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In vitro synthesis of berberine, a pharmacologically important isoquinoline alkaloid in callus cultures of *Tinospora cordifolia*

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ABSTRACT

Callus cultures were raised from young green leaf segments of the medicinal climber *Tinospora cordifolia* in Murashige and Skoog's media containing different growth regulator regime. The *in vitro* callus cultures were analysed for the accumulation of active principle through high-performance liquid chromatography. Quantitative analysis of berberine was achieved by thin-layer chromatography and UV-absorbance method. Callus cultures accumulated an appreciable amount of berberine. A maximum of 355 μg/g dry wt accumulation of berberine was noted when the callus was grown in the presence of 2 mg/l 2, 4-D and 0.1 mg/l BA. However, maximum callus growth was achieved in the presence of 0.5 mg/l BA and 2 mg/l 2,4-D. Maximum tissue growth didn't favour an increased accumulation of the berberine. Enhanced accumulation of berberine was found when callus growth was less and therefore may be associated with the stress faced by *in vitro* tissues. The experimental results indicate that it is desirable to adopt two different growth regulator regime - one for accelerated callus growth and another for enhanced *in vitro* synthesis of berberine. The *in vitro* cultures of *T. cordfiolia* also offers an ideal system for understanding the studies on elucidating alternate routes of berberine biosynthetic pathway as well as possible elite cells for scaling up of berberine.

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INTRODUCTION

Berberine is an isoquinoline alkaloid found in members of the plant families such as Menispermaceae and Berberidaceae. The alkaloid exhibits several pharmacological and biological effects (Galvez *et al.*, 2013). Berberine is accumulated in leaves, root, stem, flowers and fruit. Berberine is also the principal active principle of medicinal plants such as *Cosciniumfenestratum* (Narasimhan and Nair, 2004a,b), *Tinospora cordifolia* (Mohan *et al.*, 2017), *Berberis aristata* (Rathi *et al.*, 2013) and *Coptis Chinensis* (Brown *et al.*, 2008) . Berberine containing botanicals are used in the preparation of several ayurvedic medicines and other traditional practices (Neag *et al.*, 2018). As a result of overexploitation as well as the destructive collection, some of the berberine containing botanicals are facing a constant threat to their natural populations (Narasimhan *et al.*, 2006).

In vitro synthesis of secondary metabolites using plant cell and tissue cultures are one of the active areas of plant science research. Culturing cells offers a unique opportunity in multiple fields such as metabolic engineering (Sato *et al.*, 2001); biotransformation (Yamada and Okada, 1985); cell

cloning (Lawrence, 1981) in addition to *in vitro* synthesis and scaling up (Taticek *et al.*, 1991). Establishment of *in vitro* cultures provides a means to bypass the plant for its natural products (Mulabagal and Tsay, 2004).

MATERIALS AND METHODS

Explant preparation and sterilisation

Tinospora cordifolia grown in the greenhouse conditions served as the stock plant for the present study. Young green leaf segments from this stock plant were washed with running water for 25 minutes. Chemical sterilization was carried out with 0.1% $HgCl_2$ (w/v) solution for eight minutes. The sterilised explants were washed with sterile distilled water two times, and the ends were removed before inoculation.

Tissue culture media and incubation

In vitro cultures were raised in Murashige and Skoog's medium (Murashige and Skoog, 1962) containing different growth regulator regime of 2,4-D (2,4 Dichlorophenoxy acetic acid), NAA (Naphthalene acetic acid) and BA (Benzyl adenine). The media, glassware and the accessories required for inoculation were sterilised in an autoclave at 121^0C for 12 minutes at a pressure of 12 psi.

Extraction, identification and quantitative analysis of berberine

Callus tissue was harvested after 42 days of growth and washed well with double distilled water. The tissue was further dried in an oven at 35^0C for 24 hours, and the dry weight was noted (dry cell biomass). Extraction of berberine was achieved with the help of a soxhlet apparatus using methanol as the solvent; the concentrated extract of 5 ml served as the samples for analysis. Identification of berberine was carried out by HPLC analysis (Narasimhan and Nair, 2004a). For quantitative analysis, berberine was first separated through TLC using a silica layer of 0.5 mm thickness. The separation was done by using a mobile phase of butanol, acetic acid and water at a ratio of 7:1:1. The amount of berberine was calculated by UV-absorbance method (λ =345 nm) using a standard berberine chloride obtained from Sigma chemicals, USA. Experiments were conducted in three replications; the results were calculated as mean $\pm$ standard error. Duncan's multiple range test analysed the comparison of means.

RESULTS AND DISCUSSION

TLC exhibited better separation of berberine. The bands of berberine appeared as yellow spots which were fluorescent under UV illumination (Figure 1a, b). HPLC analysis confirmed the identity of the berberine (Figure 2). Callus culture exhibited appreciable growth in MS media augmented with plant growth regulators (Table 1). Maximum callus growth in terms of dry biomass was obtained when cultures were grown in the presence of 2mg/l 2,4-D and NAA or BA 0.5 mg/l. Growth regulators exhibited a significant effect on the *in vitro* biomass accumulation. A concentration of 0.1 mg/l BA in the presence of 2 mg/l 2,4-D elicited maximum accumulation of berberine (355 μg/g dry wt.) Cell suspension cultures of *Coscinium fenestratum* also exhibited a similar effect of growth regulators on berberine biosynthesis, but at a higher concentration of 4 mg/l NAA (Narasimhan and Nair, 2004a). The current result revealed that higher concentration of NAA or BA (0.5 mg/l) is inhibiting the accumulation of berberine in cultured tissues. However, *in vitro* cultures of *Thalictrum minus* exhibited a positive correlation of NAA with berberine accumulation (Nakagawa *et al.*, 1986). In an experiment, a beneficial effect of BA in enhancing berberine accumulation was revealed in cultured cells of *Thalictrum minus* (Hara *et al.*, 1993). However, in the current study, the effect of BA was more beneficial in inducing the berberine biosynthesis compared to NAA. According to Chintalwar *et al.* (2003); *in vitro* cultures raised from stem explants of *T. cordifolia* accumulates jatrorrhizine and berberine. Appreciable berberine production was also found using callus cultures raised from nodal explants (Pillai and Siril, 2020) and intermodal segments (Sharma, 2017).

Another important feature noted was the yellow colouration of the agar medium (Figure 1c) Similar feature was observed in callus and cell suspension cultures of *Coscinium fenestratum* and has been concluded that it is because of the release of berberine into the media (Narasimhan and Nair, 2004b). Therefore, the colouration of the media is most probably due to the release of the secondary metabolites from the cultured plant tissue.

In vitro experiments revealed that the optimum growth regulator regime for growth and berberine yield is different. This points out to the fact that a two-stage culture system. In this model, the first stage of the culture is to achieve an appreciable biomass culture the cells in growth regulator regime. The second staged is aimed at a maximum production of the metabolite. A similar two-stage

Table 1: Biomass and berberine yield from *in vitro* callus cultures of *T. cordifolia

Growth regulator regime in MS media (mg/l)			Callus	Berberine
2,4-D	BA	NAA	Dry wt. (mg)	(μg/g dry wt.)
	Control		44.67^{a} + 1.76	63.28^{a} + 7.35
2	-	0.1	93^{b} + 4.58	78.41^{a} + 4.94
2	-	0.3	46.33^{a} + 2.40	238.46^{b} + 36.56
2	-	0.5	107^{b} + 11.54	73.32^{a} + 10.24
2	0.1	-	77.66^{ab} + 17.54	355.04^{c} + 43.68
2	0.3	-	105.33^{b} + 27.05	220.83^{b} + 8.38
2	0.5	-	109.67^{b} + 12.45	124.02^{a} + 0.02

*Results are the mean of 3 replication expressed as mean ± Standard error. The same letters indicate that the results are not statistically different by Duncan's multiple range test.

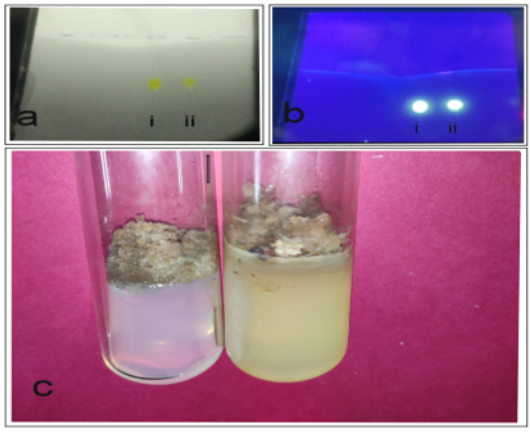

Figure 1: TLC profiling of callus culture of *T. cordifolia*. (a) Lane 1: Standard berberine 2: Methanol extract of callus – visible light (b) UV illumination (c) after 42 days of growth.

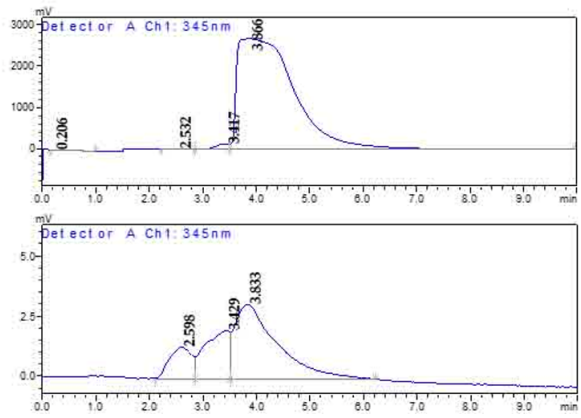

Figure 2: HPLC Profile of standard berberine (above) and methanol extract of callus culture (below). The retention time of 3.8 minutes indicates berberine.

culture system for enhanced secondary metabolite accumulation was found in the case of shikonin derivatives (Fujita *et al.*, 1981).

For successful industrial production of a secondary metabolite from cultured plant cells, the purification of metabolite must be economical and straightforward. Separation and purification of berberine can be easily achieved through TLC (Shu-Hua *et al.*, 1998). TLC is considered as the most economical method (Mahato *et al.*, 2019). Quantitative estimation of berberine can be done through UV absorbance method. This method is a reliable, cost-effective, rapid, sensitive and straightforward method (Khosa *et al.*, 2018). *In vitro* cultures of *T. cordifolia* as a source of berberine are of great potential, because of the limited availability of the traditional sources of berberine such as *Coptis* and *Coscinium* (Rama *et al.*, 2008). Further, the cultures may be utilised for understanding further mechanisms of biotransformation, biosynthesis and metabolic engineering in berberine biosynthesis. Based on these reasons, *in vitro* cultures of *T. cordifolia* can be considered as an ideal system for the source of berberine as well as further studies regarding berberine metabolism.

CONCLUSION

In vitro callus cultures of *T. cordifolia* accumulated a significant amount of berberine. Separation and quantification of the compound can be achieved through simple methods such as TLC and UV absorption. The conditions suitable for enhanced growth of the tissue did not favour berberine accumulation. Therefore, it is logical to adopt a two-stage culture system. Further, callus cultures of *T. cordifolia* offer an alternate source of berberine and maybe experimented further for identifying the biosynthetic pathway.

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Conflict of interest

The authors declare that they have no conflict of interest for this study.

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