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Research Article

Recalcitrant Nature of *Hamelia patens* Jacq. Towards Androgenic Induction

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Abstract

Hamelia patens Jacq. is an ornamental and medicinal plant commonly known as "redhead," "scarlet," or "fire bush." belongs to the Madder family (Rubiaceae). Till now, attempts to introduce androgenesis in *H. patens* were not made as evident from the research literature reviews. The focus of this study was to develop an efficient and reproducible protocol for the induction of androgenesis in this ornamental plant by optimizing microspore developmental stages, pretreatments, growth regulator combinations in nutrient media, etc. The uninucleate stages of microspore development were observed in the bud size ranging from 14 mm to 18 mm. However, only a few swellings in the cultured anthers were observed and the further calli induction leading to the androgenesis was not achieved in the *H. patens*.

Keywords: *Androgenesis, Hamelia patens, Recalcitrant, Uninucleate microspore.*

Introduction

Rubiacea family, also known as the Madder family, is known pharmaceutically to innovative drugs and used in usual medicine to cure chronic and communicable diseases (Pandal, *et al*., 2009). The plant is a good source for phytochemicals like alkaloids and flavonoids. It is indigenous to America and introduced in India as an ornamental plant (Little, *et al*., 1974). In Mexico, 42 different medicinal purposes have been known especially in menstrual disorders, stopping bleeding, and healing sores. Pimples and malaria (Yasunaka, *et al*., 2005) skin diseases, athlete's foot, relieve pain, skin lesions, blisters, eczema, stomachache, itching headache, asthma, rash, insect bites, burn, scurvy, postpartum pain, uterine, inflammation, rheumatism, nervous shock, intestinal worms, and dysentery (Reyes, *et al*., 2004). In addition, the aerial part of the plant contains rosmarinic acid, well known to have antidepressant and immune-modulating activity (Khandelwal, *et al*., 2012).

Since the *in vitro* studies on cultures of *Hamelia patens* are not performed and documented, this endeavor has been made to develop a protocol for *in vitro* androgenic induction in *H. patens* considering various aspects involved in the androgenic response of plants.

Materials and Methods

Plant Material

The *Hamelia patens* plant that is adapted to the local environment at the Departmental botanical garden was selected as a donor plant. The plant was developed at an average 35°C and 65% humidity without the application of any fertilizers and pesticides. The explant anther was selected based on floral buds having microspores at the early to late uninucleate stage of development (Figures 1 and 2). The developmental stages of microspores inside the anthers were determined by 1% acetocarmine staining (Fan, *et al*., 1988). The floral buds (Figure 1) and the microspore developmental stages (Figure 2) belong to the same plant.

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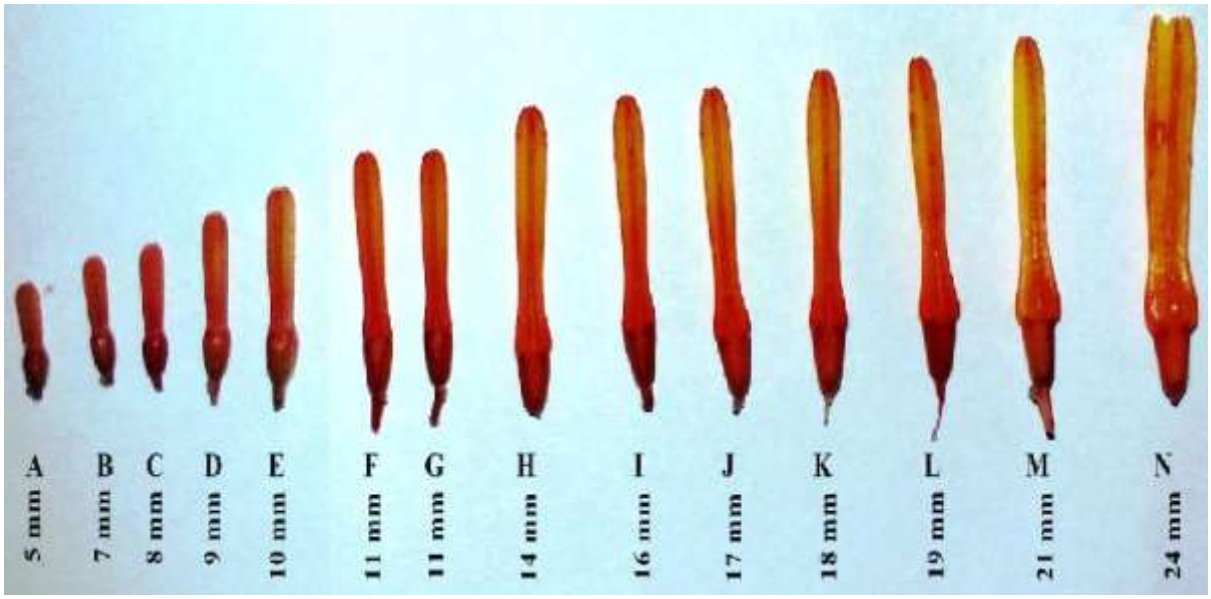

Figure 1 (A-N): Morphological variation in floral buds with respect to development stages.
(A to C: Meiotic bud; D to G: Pollen tetrad stage; H to K: Uninucleate stage; L to N: Mature pollen)

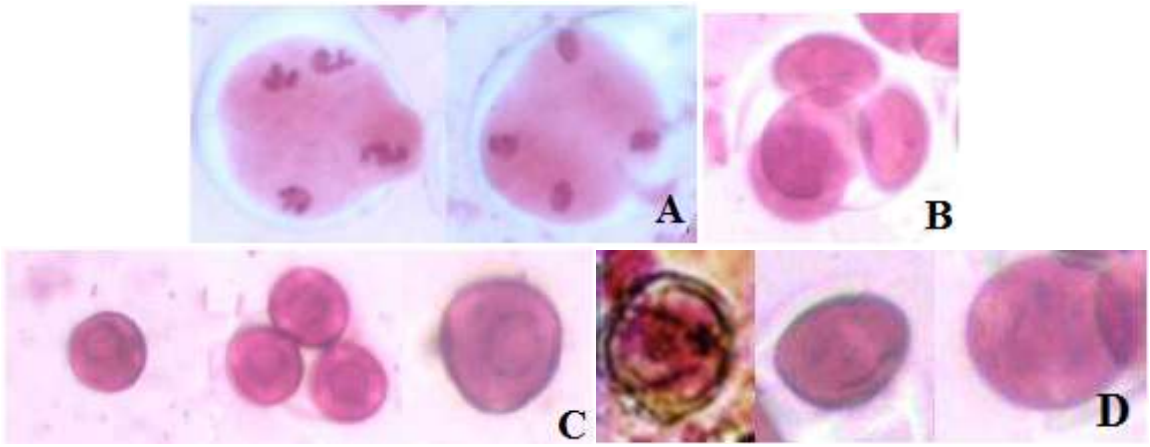

Figure 2 (A-D): Variation in pollen developmental stages with respect to bud size.
(A: Meiosis; B: Pollen tetrad stage; C: Uninucleate stage; D: Mature pollen)

Explant Preparation

The floral buds having anthers at the early to late uninucleate stage of development were subjected to optimal cold pretreatment at 8°C for 10 days [Narkhedkar, *et al*., 2016]. The pretreated floral buds were surface sterilized in 1% labogent, 0.1% mercuric chloride, and 50% ethanol for 5 minutes, 2 minutes, and 2 minutes respectively, followed by two successive washings with distilled water for 2 minutes each.

Anther Culture

Initially, the pretreated anthers were excised from surface sterilized floral buds. Anthers were cultured in MS media supplemented with a range of 2,4-D concentration as 5 mg/l, 6 mg/l, likewise to 10 mg/l with a constant combination of NAA at 0.5 mg/l and BAP 1.5 mg/l. The cultures were maintained at a temperature of 25±1°C; relative humidity was 50–60% and complete dark period. Also, the protocol for androgenic induction was set up to optimize the pretreatment of floral buds (Heat shock at 35°C for 3 hours; Heat shock at 35°C for 6 hour; Centrifugation at 2000 rpm for 3 hour; Centrifugation at 2000 rpm for 6 hour; Polyethylene glycol 4000 1.5 % for 2 days; Polyethylene glycol 4000 3.0 % for 2 days; Mannitol 0.3 M at 4°C for 2 days; Mannitol 0.7 M at 4°C for 2 days; Cold treatment at 8°C for 5 days; Cold treatment at 8°C for 10 days and a control set without any pretreatment). Later, the protocol was designed to know the effect of different

developmental stage of microspore (Tetrad to early uninucleate stage; Uninucleate stage and Late uninucleate to early binucleate stage) in MS basal media. As the androgenesis is very susceptible to the nutrients available in the media, the next level of optimization was for selection of appropriate nutrient media (Gamborg media; Nitsch media, MS media and NLN media).

Results and Discussion

Microspore Developmental Stages

Most properly, bud size for microspores at tetrad stage ranges between 8 - 11mm, for microspores at uninucleate stage ranges between 14 - 18 mm and late uninucleate to binucleate stage of pollen development is observed from 19 - 24 mm buds (Figure 1). The microsporogenesis under progress is evident in floral buds (Figure 2 A). The completion of microsporogenesis and tetrahedral tetrad formation was observed (Figure 2 B). The uninucleate stage was confirmed by central nucleus (Figure 2 C). The beginning of first mitosis is noted by initiation of unequal division at the peripheral position in microspores (Figure 2 D).

Precisely, floral buds at uninucleate stage of microspore development can be clearly observed and thus could be collected for pretreatment between 1500-1900 hrs (Figure 1 and Figure 2 C).

Anther Culture

In *Hamelia patens*, anther culture in MS media supplemented with range of 2,4-D concentration (5mg/l to 10 mg/l) with constant combination of NAA at 0.5 mg/l and BAP 1.5 mg/l did not produce any significant progressive response throughout the culture period. Also, the protocol designed for androgenic induction by providing various pretreatments yields no significant response at all set of experiments. Only, a very less swelling in cultured anthers was achieved with no further progress (Figure 3). However, such a like responses are also reported in Fabaceae where, some success in initiation of pollen embryogenesis and production of haploid plants is limited to a few species only, for example *Medicago* sp. and *Cajanus cajan* L. (Croser, *et al*., 2006). This little response was considered as may be due to mistake in selection of microspore developmental stage for culture. In later studies on investigating the effect of different developmental stage of microspore and selection of appropriate nutrient media, here also, the culture was recalcitrant.

The prolong culture showed the browning of explant (Figure 3). This browning was attributed to the presence of phenolic compounds in the *Hamelia patens*. So, the nutrient media was supplemented with antioxidant compounds, such as ascorbic acid 10 mg/l, cystein 5 mg/l and reduced glutathione 5 mg/l in basal MS media. This composition of media was found to beneficial for reducing browning to some extent but no enhancement in response of anthers towards callus formation was evident. Androgenesis in more than 200 species belonging to the various families, viz. the Solanaceae, Cruciferae and Gramineae has been reported (Dunwell 1986 and Hu and Yang 1986), while many woody plants and legumes are rather remains recalcitrant (Germana, 2009).

After observing such negative response in all the protocols, cytological observations were made after the given stress pretreatments and after two months of culture to know the effect on microspores (if any). The pretreated and cultured anthers were crushed to release microspores and slides prepared with 1% acetocarmine showed that in both cases, after pretreatment and two months of culture, the microspore shows no changes towards androgenesis. Changes like constricted cytoplasm were evident in anthers after pretreatment. But, after two months of culture, instead of microspores progressing towards sporophytic development, many microspores showed binucleate stage with unequal division, a key feature of gametophytic development (Touraev, *et al*, 1995).

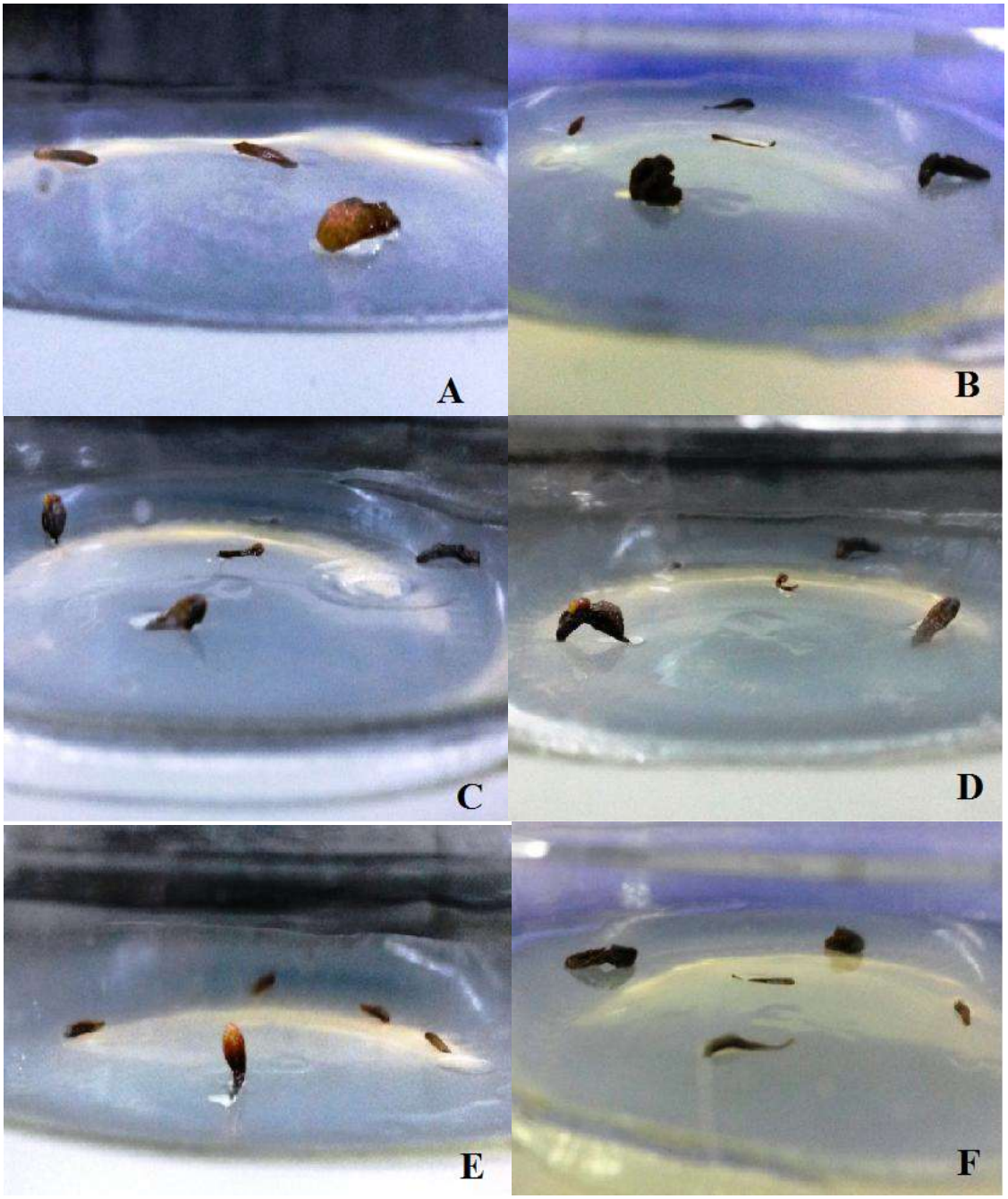

Figure 3 (A-F): Response of *Hamelia patens* Jacq. anther culture towards androgenic induction. (A to F: Variation in response showing anther swelling, curling and minute callus initiation. Images are shown irrespective of the experimental set).

Conclusions

After all the observations it was confirmed that the *Hamelia patens* is recalcitrant for androgenesis. From these reports, it can be inferred that androgenesis is not a plausible phenomenon at the studied parameters in the present investigation on *Hamelia patens*.

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