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DEVELOPMENT OF A MASS PROPAGATION PROTOCOL FOR *URARIA PICTA* AIMED AT ITS CONSERVATION AND SUSTAINABLE UTILIZATION

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ABSTRACT: *Uraria picta* (family Fabaceae) is an important woody medicinal herb. This plant has been used in traditional systems of medicine for the treatment of various diseases. It is also used in an Ayurvedic formulation of "Dashmula". The utilization of roots by uprooting the entire plant and the problems associated with seed germination threaten this plant in its natural habitat and cause a shortage of root material for Ayurvedic preparation. The present study aimed to assess seed viability and the influence of pre-sowing treatments on seed germination and seedling development of *U. picta*. The treatments were applied singly and in combination, including acid scarification (H_2SO_4), hot and rubbing with sandpaper, pre-soaking in gibberellic acid (GA_3), and the application of cuttings to the seed coat followed by pre-soaking in distilled water. The significantly highest rate (90%) of seed germination and a higher proportion of strong seedlings were observed in the seeds treated with 95% H_2SO_4. The protocol developed for seed germination and seedling development can be applied for raising a large number of *U. picta* plants, which will help in the conservation of plants and increase its availability for medicinal purposes.

INTRODUCTION: *Uraria picta (Jacq.),* usually known as "Prishnaparni", belongs to the family Fabaceae. This plant is one of the important ingredients of ten herbal formulations called "dashmula" [1]. *U. picta* is reported to be a perennial erect woody herb reaching approximately 1.0–2.5 m in height. It is widely distributed throughout India [2]. In addition to India, *U. picta* has also been reported from different parts of Africa, such as Nigeria, Egypt, Ethiopia, the Congo, South Africa [2, 3], China, Japan, Bangladesh, Pakistan, Bhutan, Nepal, and Australia [4, 5].

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The herb *U. picta* is an ingredient of Dashmula, which is used for the treatment of fever and inflammation [2]. All parts of this plant have medicinal importance and are used by certain Adivasis and native tribes [6]. Leaves are good antiseptics and are used against gonorrhoea. Fruits and pods are effective against oral sores in children, and roots are used against cough, chills, and fever [7].

The roots are used medicinally for invigorating the liver and spleen as sedatives to produce strength to the nervous system, cure all types of inflammation, detoxify the entire body, be used to treat skin diseases and heal fracture wounds [8]. Traditionally, the plant is used as an antidote against the bites of certain Indian vipers [8]. The root isolates of the plants show antimicrobial activity against fungi and both Gram-positive and Gram-negative bacteria [7].

U. picta is becoming increasingly rare because of overexploitation by various pharmaceutical industries as well as local tribes for medicine and trade purposes coupled with poor seed viability [7, 9]. Hence, there is an urgent need to develop *in-vitro* methods for the rapid production of cloned plants of *U. picta* for providing uniform raw material for medicinal purposes as well as for its rehabilitation in natural habitats for conservation and sustainable utilization. The propagation of *U. picta* via seeds is too slow because of poorly viable seeds and dormancy for several months [2]. On the other hand, since roots are important for Ayurvedic preparation, plants are uprooted on a mass scale from their natural habitats, which leads to a depletion of resources. As a result, it is becoming rare and endemic [3].

Several pretreatments have been proven to be efficient in overcoming the dormancy of leguminous and other seeds [4, 5]. Micropropagation of *U. picta* was achieved through axillary bud culture [2]. However, the micropropagation method is cost intensive and sensitive compared with the seed propagation method. Therefore, the objective of this study was to evaluate the seed viability and germination rate of seeds by applying physical treatments, acid scarification, and gibberellic acid (GA_3) to develop a viable method for large-scale plantation to increase the availability of root biomass for Ayurvedic and other medicinal preparations.

MATERIALS AND METHODS:

Seed Collection Site and Surface Sterilization of Seeds: Seeds of *U. picta* was collected from wild plants in the Gandhamardhan Hill region, where it grows abundantly **Fig. 1A**. The seed germination experiment was set up as a completely randomized design with three replications. The treatment was performed by placing one hundred seeds in a beaker. Before various treatments were applied, the seeds were washed with Tween 20 (liquid detergent). This step is intended to clean the seed surface and remove any substances that could inhibit germination. After that, the seeds were washed with Bavistin, a fungicide used to prevent fungal infections during the germination process. The seeds were then surface sterilized with 0.1% mercuric chloride and rinsed three times with double distilled water.

Seed Viability Test: The seed viability was determined by the Tetrazolium test following the standard procedure described earlier by [10]. The parts of the seeds that were viable became red or pink, and the nonviable parts remained white. For this study, a total of five hundred seeds were divided into five replicates, and each replicate included one hundred seeds. Seed viability was checked by using 0.5% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC), which was prepared by dissolving 2,3,5-triphenyltetrazolium chloride (TTC) in sodium phosphate buffer (pH 7.0).

Physical Treatment of Seeds:

Sand Paper Scarification of Seeds: The surface-sterilized seeds were directly transferred to Petri dishes and used as controls in this study. The seeds were placed in a 0-grade sand paper flap, and a slight friction force for 15 sec was applied manually to scarify the seeds. The damaged seeds were subsequently discarded by visual observation, and undamaged seeds were used for the germination test.

Hot Water Treatment of Seeds: The seeds were soaked separately in hot water (60 °C) for durations of 5 min, 10 min, and 15 min as well as in boiling water (100 °C) for durations of 1 min and 2 min. After a specific duration of treatment, the hot water was removed, and the seeds were subsequently washed with autoclaved double distilled water three times. The treated seeds were placed in Petri dishes for germination.

Cold Water Treatment of Seeds: For low-temperature treatment, the seeds were immersed in cold water (4 °C) and placed in a refrigerator at 4 °C for different durations, such as 6 h, 12 h, 18 h, and 24 h, after which germination was observed.

Chemical Treatment of Seeds:

Acid Scarification of Seeds: The seeds were immersed in concentrated H_2SO_4 and HCl (Sisco Research Laboratories Pvt. Ltd. (SRL), India) separately for different durations of 2 min, 5 min, and 10 min, with different concentrations of both acids. After treatment, the seeds were washed five times with distilled water and used for the germination test.

KNO_3 Treatment of Seeds: Seeds were soaked in potassium nitrate (KNO_3) solutions at three different concentrations, 0.1%, 0.2%, and 0.3%, followed by germination.

GIbberllic acid (GA_3) in Combination with Sulfuric Acid (H_2SO_4) Treatment of Seeds: Seeds underwent a two-step treatment process. First, they were pretreated with different concentrations (25, 50, and 90%) of sulfuric acid (H_2SO_4) for 5–10 min at 10 min intervals. The seeds were subsequently dipped in gibberellic acid (GA_3) solutions with concentrations ranging from 1.0 mg/L to 5.0 mg/L in a Petri dish.

Micropropagation of *U. picta*:

Selection of Explants: The Gondhamardhan hill range has unique ecological characteristics or environmental conditions that influence the traits of the collected *U. picta* plants. Mature seeds were collected from November to January 2021 from the Gandhamardan and used for the experiment. Mature seeds are often preferred for collection, as they are more likely to be viable for germination.

Surface Sterilization of Explants: The mature seeds were collected and treated with an optimum concentration of H_2SO_4, i.e., 25% for 5–10 min, and soaked in warm water for 1 h, followed by treatment with an aqueous solution of Teepol, a liquid detergent (5%, v/v; Reckitt Benckiser Ltd., India), for 5 min. Subsequently, the seeds were subjected to treatment with the fungicide Bavistin (2%, w/v) (BASF, Mumbai, India) for 5 min and then rinsed with double distilled water (3–4 times). Finally, surface sterilization of the seeds was carried out under a laminar hood with 0.1% (w/v) $HgCl_2$ (Hi-Media, India) for 5 min, followed by proper washing in autoclaved double distilled water.

Seed Germination: Surface sterilized seeds were inoculated on plant growth regulator-free Murashige and Skoog (1962) (MS) media, half-strength (½ MS) media, or ½ MS media supplemented with various concentrations of gibberellic acids (0.5–4.0 mg/L). All the media contained 3.0% sucrose as a source of carbon and 0.6% agar as a gelling agent. The pH of the medium was adjusted to 5.8 ± 0.1 prior to the addition of agar, and the mixture was sterilized at 15 psi and 121 °C for 15 min via an autoclave.

Shoot Proliferation: *In-vitro* seedling explants were inoculated in MS media alone or MS media supplemented with different concentrations and combinations of plant growth regulators, such as meta-topolin (mT) (1.0-5.0 mg/L), N^6-benzyladenine (BA) (1.0 - 5.0 mg/L), kinetin (KIN) (1.0 - 5.0 mg/L), and zeatin (Z) (1.0 - 5.0 mg/L), for multiple shoot proliferation. All the media contained 3.0% sucrose and 0.6% agar (Himedia, Mumbai, India). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min. The cultures were incubated under controlled conditions, such as 25±2 °C, 60±10% relative humidity, and an 8 h photoperiod (PFD 40 µmol $m^{-2}s^{-1}$) provided by white, fluorescent tubes (Philips, Kolkata, India). The number of shoots obtained per *in-vitro* seedling was recorded after six weeks of culture.

Rooting: Well-developed shoots (4–6 cm in height) were separated from explants and transferred for rooting on MS basal media and ½ MS media supplemented with growth regulator media (i.e., ½ MS + IBA 0.5 mg/L and ½ MS + IBA 1.0 mg/L), 3.0% sucrose and gelled with 0.6% agar at pH 5.8).

Acclimatization and field Establishment of *In-vitro*-generated Plants: The roots were carefully washed with tap water to clean the agar. These plantlets were planted in small pots of planting trays containing forest soil:organic compost:sand (1:1:1) in green house at a controlled environment (temperature, 30±1 °C; humidity, 80±10%). Finally, the pots were transferred to field conditions (temperature, 18–32 °C; humidity, 55–80%).

Data Analysis: All the experiments were repeated three times. The data are presented as the means ± standard deviations.

RESULTS AND DISCUSSION: The 2,3,5-triphenyltetrazolium chloride (TTC) test is used primarily to obtain rapid results on seed viability for both nondormant and dormant seeds [10]. A number of reports have documented the evaluation of seed viability using tetrazolium test in several medicinal plants, including *Rhodiola imbricate* [11], *Triticum aestivum* [12], and *Anacamptis laxiflora* [13]. In the present study, intact seeds were soaked in TTC solution and incubated for 24 h in the dark;

those that did not stain were due to the impermeability of the solution in the seeds through the seed coat. However, the embryos of seeds with a seed coat cut at the micropylar end soaked in TTC solution for 24 h turned dark red. This study revealed that all the seeds (100%) were viable. In contrast [2], reported that the seeds of *U. picta* were poorly viable (although not specified the tests used to check the viability of seeds). This variation in seed viability might be due to differences in seed source usage. Viability might be affected by the nutrient status of the mother plant, environmental factors, and the age of the seeds [5]. However, the seeds were placed on moist germination paper in Petri dishes, and the seeds sown in moist soil did not show any signs of germination after 30 days of incubation. These results indicate that some kind of dormancy exists in the seeds of *U. picta* due to the physical barrier of the seeds because of the presence of a hard seed coat.

To improve seed germination, breaking dormancy or the physical barrier of the seed is necessary. Thus, seeds were subjected to physical treatments (i.e., sand paper, hot water, and cold-water treatments) and chemical treatments (e.g., KNO_3, HCL, and H_2SO_4 treatments) at different concentrations for different durations. Chemical treatment helps in breaking down the seed coat, allowing water to penetrate more easily and initiate germination. In particular, seeds were treated with different concentrations of sulfuric acid (25%, 50%, and 90%), and the percentage of seed germination was recorded. Compared with the other acids, 90% of the seeds were germinated with sulfuric acid **Table 1.**

For the study of comparative *in-vitro* seed germination of *U. picta*, seeds were surface sterilized and inoculated on MS media, ½ MS media, or ½ MS media supplemented with different concentrations of GA_3 (0.5–4.0 mg/L). Approximately 8.0% of the seeds were germinated on ½ MS media supplemented with 2.0 mg/L GA_3. To improve the percentage of seed germination, seeds were treated with different concentrations of H_2SO4 (25–90%) followed by surface sterilization and inoculated on MS media, ½ MS media, or ½ MS media supplemented with different concentrations of GA_3 (0.5–4.0 mg/L). After treatment with H_2SO_4 (90%) followed by surface sterilization, the percentage of seed germination improved, and the 90% highest percentage of seed germination was achieved on ½ MS medium supplemented with 2.0 mg/L GA_3 among all the H_2SO_4 treatments **Fig. 1B, Table 2.**

TABLE 1: EFFECT OF PHYSICAL TREATMENTS AND ACID SCARIFICATION ON SEED GERMINATION OF *U. PICTA*

Physical treatment	Germination (%)	Chemicals treatment	Germination (%)
Sand paper treatment	1.0± 0.30	KNO_3 (0.1%)	1.5±0.93
		KNO_3 (0.2%)	1.8±1.13
		KNO_3 (0.3%)	2.5±1.21
Hot water treatment	1.5±0.50	HCL (25%)	4±0.83
		HCL (50%)	5.7±0.91
		HCL (90%)	7.1±1.00
Cold water treatment	1.0±0.30	H_2SO_4 (25%)	15±1.13
		H_2SO_4 (50%)	42±1.21
		H_2SO_4 (90%)	62±1.12

TABLE 2: EFFECT OF SULPHURIC ACID WITH MS MEDIUM TREATMENTS ON SEED GERMINATION OF *U. PICTA*

Medium for germination untreated seeds	Germination (%)	Medium for germination seeds treated with 25% of H_2SO_4	Germination (%)	Medium for germination seeds treated with 50% of H_2SO_4	Germination (%)	Medium for germination seeds treated with 90% of H_2SO_4	Germination (%)
MS	1±1.00	MS	4±1.23	MS	7±1.23	MS	15±1.23
½ MS	2±1.02	½ MS	8±1.11	½ MS	10±1.31	½ MS	20±1.02
½ MS + 0.5 mg/L GA_3	4±1.05	½ MS + 0.5 mg/L GA_3	25±0.87	½ MS + 0.5 mg/L GA_3	18±0.87	½ MS + 0.5 mg/L GA_3	44±1.34
½ MS + 1.0 mg/L GA_3	6±1.02	½ MS + 1.0 mg/L GA_3	30±0.91	½ MS + 1.0 mg/L GA_3	50±1.45	½ MS + 1.0 mg/L GA_3	80±1.31

½ MS + 2.0 mg/L GA_3	8±0.98	½ MS + 2.0 mg/L GA_3	40±1.34	½ MS + 2.0 mg/L GA_3	60±1.23	½ MS + 2.0 mg/L GA_3	95±1.45
½ MS + 3.0 mg/L GA_3	3±1.42	½ MS + 3.0 mg/L GA_3	37±1.21	½ MS + 3.0 mg/L GA_3	52±1.24	½ MS + 3.0 mg/L GA_3	82±1.32
½ MS + 4.0 mg/L GA_3	2±0.92	½ MS + 4.0 mg/L GA_3	34±1.15	½ MS + 4.0 mg/L GA_3	48±1.56	½ MS + 4.0 mg/L GA_3	78±0.92

In-vitro seedling explants of *U. picta* were cultured on MS media and MS media supplemented with mT, BA, KIN, and Z for multiple shoot formation. On MS media, explants failed to respond to shoot multiplication. When explants were inoculated on MS media supplemented with different concentrations of cytokinins, the rate of shoot multiplication increased.

Among all the tested concentrations of cytokinins, a maximum number of shoots were produced on MS media supplemented with 2.0 mg/L mT. In this medium, approximately 21 shoots were produced with a mean shoot length of 7.1 cm **Fig. 1D**, followed by 16 shoots per explant with an average shoot length of 6.9 cm on MS media supplemented with 2.0 mg/L BA **Table 3.** On MS media supplemented with 2.0 mg/L Z, approximately 10 shoots were produced per explant, with a shoot length of 3.0 com **Fig. 1C, Table 3.** Compared with other cytokinins, metacoopolin is an aromatic cytokinin that has the potential to produce more *in vitro* shoots [14, 15, 16]. *m*T is also known to promote shoot growth and development while preventing uninvited morphophysiological side effects such as hyperhydricity and shoot tip necrosis [14, 17, 18, 19, 20].

The metabolism of *m*T is faster than that of BAP because of the easy translocation of its metabolic product (O-glucoside) to various plant parts. However, BA tends to remain restricted to the basal part of plants and exerts an inhibitory effect on rooting [21]. The absence of cytokinin in the MS medium was unfavourable for the induction of multiple shoot formations in *U. picta*.

TABLE 3: EFFECT OF CYTOKININ AND MEASUREMENT OF SHOOT NUMBER AND SHOOT LENGTH *OF U. PICTA*

MS medium supplemented with plant growth regulators (mg/L)				Shoot regeneration (%)	Mean No. of shoots/explant	Shoot length (cm)
mT	BA	KIN	Z			
0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.0				80.0	17.0	4.0
2.0				95.0	21.0	7.1
3.0				85.0	17.0	2.8
4.0				70.0	11.0	1.9
5.0				65.0	9.0	1.3
	1.0			85.0	15.0	5.9
	2.0			95.0	16.0	6.9
	3.0			80.0	12.0	4.5
	4.0			77.0	7.0	2.1
	5.0			67.0	6.0	1.7
		1.0		85.0	11.0	3.0
		2.0		70.0	8.0	3.2
		3.0		65.0	6.0	3.0
		4.0		55.0	5.0	2.5
		5.0		50.0	3.0	2.0
			1.0	65.0	9.0	2.4
			2.0	70.0	10.0	3.0
			3.0	55.0	7.0	2.5
			4.0	45.0	5.0	1.9
			5.0	40.0	3.0	1.6

For root induction, *in-vitro* regenerated shoots were excised and inoculated on ½ MS media and ½ MS media supplemented with 0.5 and 1.0 mg/L IBA. The roots of *in-vitro* shoots were not induced on ½ MS media. When shoots were inoculated on ½ MS media supplemented with IBA, the number of roots increased, and a maximum of 7.0 roots per shoot with an average root length of 2.8 cm was observed

on shoots in ½ MS media supplemented with 1.0 mg/L IBA **Fig. 1E, Table 4.** All *in-vitro* regenerated plantlets were acclimatized and subsequently transferred to the field with a survival rate greater than 95% **Fig. 1F, G, and H.**

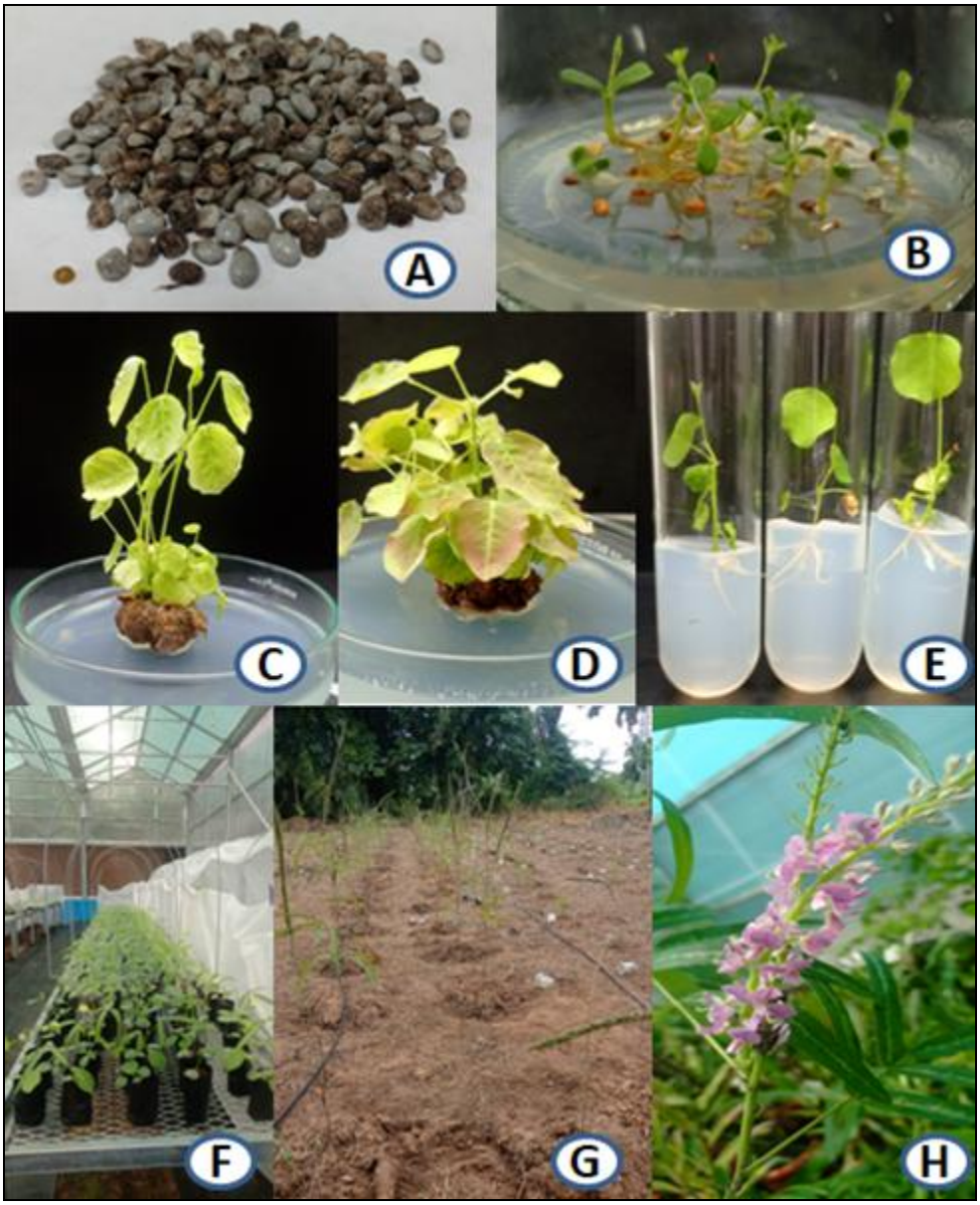

FIG. 1: MICROPROPAGATION OF *URARIA PICTA* THROUGH *IN VITRO* SEEDLING: (A) Matured seeds; (B) Seed germination in culture medium; (C) Multiple shoot proliferation on MS medium supplemented with 2.0 mg/L Z; (D) Multiple shoot proliferation on MS medium supplemented with 2.0 mg/L mT; (E) Rooting of *in-vitro* shoots on ½ MS medium supplemented with 1.0 mg/L IBA; (F) Acclimatization of in vitro regenerated plants; (G) Field establishment of tissue cultured plants; (H) Flowering of field established plants.

TABLE 4: EFFECT OF AUXIN AND MEASUREMENT OF ROOT NUMBER AND ROOT LENGTH OF *U. PICTA*

Medium for rooting	Mean No of roots/shoot	Mean root length (cm)
½ MS	0.0	0.0
½ MS + 0.5 mg/L	5.0	2.0
½ MS +1.0 mg/L	7.0	2.8

CONCLUSION: In the present investigation, the application of a superfine cut to the seed coat followed by soaking in distilled water resulted in 100% germination. As the seeds are small in size, the application of a superfine cut to the seed coat is tedious and time-consuming manual work. Therefore, the alternative is treatment with concentrated H_2SO_4 for 5 min, which results in

approximately 95% germination. These results indicate that the seeds of *U. picta* might experience physical dormancy imposed by the impermeable seed coat, which could be overcome either by the application of cuttings to the seed coat or treatment with concentrated H_2SO_4. The results of the present investigation on the TTC test and 100% germination with the application of a slight cut to the seed coat at the micropylar end of the seeds of *U. picta* might be impermeable to water and gases, causing unpredictable germination. The protocol described in the present investigation can be utilized for large-scale cultivation of *U. picta*, a medicinally important woody herb.

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Data Availability: All the data generated during this experiment are included in this article.

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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