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continued on the back inside cover

**Cover: The nine vultures of India, digital art made on Krita by Dupati Poojitha.**

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ARTICLE

# Propagation through stem cutting and air layering of a Critically Endangered tree *Humboldtia unijuga* Bedd. var. *trijuga* J.Joseph & V.Chandras. (Magnoliopsida: Fabales: Fabaceae)

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**Abstract:** *Humboldtia unijuga* var. *trijuga* is an evergreen tree, endemic to the southern Western Ghats of India belonging to the family Fabaceae, and is categorized as 'Critically Endangered'. High rates of flower & fruit predation and the recalcitrant nature of seeds have detrimental effect on regeneration, and individual recruitment in the wild. Therefore, the present study aimed to produce saplings through conventional propagation methods of stem cuttings and air layering by exogenous application of auxins, with various concentrations of Indole-3-butyric acid (IBA), Indole-3 acetic acid (IAA), and α-Naphthalene acetic acid (NAA). The study revealed that both IBA and IAA had developmental effects on stem cuttings and air layering, but maximum rooting was observed at 1,500 mg/l of IBA. This concentration may be used for mass multiplication and conservation of this endangered tree species.

**Keywords:** Agasthyamalai, auxin, conservation, conventional propagation method, evergreen tree, southern Western Ghats, Thiruvananthapuram.

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**Competing interests:** The authors declare no competing interests.

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**Author contributions:** PSJ conceptualised and designed the work, analysed the data, SS carried out the fieldwork, data collection, and drafted the manuscript. PSJ corrected and edited the manuscript in the final form. PSJ and SS approved the final manuscript.

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## INTRODUCTION

*Humboldtia unijuga* var. *trijuga* J.Joseph & V.Chandras. is a medium-sized evergreen tree of the family Leguminosae, endemic to the southern Western Ghats. Its distribution is highly restricted to the hillocks of the Agasthyamala forests within the altitude range of 490–1,050 m (Sanjappa 1986). This species has been categorized as 'Critically Endangered' by the IUCN Red List (WCMC 1998). Traditional healers use the bark and leaves of this tree for skin treatment (Vijayan et al. 2007). Its crimson-coloured cauliflorous flowers, with their strikingly attractive appearance, make this plant as a potential ornamental choice for gardens, and avenues. Being a Critically Endangered species and a potential economically important plant, conservation of this species is highly essential. Studies showed that flower, fruit, and seed predation by animals along with the recalcitrant nature of seeds negatively affect the natural regeneration, and recruitment of this species (Jothish & Anilkumar 2023). Hence, it is very important to multiply the plant and reintroduce into its natural habitat. Vegetative propagation is an easy and advantageous method to obtain saplings of species with ineffective sexual reproduction (Honnay & Bossuyt 2005; Jose et al. 2011).

Vegetative propagation methods are considered technically simple and cost-effective for developing exact copies and conserving the stock plants with the same genetic identity (Carmona et al. 2022). Treating with rooting hormones is an effective approach for the multiplication and vegetative propagation of any plant species (Abidin & Metali 2015). The positive effects of auxin treatment for vegetative propagation were reported by various workers (Ali et al. 2008; Kharkwal et al. 2008; Jeruto et al. 2010; Kamila & Panda 2019). Among the propagation methods, air layering has an added advantage over other techniques, as it ensures early blooming, and fruit set (Jose et al. 2010). In this context, simple vegetative propagation trials of air layering and stem cuttings were conducted with rooting hormones for the production of more planting materials for the reintroduction, and conservation programs of this species.

## MATERIALS AND METHODS

### Study species

*Humboldtia unijuga* var. *trijuga* is an evergreen under-storey species growing up to 12 m. It is located in Bonacaud of Thiruvananthapuram District of Kerala State. The tree naturally exhibits slow growth. Leaves are compound with three to four pair of leaflets. Flowering is observed in August–January. Flowers are borne in compact racemes on branches and main trunk, and are crimson red in colour. Fruit is a legume with one or two seeds and fruits are observed in December–April. Insect larvae and arboreal animals like monkeys and squirrels predate flowers and fruits. Seeds are large and recalcitrant.

### Stem cuttings

The stem-cutting experiment was conducted during August–September at the central nursery of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, in 2022. Each experiment had 25 cuttings for each treatment. Healthy and disease-free stem cuttings of semi-hard type having 15–20 cm of length, 8–10 mm diameter with 3–5 nodes, and leaves were collected from adult individuals from the natural habitat. Immediately after collection, the cuttings were brought to the nursery and treated with 1% Bavistin to avoid fungal infection. The basal portion of each cutting was cut at right angles and dipped in four different concentrations of auxins (500, 1,000, 1,500, and 2,000 mg/l), viz., Indole-3-butyric acid (IBA), Indole-3 acetic acid (IAA), and α-Naphthalene acetic acid (NAA) for five minutes. The cuttings dipped in distilled water were treated as a control. The cuttings were planted on the same day of collection as early as possible. To avoid / reduce the transpiration rate, the leaf's surface area was subjected to a half-cut. Immediately after the treatment, the whole set was planted into the sand bed in the mist house having a temperature of 28 ± 2 °C, and 70–80 % relative humidity. Intermittent mist was supplied for 40 seconds six times in a day. The experiment was observed twice a week for the first six weeks, and the cuttings that showed wilting were removed. After 12 weeks the sprouted plants were observed. Root parameters like percentage of rooting, number of roots per cutting, and root length were recorded. Cuttings with roots of ≥ 1 mm were considered as rooted and used for calculating rooting percentage (out of total treatments) and root length of ≥ 1 cm was considered for calculating mean number of roots. After measurements, they were planted in polythene bags containing a potting mixture of river sand, dried cow dung, and garden soil in a 2:1:1 ratio. Survival percentage was calculated after six months.

### Air layering

Air layering was done on 16 randomly selected

disease-free, healthy individuals of *H. unjiuga* var. *trijuga* growing in its habitat. The layering experiments were conducted in August–September of 2022, which experienced an active growth phase of the plant with favorable climatic conditions. Actively growing stems were selected, and from these branches, and lateral branches of 30–50 cm length, and 1–3 cm diameter with leaves were randomly selected from each tree for layering treatments. A small strip of bark (3 cm) from the selected branches was girdled out below the nodal region using a sharp budding knife. Various concentrations (500, 1,000, 1,500, and 2,000 mg/l) of growth hormones such as IBA, IAA, and NAA were applied (absorbent cotton dipped in respective concentrations of auxins) on the girdled region, and were covered with polythene sleeves containing moist rooting compost. The rooting medium was prepared by mixing cocopeat, river sand, and dried cow dung in a ratio of 2:1:1. Both ends of the layering were tied tightly with thread to avoid drying of the medium. Small holes were made in the polythene sleeves to permit limited air exchange. The trials were carried out in such a manner that a single tree was layered with the application of a single concentration (500 or 1,000 or 1,500, or 2,000 mg/l) of different hormones including one control without hormones (4 treatments + 1 control). Accordingly, a total of 80 layers (experiment) were created under four different auxin treatments. The air layers were labelled properly and left undisturbed for eight weeks. Observations were made weekly for root emergence and the treatments were sprayed with water to maintain moisture content. After eight weeks, the air layers were cut from the parent plant and the rooting mixture was gently removed. The success of layers was assessed by recording the presence of callus, rooting percentage, number of roots, and root length as in stem cuttings. The number of roots initiated from each treatment was counted and averaged. Similarly, lengths of roots formed in a layer were measured, and mean root length was calculated. After measurements, air-layered plants were planted in polythene bags containing potting mixture of river sand, dried cow dung, and garden soil in 2:1:1 ratio and kept in the nursery. Survival percentage was calculated after six months.

### Statistical analysis

Each of the five treatments, including the control, was replicated five times. The results of root length and number of roots were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, $p \leq 0.05$ with SPSS software v.16. Data for mean root length and number of roots are given as mean ± standard error of the replicates.

## RESULTS

### Propagation through stem cuttings

The stem cuttings treated with IBA and IAA only showed developmental response**.** The rooting percentage was varied 16–66.6 under different auxin concentrations. All IBA-treated cuttings showed rooting, while cuttings treated with 1,000 and 1,500 mg/l IAA only showed rooting. Those cuttings treated with NAA and control cuttings showed no rooting response at all (Table 1). Cuttings treated with 1,500 mg/l of IBA showed maximum rooting (66.6%), whereas 1,500 mg/l IAA resulted in 54.5% rooting. The mean root number and mean root length of IBA set ranged from 2.4 ± 0.24–7.2 ± 0.58 and 1.8 ± 0.05–6.4 ± 0.12 cm and were significantly higher than other treatments (one-way ANOVA). The IAA set ranged from 2.2 ± 0.37–5.2 ± 0.48 and 2.3 ± 0.18–4.7 ± 0.12 cm, respectively (Image 1A–C). The bud initiation was observed on the fourth week after planting in control cuttings, however after the bud break it dried off. The stem cuttings treated with 1,500 mg/l of IBA and IAA showed bud initiation after two weeks, and responded with maximum rooting, and shooting. After six weeks the buds were transformed into fully functional leaves. Finally, after 12 weeks the regenerated stem cuttings were transplanted into separate pots.

### Air layering

Air layering samples responded positively to auxins such as IBA and IAA. The root initiation was observed in hormone-treated samples after 3–4 weeks. Rooting percentage varied 20–52.6 %. The number of roots and root length were measured after eight weeks (Image 1D–E). Data indicated that layering of samples treated with 1,500 mg/l of IBA recorded higher percentage of rooting success (52.6%) and was at par with 1,500 mg/l of IAA (46.1%). The mean root number and the mean root length of IBA set ranged from 3.2 ± 0.20–10.2 ± 0.37and 1.6 ± 1.01–7.2 ± 0.19 cm, and significantly higher than other treatments (one-way ANOVA). The IAA set was 3.2 ± 0.20–6.8 ± 0.20 and 2.6 ± 0.08–4.6 ± 0.13 cm, respectively (Table 2). After 15 weeks the layered samples were transplanted into separate pots and placed under a mist house before reintroduction.


**Image 1. *Humboldtia unijuga* var. *trijuga*: A–C—Stem cuttings. A—1,500 mg/l of IBA treated stem cutting | B—1,500 mg/l of IAA treated stem cutting | C—Stem cutting established on polythene bag | D—Air layering | E—early flowering on air layered sapling. © Shintu S.**

## DISCUSSION

The present study aimed to develop a vegetative propagation protocol for the multiplication and ex situ conservation of the 'Critically Endangered' species *H. unijuga* var. *trijuga*. When effective seeding is not available, exact copies of the parent plant can be produced in large numbers using conventional propagation methods, which can be made more successful through the application of exogenous auxin, within a shorter period. The present study showed that exogenous application of specific auxins resulted in rooting in stem

**Table 1. Effect of different concentrations of auxins on rooting percentage, number and length of roots, and survival percentage of stem cuttings of *Humboldtia unijuga* var. *trijuga* after 12 weeks.**

| Treatments / hormone | Concentration (mg/l) | Callus formation (%) | Number of roots (Mean ± SE) | Root length (cm) (Mean ± SE) | Rooting (%) | Survival (%) |
|---|---|---|---|---|---|---|
| Control | NA | 16 | NR | NR | NR | NA |
| IBA | 500 | 32 | $2.4 \pm 0.24^{d}$ | $1.8 \pm 0.05^{f}$ | 25 | 25 |
| | 1000 | 56 | $3.8 \pm 0.37^{c}$ | $2.7 \pm 0.12^{d}$ | 54.5 | 36.3 |
| | 1500 | 68 | $7.2 \pm 0.58^{a}$ | $6.4 \pm 0.12^{a}$ | 66.6 | 40 |
| | 2000 | 36 | $4.4 \pm 0.89^{bc}$ | $3.5 \pm 0.15^{c}$ | 16 | NR |
| IAA | 500 | 24 | NR | NR | NR | NA |
| | 1000 | 40 | $2.2 \pm 0.37^{d}$ | $2.3 \pm 0.18^{e}$ | 44.4 | 25 |
| | 1500 | 56 | $5.2 \pm 0.48^{b}$ | $4.7 \pm 0.12^{b}$ | 54.5 | 33.3 |
| | 2000 | NR | NA | NA | NA | NA |
| NAA | 500 | NR | NA | NA | NA | NA |
| | 1000 | NR | NA | NA | NA | NA |
| | 1500 | NR | NA | NA | NA | NA |
| | 2000 | NR | NA | NA | NA | NA |

Note: Stem cuttings with at least one root were considered for calculating percentage of rooting: SE—standard error | ANOVA Df (n-1) = 12, F = 74.6***, F = 573.9***, level of significance $P < 0.05$, $n = 25$ | different letters indicate significant differences between treatments based on $p$ value < 0.05 and same letter are not significantly different from each other at the $p < 0.05$. NR—Not responded | NA—Not applicable.

**Table 2. Effects of different concentrations of auxins on rooting percentage, number and length of roots, and survival percentage of air-layered branches *Humboldtia unijuga* var. *trijuga* after 12 weeks.**

| Treatment / hormone | Concentration (mg/l) | Callus formation (%) | Number of roots (Mean ± SE) | Root length in cm (Mean ± SE) | Rooting (%) | Survival (%) |
|---|---|---|---|---|---|---|
| Control | | 30 | $1.4 \pm 0.24^{d}$ | $1.04 \pm 0.05^{e}$ | 33.3 | NR |
| IBA | 500 | 20 | $3.8 \pm 0.37^{c}$ | $2.4 \pm 0.14^{c}$ | 20 | NR |
| | 1000 | 52 | $6.6 \pm 0.50^{b}$ | $4.3 \pm 0.11^{b}$ | 46.1 | 50 |
| | 1500 | 76 | $10.2 \pm 0.37^{a}$ | $7.2 \pm 0.19^{a}$ | 52.6 | 60 |
| | 2000 | 36 | $3.2 \pm 0.20^{c}$ | $1.6 \pm 0.06^{d}$ | 22.2 | NR |
| IAA | 500 | NR | NA | NA | NA | NA |
| | 1000 | 36 | $3.2 \pm 0.20^{c}$ | $2.6 \pm 0.08^{c}$ | 33.3 | 33.3 |
| | 1500 | 52 | $6.8 \pm 0.20^{b}$ | $4.6 \pm 0.13^{b}$ | 46.1 | 42.8 |
| | 2000 | NR | NA | NA | NA | NA |
| NAA | 500 | NR | NA | NA | NA | NA |
| | 1000 | NR | NA | NA | NA | NA |
| | 1500 | NR | NA | NA | NA | NA |
| | 2000 | NR | NA | NA | NA | NA |

Note: Air layering with at least one root was considered for calculating the percentage of rooting: SE—standard error; ANOVA Df (n-1) = 12, F = 203.7***, F = 683.5***, level of significance $P < 0.05$, $n = 25$ | different letters indicate significant differences between treatments based on $p$ value < 0.05 and same letter are not significantly different from each other at the $p < 0.05$. NR—Not responded | NA—Not applicable.

cuttings and air layering in *H. unijuga* var. *trijuga*, and the saplings survived successfully. Many studies revealed the effectiveness of vegetative propagation of endemic and endangered species used for the restoration of vegetation (Lemay et al. 2009; Ramos-Palacios et al. 2012; Duarte et al. 2018) and ex situ conservation practices for species with inherent problems of seed germination and seedling establishment in wild (Kamila & Panda 2019). Also, this will allow large-scale production of planting materials for reintroduction programmes. The precision of auxins was found critical for the vegetative propagation success in this study. Auxins are particularly

crucial for plant cell growth and are involved in numerous biological processes including initiation of leaf primordia, and lateral root production (Bertoni 2011; Pacurar et al. 2014).

The present study confirmed that the application of auxins IBA and IAA were found ideal for *H. unijuga* var. *trijuga* which promote root formation both in air layering, and stem cuttings like *Syzygium caryophyllatum* (Hussain & Anilkumar 2016) and *Dysoxylum malabaricum* (Hussain et al. 2013), as they found that stem cuttings treated with 1,500 mg/l of IBA recorded the highest rooting success (53.3%) and 63% of success in air layering. Although rooting was observed in all the treatments, especially of IBA treatments, data revealed the maximum significant rooting response by higher concentration of IBA followed by higher concentration of IAA (Tables 1 & 2). The application of IBA may enhance the translocation of sugar to the base of cuttings and stimulate rooting in the layering process. Nanda (1975) reported that auxins promote the activity of hydrolytic enzymes, which in turn promotes stem cuttings to root by enhancing the mobilization of reserve food supplies. The present experiment revealed that stem cuttings treated with 1,500 mg/l IBA resulted in significantly higher values for sprouting of 66.6% and 54.6% survival. The application of IBA was found ideal for the allied species *Humboldtia vahliana* as reported by Jose et al. (2010). Behera et al. (2020) reported that cuttings of *Commiphora wightii* treated with concentrations of IBA shows better result at concentration of 1,000 mg/l. The present study as well as other studies showed that IBA is one of the most effective and widely used auxins in vegetative propagation over a wide range of concentrations and is effective in stimulating root growth in a large number of plant species (Hartman et al. 2011). IBA was found to be a better rooting hormone in comparison with IAA & NAA, and is nontoxic to plants (Eganathan et al. 2000). In this study, no response was found against the application of NAA in both stem cuttings and air layering. Jose et al. (2011) recorded a high percentage of rooting in *Humboldtia bourdillonii*, an allied endemic species, using 500 mg/l of NAA. This may be due to the inherent physiological differences of the species.

It was observed that the rooting response treated with 1,500 mg/l of IAA and IBA were recorded with formation of maximum number of roots and survival. The highest survival percentage of air layers (60%) was recorded in stem treated with 1,500 mg/l IBA and 42.8 % in IAA. A similar type of result was reported in *Elaeocarpus venustus* (Soorangkattan et al. 2021). According to Eganathan et al. (2000), saplings raised via air-layering exhibit greater adaptability to field conditions. A higher rooting success was reported by Kamila & Panda (2019) when using 5,000 mg/l of IBA in *Lasiococca comberi.* Woody forest species like *Myrica esculenta* (Purohit et al. 2004) and *Quercus glauca* (Purohit et al. 2005) were easily multiplied through air layering. The rooting response varies depending on the type of cuttings used and the season may be due to the activities of hydrolytic enzymes which are reported to be highly active during monsoon and post monsoon months (Nanda 1975; Blake & Bentley 1985).

The present study showed that the application of IBA and IAA may result in rooting of stem cuttings, and air layering experiments of the endangered woody species *Humboldtia unijuga* var. *trijuga*, which could be used successfully for mass propagation, and for reintroduction programs. Also, the plants survived well in the nursery (Tables 1 & 2) and even some of the air-layered saplings flowered (Image 1E). This feature showed a good indication that these plants reintroduced in their native habitat, may survive.

## CONCLUSION

The present study revealed a mass multiplication technique through stem rooting and air layering by the application of auxins to conserve this critically endangered species. The stem cuttings and layering samples pre-treated with 1,000 & 1,500 mg/l of IBA, and IAA showed the maximum response. The present study may provide a cost–effective technique for mass production of genetically identical mature planting materials for reintroduction programmes. This technique could support the long-term survival of this species in the wild.

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