node (E) displaying partially differentiated leaf traces and developing vascular connections (VC) between bundles (scale bar: 200 µm). Transverse

section of mother plant node (F) exhibiting well-developed leaf traces, prominent vascular bundles, and organized tissue structure with complete vascular integration (scale bar: 200 μm).