ISSN (Online): 2349-1183; ISSN (Print): 2349-9265
TROPICAL PLANT RESEARCH
The Journal of the Society for Tropical Plant Research
9(3): 96–102, 2022
DOI: 10.22271/tpr.2022.v9.i3.015

Research article

In Vitro sterilization protocol for callus induction of *Baccaurea courtallensis* (Wight) Müll.Arg. - An endemic tree of Southern Western Ghats, India

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Abstract: Experiments were carried out to establish *in vitro* culture using terminal and lateral buds of the endemic tree *Baccaurea courtallensis* of Phyllanthaceae which presented formidable difficulties. Besides the profuse phenol exudation, cultures were infected with intense fungal contamination. Efforts directed to control the schedule by adjusting the concentration of disinfectants NaOCl and $HgCl_2$ with additional rinsing of explants with 70% ethanol and pre-soaking of shoots in various concentrations of the fungicide Bavistin never fetched any success. The intricately connected fungus seems to have found a niche amidst the leaf primordia in shoot apices and the axillary crevices of the nodal regions completely establishing itself in cultures. Perplexed with no remedy in sight, an interesting attempt of quick surface washing with 70% acetone was introduced in the surface sterilization schedule. Fresh explants were quickly rinsed with 70% acetone, followed by 0.1% mercuric chloride, 1% bavistin along with 4% PVP in succession and later wash with sterile distilled water enabling effective establishment of culture. Callus formation was observed on MS and WPM supplemented with auxin and cytokinin. Callus induction from the nodal region was observed at all concentrations and combinations of PGRs supplemented. Maximum induction of callus was obtained from MS medium augmented with BAP 4µM alone and in combination with IBA 1 µM +TDZ 4 µM.

Keywords: Shoot tip - Endemic - Acetone - Bavistin - PVP - WPM - Callus induction - TDZ.

[Cite as: Nandhini K, Ravichandran P, David VJ & Manimekalai V (2022) In Vitro sterilization protocol for callus induction of *Baccaurea courtallensis* (Wight) Müll.Arg. - An endemic tree of Southern Western Ghats, India. *Tropical Plant Research* 9(3): 96–102]

INTRODUCTION

Baccaurea courtallensis (Wight) Müll. Arg**.** is a moderately sized evergreen tree of the Phyllanthaceae family, distributed from the South Konkan to South Kerala and adjoining Western parts of Tamil Nadu in the evergreen forests up to an altitude of 700 to 1000 m. *Baccaurea courtallensis* is one of the lesser-known wild edible fruit-yielding tree, used by various ethnic communities and local folks of the Western Ghats, especially Muthuvan, Kani, Kadar tribes. Fruits are crimson colour and acidic. Only the local tribal population of the Western Ghats region consume ripe fruits and it is not widely known to others. Fruits are harvested for their medicinal value and/or for pickling, leaving less chance for natural regeneration. The Food and Agricultural Organization estimated that around one billion people use wild foods in their diet. Wild fruits provide more nutrition compared to other cultivated fruits. Fruits of *Baccaurea courtallensis* are also rich in their nutritive value (Nazarudhin 2010).

The tribal's and local communities eat ripen fruits. Aril is sweetish sour and the rind is pickled (Jose *et al*. 2016). Fruits are acidic and edible used for infertility problems. Consumption of ripened fruits also cures mouth and stomach ulcers (Karthick Prabu *et al*. 2013). Rock-rubbed root and leaf paste have been used to treat piles, poison, heal wounds and to control diabetes. The bark of this plant has biologically active compounds, such as

Received: 21 May 2022 Published: 31 December 2022
https://doi.org/10.22271/tpr.2022.v9.i3.015

tannins, saponins, terpenoids and flavonoids, and stigmasterol (Sujatha *et al.* 2009). Dry seeds (kernels) contain 22% of oil. There are two major fatty acids are present in seed oil, namely palmitic acid 42.59%, oleic acid 36.15% and two minor acids are also present lauric acid - 0.40%, linoleic acid - 0.38% other than these oils, stearic acid content was 16.20% and myristic acid was 4.28% and also including some traces of linolenic acid present in the seed oil (Mohan 2009). The fruit rind possesses steroids, coumarins, tannins, flavonoids, phenols, quinines and volatile oils (Abishek *et al*. 2011). Methanolic extracts of the bark of *Baccaurea courtallensis* were reported to have antibacterial and antifungal activities (Sujatha *et al.* 2009).

In vitro conservation of plants comprises of a selection of explants, aseptic culture establishment, multiplication of micro shoots, and rooting followed by acclimatization of the plantlets. Amidst these stages, the challenging step is standardizing the sterilization procedures of explants for aseptic culture establishment. On an average, losses due to contamination under *in vitro* conditions are between 3–15% in the majority of commercial and scientific plant tissue culture laboratories (Cassells 2001). Plant tissue cultures may be infected by a variety of organisms, including bacteria and fungi, which can drastically lower their production or even stop them from growing at all. There are several pathogens (microbial contaminants) which have been a major threat to *in vitro* cultures due to their rapid proliferation characteristics. Explants may be contaminated during laboratory manipulations by endophytic bacteria, micro-arthropod vectors, or combination (Leifert 1992). Fungus also arrives with explants or airborne or either culture. Obuekwe & Osagie (1989) reported that fungi such as *Aspergillus niger* Tieghe. and *Aspergillus flavus* Link produce oxalate and aflatoxin poisons respectively, that can cause lethal to plant cultures (Obuekwe *et al*. 1989). A successful tissue culture protocol starts with effective explants sterilization (Dodds & Roberts 1985). Different methods are used to eliminate fungal and bacterial contamination, including use of antibiotics, fungicides and inactivation by heat and light.

As of now there are no reports on surface sterilization of *Baccaurea courtallensis*, which is a prerequisite for further tissue culture techniques like organogenesis or any genetic manipulation of this endemic plant. The most important step in explant preparation for further processes are keeping the explant alive and overcoming the problem of contamination. Therefore, in this experiment, an attempt has been made to establish an efficient surface sterilization protocol for the *in vitro* propagation of *Baccaurea courtallensis,* using different sterilant and varying their concentrations and durations of exposure as a best alternative to curb contaminations during *in vitro* culture of this lesser known, underutilized, medicinal, fruit yielding endemic species.

MATERIALS AND METHODS

Sources of plant material and nutritional medium

A few healthy five-year-old trees were identified from the campus of Sri Parasakthi College, and forest areas of Courtallam, Tamil Nadu. Fresh, healthy and juvenile explants were obtained from these sources. Explants such as shoot tip, internode and petiole were collected fresh whenever required.

Surface sterilization

Surface sterilization of explants such as leaves, shoot tips, and internodes was performed by the following series of steps. Explants were first washed with running tap water for 3 minutes to remove dust particles and again washed thrice with distilled water and then soaked in liquid commercial bleach (10% of NaOCl) containing a drop of tween-20 and 0.4% of PVP for 5 minutes. Explants were incubated in different concentrations and durations in Bavistin, $HgCl_2$, Anti mycotic solution, Acetone and Ethanol followed by wash with sterile distilled water to remove any traces of sterilant for 10 minutes. Sterile explants were placed on a grooming pad and cut into the required sizes. Generally, one explant was placed per culture vial and 20 replicates were made for each treatment.

Culture conditions

Explants were inoculated on WPM (Lloyd McCowns 1980) medium and MS medium (Murashige & Skoog 1962) containing 2 to 3% sucrose and gelled with 0.8% to 1.0% agar supplemented with PGRs. pH of culture medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 min at 120˚C for 15 lbs pressure for 15 minutes. Cultures were maintained under a 16/8 h light/dark regime under 4,000 lux light intensity provided by cool white fluorescent tubes. The temperature of culture room was maintained at 25±2°C.

Data recording and analysis

The percentage of survival rate of explants, growth type and explants colour were recorded. Contamination was evaluated on 5^{th} day of incubation. Numbers of contaminated cultures were counted. Following formula was applied to calculate of contamination percentage.

$$\text{Contamination (\%)} = \frac{\text{Number of explants contaminated}}{\text{Total number of explants inoculated}} \times 100$$

RESULTS AND DISCUSSION

Plant tissue culture techniques are always linked with microbial contamination. Multiplication of microbes and their competition with explant growth for nutrients may alter the culture environment. To avoid this kind of contamination, the explants must be sterilized before inoculation into a tissue culture medium. In this experiment, different concentrations of sterilizing agents were used (Table 1).

Table 1. Effect of different concentrations and combination of surface sterilant on explants.

S.N.	Surface sterilizers	Concentration	Duration of exposure in secs.	$HgCl_2$ treatment conc. and duration	Survival rate (%)	Contamination rate (%)	Growth of the explant
1	Ethanol	70%	30–50 sec	0.05% 5 min	10 %	90%	++
2	Fungicide	0.1%	5	0.05% 5 min	10 %	90%	++
3	Acetone	70%	30–40	0.1% 5 min	20 %	80%	++
4	Antimycotic solution	10%	5 min	0.1% 5 min	20 %	80%	++
5	Acetone & fungicide	70% 1%	30–50 10	0.1% 5 min	90 %	10%	+++

Note: ++ - Moderate, +++ - Good.

In culture of *Baccaurea courtallensis* besides the profuse phenol exudation browning was also occurred. Standardization of surface sterilization methods and restrict contaminants of explants in this plant was a major hurdle for the establishment of cultures and maintenance. Efforts directed to control the schedule by adjusting the concentration of disinfectants sodium hypochlorite and Mercuric chloride, additionally rinsing of tissues with 70% ethanol and pre-soaking of shoots in various concentrations of the systemic fungicide Bavistin was never succeed. Bavistin 1% along with 0.1% solution of $HgCl_2$ for 5 min were used for sterilization. After inoculation, it was observed that when explants were treated with 1% Bavistin for 5 minutes, 10% of explants were found contamination-free, showed slow growth and colour of the explant were normal. At higher concentrations it showed maximum effects against contamination but survival percentage of explants was very low. The intricately associated fungus grows and establishes itself in the leaf primordia in shoot apices and the axillary crevices of the nodal regions and interferes the development of explants in cultures. To overcome this interesting attempt of quick surface washing with Acetone was introduced in the surface disinfection schedule. Fresh explants were quickly rinsed with 70% Acetone for 50 to 60 seconds, before treatment with 1% Bavistin for 10min followed by 0.1% solution of $HgCl_2$ for 5 minutes along with 4% PVP in succession and washed with freshly prepared chilled sterile distilled water enabled effective establishment of culture without contamination. The explants were wiped with pre-sterilized tissue paper, trimmed and inoculated on MS and WPM supplemented with various concentrations of auxins and cytokinins. Nearly 90% of contamination-free cultures were recorded. The combination of Acetone, Bavistin and $HgCl_2$ proved a good surface sterilant and inhibiting fungal contaminants in *Baccaurea courtallensis*.

Callus formation in vegetative explants

Experiments were focused to achieve callus induction and regeneration of entire plantlets, cultures were initiated using shoot buds, nodal regions and petiole explants. Significant observations were made on the callusing potential of shoot buds and nodal segments. Contrary to the expectations shoot tip and nodal buds inclined more towards callus development. The bud at the nodal region resisted organized growth and re-differentiated at a very low frequency. Efforts were taken to control callus by eliminating endogenous auxin supplementation and an effort to add anti-auxin TIBA as a medium component did not realize the expected results.

In addition, wound callus developed prominently on excised region of node (Fig. 1A). Callus formation was exhibited regularly in the region where the petiole gets connected the main stem. Callus formation was observed in all types of explants such as shoot tips, nodal segments and petiole. Nodal explants showed 70% of callus on MS+BAP 4µM augmented medium (Fig. 1D). Friable, soft callus emerged even in basal medium however the frequency of callus was low. Nodal explants were best at MS+TDZ 4µM, BAP 4µM and IBA 1µM combination

(Fig. 1E). Auxin and cytokinin showed a synergistic effect on nodal explants to produce high amount of callus. Pale green, friable and wet callus was formed in these treatments (Fig. 1F). In addition, with that same concentration of BAP, TDZ along with IBA augmented medium showed callus formation, in petiole explants. Callus formation was observed within five days from the petiole excised region. The callus was friable, soft and pale green. Calli formed in all media combinations and concentrations but did not develop further and turned brown, due to the phenolic exudation in plant tissues (Fig. 1B & C). Regeneration of the shoot tip was investigated in WPM medium containing various concentrations of BAP. All treatments showed callus formation, yet BAP 14, 16, 20 µM produce new shoots. Control WPM medium did not support the shoot

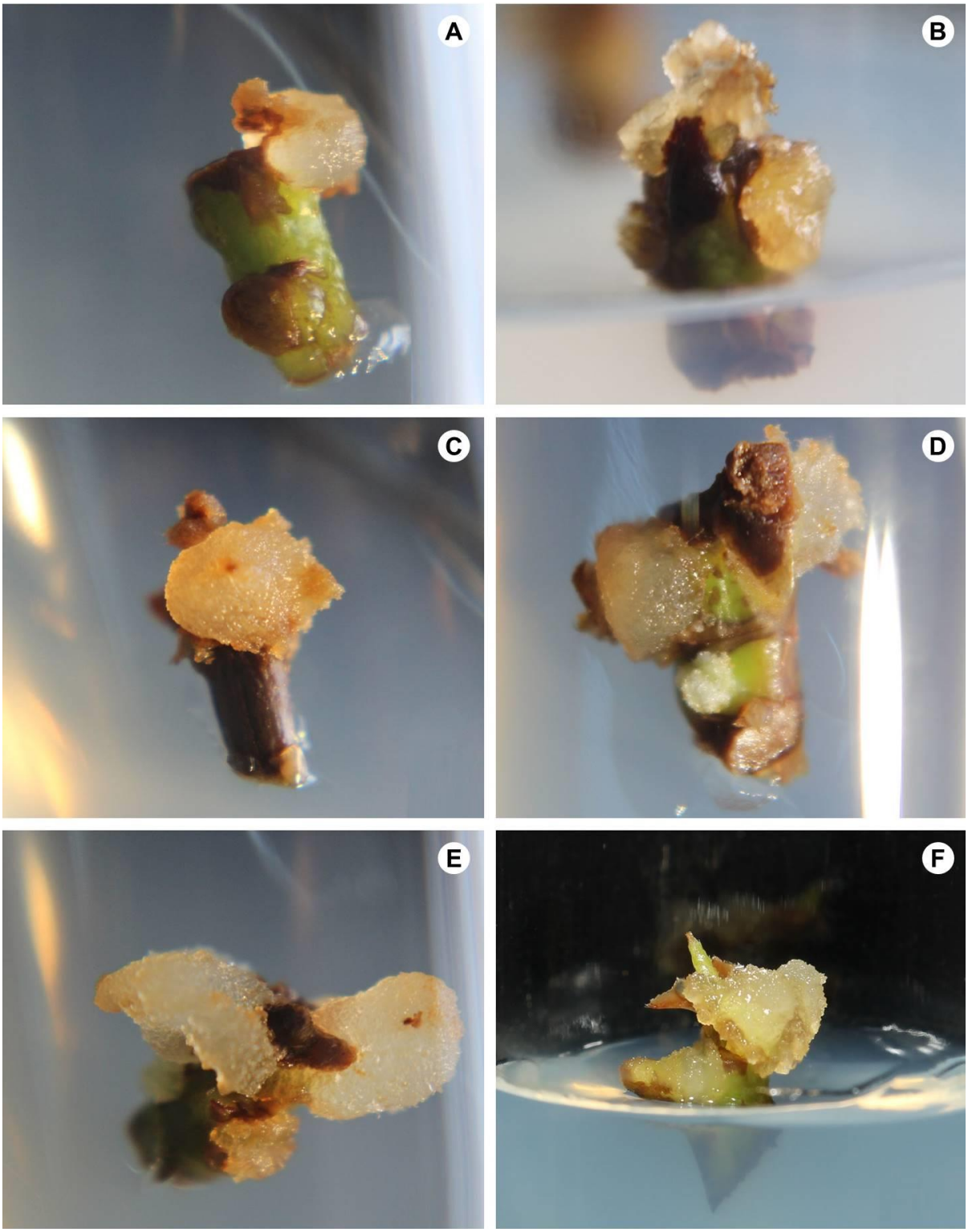

Figure 1. A, Early and regular feature of callus formation exhibited by an axillary bud in contaminated free culture - MS medium augmented with BAP 4 µM; **B-F,** Copious amount of friable callus formed on MS medium supplemented with TDZ 4 µM+BA 4+IBA 1 µM; **C,** A well-developed callus showing necrotic region, browning due to phenolic exudation.

induction. Only one shoot formed from each explant. The entire explants produced callus at the tip of shoot, colour of the callus was white and friable, wet in texture. The highest frequency of callus was observed in WPM medium fortified with BAP 14 µM (Table 2 & 3).

It has been shown in the past that legumes, monocotyledonous plants and trees possess resistance in displaying morphogenetic responses in culture (Mathews 1987). Plants containing latex such as members of Asclepeidaceae and Euphorbiacae have not been quite amenable to be installed as productive cultures (Karthick Prabu *et al*. 2013). Though, there are species that are non–laticiferous despite being members of these families, documented report shows it was never easy to bring explants to be groomed in cultures from these taxa. The annoying issue of phenolic exudation and browning of the medium could be overcome after much struggle in arriving at a complex surface sterilization schedule that could provide a method involving the use of unusual sterilant. The disinfectants used to keep the cultures alive good enough to form a callus but it is not clear if the primordial buds present in the shoot tip and nodal explants were blocked in evincing their natural growth responses.

Paradoxically even such an elaborate schedule described in table was unable to quell the fungal contamination in shoot tip cultures. Even though, the microbial interference is brought under control in most of the explants, results of this study show the terminal regions of shoots that are fragile and unique in harbouring a microcosm in itself with the tender apical meristem and the subjacent leaf primordia that protect the growing tip remains unprotected from fungal contamination. In all cultures regardless of the surface sterilization method adopted shoot tip explants were infested with a white cottony mycelial growth. It might possible that the spore or nascent mycelium intricately associated with the organ either as phyllosphere flora or as endophytes find the illuminated humid *in vitro* environment ideal for its incubation that it eventually turns out to menace to the tissue culturist (Gopal *et al*. 2016).

Table 2. Effect of BAP on vegetative explants of *Baccaurea courtallensis* (Wight) Müll. Arg.

S.N.	Medium	Response	Shoot regeneration	Colour of the callus	Texture of the callus	% of callus formation
			Response of nodal cultures			
1	MS+BM	Callus formed	-	Off white	friable	50 %
2	MS+BAP 2 µM	Callus formed	-	White	friable	60 %
3	MS+BAP 4 µM	Callus formed	-	Pale green	friable	70 %
4	MS+BAP 6 µM	Callus formed	-	White	friable	50 %
5	MS+BAP 8 µM	Callus formed	-	White	friable	60 %
6	MS+BAP 10 µM	Callus formed	-	White	friable	60 %
			Response of shoot tip cultures			
7	WPM+BM	Callus formed	-	Initially white then became brown	Friable and wet	12.5 %
8	WPM+BAP 12 µM	Callus formed	-	Initially white then became brown	Friable and wet	25 %
9	WPM+BAP 14 µM	Callus formed	Only one shoot tip formed	Initially white then became brown	Friable and wet	75 %
10	WPM+BAP 16 µM	Callus formed	Only one shoot tip formed	Initially white then became brown	Friable and wet	37.5 %
11	WPM +BAP 18 µM	Callus formed	-	Initially white then became brown	Friable and wet	50 %
12	WPM +BAP 20 µM	Callus formed	Only one shoot tip formed	Initially white then became brown	Friable and wet	62.5 %
			Response of petiole cultures			
13	MS+BM	Callus formed	-	White	Friable	10 %
14	MS+TDZ 4 + BAP 4+IBA 1 µM	Callus formed	-	Pale green	Friable	30 %

It has been pointed out that the generalization on auxin - cytokinin manipulation can fetch control overshoot and root production in callus and organ cultures and in revering plants through somatic embryogenesis the intricacies and nuances has always been species and case specific (Ngomuo *et al*. 2013). Though, recalcitrance of *Baccaurea courtallensis* is a disappointing and disturbing element in this study, the subtle variations seen in the nature and texture of the callus in different explants and more specifically regions within the explant should be considered as a positive signal to pursue further studies. Variations in nutrient composition introduced by using of two medium recipes namely MS medium and WPM and finer variations presented by adding adjuvants or altering nitrogen and other nutrient supply never provided for a change in response show that *Baccaurea*

courtallensis is a tough system to work. In other amenable species these modifications have been reported to reel out interesting and useful leads to achieve plant regeneration.

Table 3. Effect of cytokinin along with auxin on nodal buds.

S.N.	Medium	Response	Shoot regeneration	Colour of the callus	Texture of the callus	% of callus formation
1	MS+BM	Callus formed	-	Pale yellow	Friable	40 %
2	MS+KIN 4 µM + NAA 1 µM	Callus formed	-	Pale green	Friable	66.6 %
3	MS+TDZ 4 + BA 4 +IBA 1 µM	Callus formed	-	Pale green	Friable	80 %

CONCLUSION

Based on this study it can be concluded that sterilizing the explants in 70% acetone for 50 to 60 seconds followed by 1% Bavistin for 10 minutes and 0.1% $HgCl_2$ solution of for 5 minutes is the most effective treatment to inhibit fungal infections in *Baccaurea courtallensis*. However, the selection of an appropriate sterilant or disinfectant and proper disposal of the used disinfectants in a plant cell, tissue and organ culture laboratory are important. BAP 4 µM and TDZ 4 µM+BA 4+IBA 1µM enhance good intensity of callus growth in nodal explants. Similarly higher concentration of BAP 14, 16 and 20 µM promotes apical bud growth in shoot-tip explants of *Baccaurea courtallensis*. The protocol of surface sterilization and callus induction of *Baccaurea courtallensis* opens new vistas that could facilitate *in vitro* plant regeneration and conservation of this endemic plant.

ACKNOWLEDGEMENTS

The authors are thankful to the Department of Biotechnology (DBT), Ministry of Science and Technology, New Delhi, India for providing financial assistance through a research project. Project No. BT/PR29631/FCB/125/13/2018 dt.25.02.2019. The Principal Investigator (author 2) and JRF (author 3) are also extending thanks to Tamil Nadu Forest department for providing permission (WL5 (A)/49087/2018 dated. 06/12/2018) to collect explants and carry out field visits.

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