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A REVIEW OF THE *IN VITRO* PROPAGATION OF *BAUHINIA* SPP.

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ABSTRACT

Bauhinia species (including *B. acuminata, B. variegata, B. purpurea*, *B. monandra, B. galpinii, B. blakeana* and *B. acuminata*) are popular ornamental plants, usually woody ornamentals or herbaceous lianas, with attractive flowers typical of the Leguminosae of arid, temperate, sub-tropical and tropical zones. *Bauhinia* species also serve as fodder and many have multiple medicinal and biological properties. There is an interest in commerce and amongst collectors to clonally propagate species from this genus. This review highlights protocols that currently exist for the *in vitro* culture of *Bauhinia* species as a means to clonally propagate material.

Key words: *Bauhinia*, liana, woody ornamental

Multi-purpose nature of *Bauhinia* species

Bauhinia species, named as such after Jean Bauhin and Gaspard Bauhin (Houghton Mifflin Co. 2009), are important ornamental, forest and medicinal plants, including climbers and trees. *Bauhinia variegata* Linn. Benth (Caesalpiniaceae), commonly referred to as the orchid tree in English, serves as a source of fire-wood and is thus important for agro-forestry, and is the most studied *Bauhinia* species. *B. variegata* is most frequently found in tropical climates and is known to restore fertility to acid and degraded soils because of its ability to fix nitrogen (Acharya and Kafle 2009). *B. vahlii* is also used as a phytoremediative species in the Himalayan region (Dhar and Upreti 1999). In Nepal, the flowers are used in curries and as fodder for cattle (Singh *et al*. 2012). *B. variegata* has numerous medicinal properties; among them anti-inflammatory and antibacterial activity (Rajkapoor *et al.* 2006; Parekh and Chanda 2007). *Bauhinia racemosa* is a sacred Hindu tree whose bark is used to treat inflammation, chronic dysentery, diarrhea, glandular inflammations, ulcer, goiter and some skin diseases (Rajanna *et al.* 2011). Bioactivities include anti-inflammatory, analgesic, antipyretic (Gupta *et al*. 2005), antitumor, antioxidant, antimicrobial (Gupta *et al*. 2004; Kumar *et al*. 2005), antiulcer (El-Hossary *et al*. 2000) and antibacterial (Dahikar *et al*. 2011), mainly by the leaves and bark. The aerial parts of *Bauhinia cheilantha* (Bong.) Steud., known as *mororó* or *pata-de-vaca* in Portuguese, is a common leguminous plant in the *Caatinga* (principal ecosystem in Brazil's semi-arid regions) that has value as a fodder crop and for its medicinal properties: anti-inflammatory, antidiabetic, sedative, antiparisitic, digestive and expectorant (Lorenzi and Matos 2008). In addition to their medicinal properties, many *Bauhinia* species have tremendous horticultural and ornamental potential, and are commonly used as garden shrubs or pot plants due to their exuberant flowers. They are particularly useful as a spreading liana-type bush for garden walls, or, in a tree form, as a landscape plant. There are no documented studies on postharvest management of cut flowers of any *Bauhinia* species, but cut flowers make an attractive bouquet with an estimated life-span of a few days without any special treatment.

Bauhinia species can be propagated through conventional sexual and vegetative means, although the process is time-consuming and has several restrictions, including a long time gap between pod formation and maturation (Jorge *et al*. 2005), and strong inhibition of germination imposed by the testa (Prasad and Nautiyal 1996).

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Considering the importance of various *Bauhinia* species, either in agro-forestry or in medicine, there is interest in the *in vitro* culture and micropropagation of these species (Bhattacharya *et al*. 2000). Even though such studies are limited, this review compiles the current literature on *Bauhinia* species with the aim of providing a useful platform for further *in vitro* studies and applied biotechnological applications. Currently very few molecular studies exist on *Bauhinia* species (Lau *et al*. 2005), and providing a tissue culture protocol would allow studies on molecular and biotechnological aspects to be expanded.

In vitro seed germination, culture and micropropagation of *Bauhinia* species

1. General considerations

Mathur and Mukunthakumar (1992) first noted the importance of micropropagation of *B. variegata* as a rapid means of producing clonal plant stock for afforestation, woody biomass production and conservation of elite germplasm. Kumar (1992) used mature *ex vitro* material and, using the protocol noted in Fig. 1, could induce callus and shoots, which could be rooted *in vitro*. *Bauhinia* species can be propagated *in vitro* through microshoots proliferation (Upreti and Dhar 1996) or by elongation of nodal buds (Singh *et al*. 2012). In addition, *Bauhinia* species can be regenerated from calli formed on *in vitro* hypocotyls through organogenesis (Mello *et al*. 2001) or by somatic embryos (Banerjee *et al*. 2012), the latter making automation of *B. variegata* micropropagation possible. Most commonly, *in vitro* propagated *Bauhinia* plants are derived from *in vitro* seed germination, and Singh *et al*. (2012) provide a simple, but apparently effective, protocol for seed sterilization for germination *in vitro*. This protocol can serve for the *in vitro* germination of other *Bauhinia* species, including *Bauhinia galpinii* (Teixeira da Silva, unpublished results; Fig. 2). Although more difficult, *Bauhinia* cultures can be also initiated from mature plants (Kumar 1992; Mathur and Mukunthakumar 1992; Dhar and Upreti 1999; Kumar *et al*. 2005), but browning of explants may be a problem (Dhar and Upreti 1999).

2. Species-by-species description

An alphabetical description is provided next of the *in vitro* response of different *Bauhinia* species. All *in vitro* studies were performed at 25 °C, unless specified otherwise.

Akhter *et al*. (2012) could germinate 95% of *B. acuminata* seeds on Murashige and Skoog (1962) (MS) basal medium containing 3% (w/v) sucrose and supplemented with 1 $mg \cdot dm^{-3}$ gibberellic acid (GA_3) under a 12-h photoperiod at 3000 Lux. When the nodes of 4- or 5-week-old seedlings were transferred to MS containing 0.5 $mg \cdot dm^{-3}$ 6-benzyladenine (BA; equivalent to BAP or 6-benzylaminopurine; Teixeira da Silva 2012) and 0.1 $mg \cdot dm^{-3}$ α-napthaleneacetic acid (NAA), 2.5 shoots per explant formed and when shoots were transferred to MS containing 0.6 $mg \cdot dm^{-3}$ NAA and 5% (v/v) coconut water (CW), 3.44 roots formed per shoot.

Gutiérrez *et al*. (2011a, 2011b), working with *B. cheilantha*, used Woody Plant Medium (WPM; Lloyd and McCown 1980) basal medium containing 3% (w/v) sucrose to regenerate shoots from nodal segments of 20-day-old *in vitro* seedlings under 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) and a 16-h photoperiod. When 2 $mg \cdot dm^{-3}$ BA (2011a study) or 1 $mg \cdot dm^{-3}$ thidiazuron (TDZ) and 2 $mg \cdot dm^{-3}$ BA were added to the medium (2011b study), 2.84 shoots formed per nodal explant, 2.4-fold more than per cotyledon. The addition of 0.5 $mg \cdot dm^{-3}$ indole-3-acetic acid (IAA), 1.0 $mg \cdot dm^{-3}$ NAA or 0.25 $mg \cdot dm^{-3}$ indole-3-butyric acid (IBA) resulted in best rooting percentage (~ 65.0%) while 0.5 $g \cdot dm^{-3}$ activated charcoal (AC) improved shoot and root formation. The authors also found that forced ventilation through a polyvinylchloride film during rooting increased the percentage of seedling survival during greenhouse acclimatization.

In *B. forficata*, two studies exist. Shoots have been shown to originate from cotyledonary node epidermal and subepidermal tissue through a process of indirect organogenesis and amitosis (nuclear fragmentation) (Appezzato-da-Glória and Machado 2004). In that study, half-strength MS (½ MS) basal medium to which 3% (w/v) sucrose and 4 $mg \cdot dm^{-3}$ BA were added allowed callus to form from 1 cm long hypocotyls after 30 days when cultured under 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD with a 16-h

photoperiod. Mello *et al*. (2000) obtained adventitious buds of *B. forficata* from callus grown on hypocotyl segments. Callus was initiated on ½ MS medium with 30 g·dm^{-3} sucrose, 2 and 4 mg·dm^{-3} BA, at 30 µmol·m^{-2}·s^{-1} PPFD and a 16-h photoperiod, with a frequency of 5-6 buds/0.5 g callus. Shoots elongated when transferred to BA-free medium. In a subsequent study, Mello *et al*. (2001) found that exposure of seedling-derived hypocotyls to 17.7 µM BA induced callus on MS basal medium with 3% (w/v) sucrose under a 16-h photoperiod at 30 µmol·m^{-2}·s^{-1} PPFD. Cell suspension cultures could be induced from 5 g of callus placed in 100 ml of liquid callus-inducing medium at 60 rpm in 250-cm^3 Erlenmeyer flasks, when subcultured every 3 weeks. Galactose, sorbitol and glycerol could not support cell suspension cultures, only sucrose.

Kumar (1992) used *ex vitro* stem cuttings from young branches of 15-18-year old *B. purpurea* trees which, after surface sterilization (Fig. 1), were trimmed to 0.5-cm explants. When placed on MS basal medium containing 3% (w/v) sucrose under 40 µmol·m^{-2}·s^{-1} PPFD and a 12-h photoperiod, callus could be induced with 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and after subculture every 30 days, shoots could be induced with 5 µM kinetin (Kin), while roots could be induced within 30 days in the presence of 5 µM NAA.

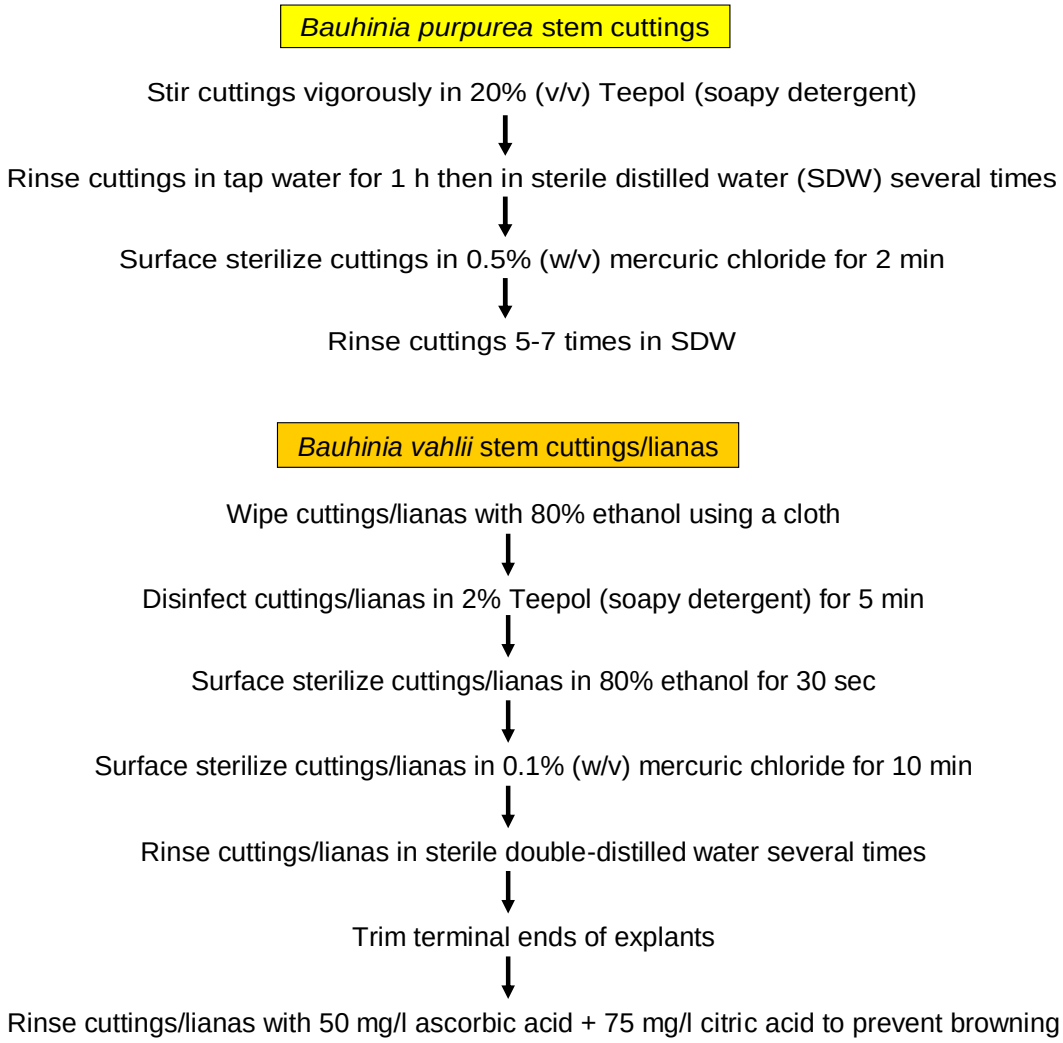

Figure 1. Surface sterilization protocol for *Bauhinia* spp. *ex vitro*-derived stem cuttings (1 cm long) from actively growing branches of mature (15-18 years old) trees based on Kumar's (1992) protocol for *B. purpurea* or for mature stem cuttings or lianas (3-4 cm long, each with one node) from *B. vahlii* (Dhar and Upreti 1999).

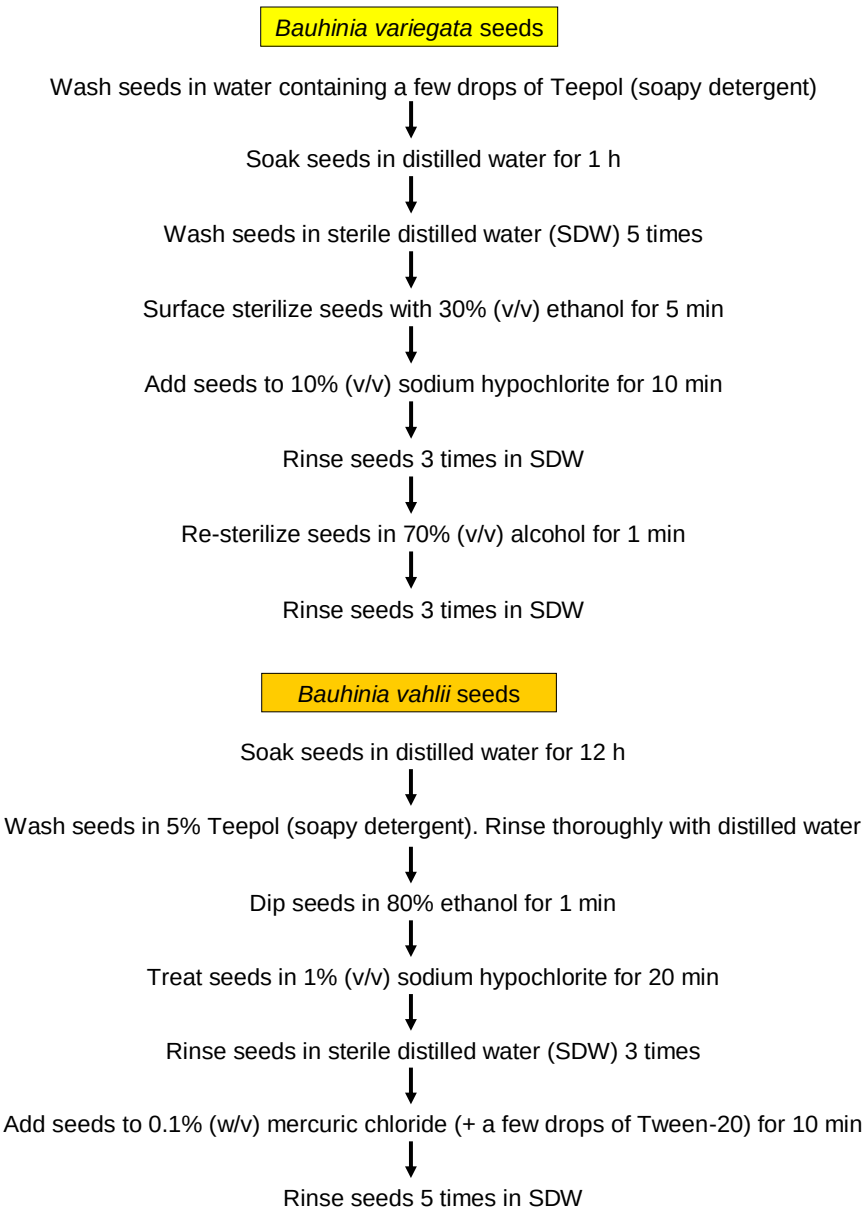

Figure 2. Surface sterilization for *Bauhinia* spp. seeds based on Singh *et al*.'s (2012) protocol for *B. variegata* and on a protocol for *B. vahlii* (Upreti and Dhar 1996). This *B. variegata* protocol works well for *B. galpinii* seed (Teixeira da Silva, unpublished data).

Only a single study exists for *B. tomentosa* (Naz *et al*. 2012). Nodal explants from *in vitro* seedlings could form 12 shoots per explant after 6 weeks on MS medium containing 50 mg·dm^{-3} adenine sulphate (AS), 5.0 µM BA and 0.5 µM NAA. Roots were induced in the presence of 5.0 µM chlorogenic acid and 2.5 µM IBA from 70% of shoots within 4 weeks. MS was more effective than WPM as a basal medium. Polyvinylpyrrolidone and ascorbic acid were able to reduce polyphenolics *in vitro*.

B. vahlii cotyledonary nodes from 15-d old *in-vitro*-germinated seedlings could form 5.5 shoots per explant on MS medium containing 20 g·dm^{-3} sucrose, 1 µM TDZ, at 40 µmol·m^{-2}·s^{-1} PPFD under a 16-h photoperiod (Upreti and Dhar 1996). This medium served equally well for shoot induction and proliferation. MS as basal medium was more effective than B5 (Gamborg *et al*. 1968), WPM or ½ MS. Some success in inducing shoots was possible with 1 µM BA, 1 µM Kin, or 2.5 µM zeatin.

55% of shoots formed roots on ½ MS medium with 1 μM NAA. Dhar and Upreti (1999) used nodes of mature lianas as explants. They were initially incubated in the dark at 4 °C for 48 h then plated on MS basal medium containing 100 mg·dm^{-3} AS and 2.5 μM Kin to induce and proliferate shoots, forming a maximum of 4.5 shoots per explant at 40 μmol·m^{-2}·s^{-1} PPFD and under a 16-h photoperiod. Browning could be reduced by soaking sterilized explants in a solution of 50 mg·dm^{-3} ascorbic acid and 75 mg·dm^{-3} citric acid. *In vitro*-derived rootless plantlets formed roots in ½ MS liquid medium with NAA and IBA (10 μM of either) in which 55.5% of plantlets rooted. Bhatt and Dhar (2000) induced 12.6 shoots per cotyledonary node in the first culture, dropping slightly to 12.0 by the fourth subculture. Shoot formation was possible on MS basal medium containing 3% (w/v) sucrose, 1 μM TDZ and 1 μM Kin (with or without 1 μM GA_3). 83% of plantlets could form roots on the same basal medium containing 1 μM NAA. Both roots and shoots were formed under 40 μmol·m^{-2}·s^{-1} PPFD and a 16-h photoperiod.

B. variegata is the most studied *Bauhinia* species. Mathur and Mukunthakumar (1992) used nodal explants of mature trees to induce 7.8 shoots per nodal segment in the presence of 13.3 μM BA on MS basal medium containing 3% (w/v) sucrose and 100 mg·dm^{-3} *myo*-inositol at a PPFD of 36 μmol·m^{-2}·s^{-1} and under a 16-h photoperiod at 28 °C. 96% of shoots rooted in the presence of 4.9 μM IBA. Thonnalak (2012) used axillary meristems as explants to induce shoots on MS basal medium containing 2.5 mg·dm^{-3} BA, although callus formed when the BA concentration exceeded 5 mg·dm^{-3}. Shoots could be proliferated with 1-2.5 mg·dm^{-3} BA at 2500 Lux and a 16-h photoperiod and formed roots when exposed to 5 mg·dm^{-3} NAA. Papafotiou *et al*. (2010) chose to use *ex vitro*-derived shoot tips and single node explants from young shoots (25 or 80 cm long) with four apical nodes, which developed after pruning one-year old seedlings, as explants. The authors also tested shoot tip or single node explants from 3-leaf (two-week old) seedlings. Explants of the 3rd, 4th and 5th node had a 70-100% response and 1.5-2.0 cm shoots with 3-5 leaves represented the most effective shoot formation on MS basal medium containing 3% (w/v) sucrose and 0.5-1.0 mg·dm^{-3} BA. Explants excised from 25 cm long shoots or from young seedlings containing three leaves formed more shoots than 80 cm long shoots. Leaf lamina explants formed callus on MS containing 2,4-D and BA, but callus failed to form shoots. Roots were induced on ½ MS medium with 2 mg·dm^{-3} IBA. In *B. variegata*, both picloram (Pic) and 2,4-D successfully induced somatic embryos either through a direct route or an indirect route (i.e., *via* callus) from the sub-epidermis of cotyledons or hypocotyls (Banerjee *et al*. 2012). Sterilized seeds were germinated *in vitro,* and cotyledons and hypocotyls were used as explants. Somatic embryogenesis was possible on MS basal medium with B5 vitamins at 40 μmol·m^{-2}·s^{-1} PPFD and a 16-h photoperiod. Basal medium contained 4-6 mg·dm^{-3} Pic for cotyledons and hypocotyls, 6-8 mg·dm^{-3} 2,4-D for cotyledons but 12 mg·dm^{-3} for hypocotyls. Using 4 mg·dm^{-3} Pic, 84 somatic embryos (SEs) formed per cotyledon and 49 SEs per hypocotyl. Using 8 mg·dm^{-3} 2,4-D, 52 SEs formed per cotyledon and 12 SEs per hypocotyl. SEs could also be induced from friable callus that formed on Pic- and 2,4-D-containing medium. Using callus induced on 6 mg·dm^{-3} Pic, 84 SEs were induced per cotyledon and 49 SEs per hypocotyl. Using callus induced on 8 mg·dm^{-3} 2,4-D, 53 SEs were induced per cotyledon and 19 SEs per hypocotyl. 0.2 mg·dm^{-3} BA was required for best rooting of SE-derived plantlets. Singh *et al*. (2012) decided to use nodes of seedlings germinated *in vitro* as explants. On MS basal medium containing 3% (w/v) sucrose and supplemented with 1 μM BA and 0.05 μM NAA, and under 40 μmol·m^{-2}·s^{-1} PPFD and a 16-h photoperiod, 7.45 nodes formed per shoot within 8 weeks. Callus was profuse and as wide as 11 mm.

Acclimatization of *Bauhinia* species

According to Singh *et al*. (2012), shoots that form *in vitro* and that have rooted well can be trimmed to plantlets containing 3-4 nodes 2-3 cm in size and the plantlets can be rooted in a mixture of 1 : 1 sand and soil (based on Kumar 1992) with fungicides added. For rooting, the plantlets were kept at high humidity for 2 weeks. Watering with 1/10-

strength MS medium was sufficient for the survival of *B. variegata* plantlets in soil (Mathur and Mukunthakumar 1992). Thonnalak (2012) showed 64% plantlet survival after transfer to peat moss in the field. A 1:1 mixture of soil and vermiculite was optimal for *B. vahlii* (Dhar and Upreti 1999), although Bhatt and Dhar (2000) suggested a 2:1:1 mixture of soilrite, sand and soil.

Future perspectives

Studies on the *in vitro* culture of *Bauhinia* species are still at a nascent phase of exploration. As their medicinal and ornamental value increase, greater attention will be paid to tissue culture. Reliable protocols exist thus far for the micropropagation of several *Bauhinia* species but several options for germplasm improvement through biotechnology should be explored in the future. The use of thin cell layers (TCLs) (Teixeira da Silva and Dobránszki 2013a, b) and rigorous attention to explant size and sampling would allow for greater control of organogenesis while synthetic seed technology (Sharma *et al*. 2013) would allow for short- to long-term storage of important germplasm. The use of phloroglucinol to improve rooting (Teixeira da Silva *et al*. 2013) could further enhance acclimatization.

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