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ORIGINAL RESEARCH PAPER

Two-stage culture for producing berberine by cell suspension and shoot cultures of *Berberis buxifolia* Lam

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Abstract In vitro cultures of *Berberis buxifolia* were established using thidiazuron (4.5, 23 and 45 mM) or picloram (4 and 40 mM) as plant growth regulators for sustaining growth. For producing berberine, a two-stage culture was performed. In the first step, thidiazuron or picloram were used for biomass production followed by the production stage where benzylaminopurine (4.4 mM) was added as a plant growth regulator. Berberine yields (102 mg g^{-1} DW) and in vitro shoot cultures (200 mg g^{-1} DW) were significantly lower than those of whole plants in the field (416 mg g^{-1} DW). The highest productivity (0.18 mg 1^{-1} day^{-1}) was attained using picloram (either 4 on 40 mM) in the first stage for producing biomass.

Keywords Berberine · *Berberis buxifolia* · Picloram · Thidiazuron

Introduction

Berberine is an isoquinoline alkaloid that is found in the roots, rhizome and stem bark of a number of medicinal plants (e.g. *Berberis vulgaris*, *B. aquifolium*, *B. aristata* and *Tinospora cordifolia* etc.). It has multiple pharmacological properties (Kulkarni and Dhir 2008; Zeng et al. 2003; Birdsall and Kelly 1997; Huang et al. 1991) and has potential as a drug for several pathologies (Kong et al. 2006; Lee et al. 2006; Yoo et al. 2006; Zhu and Qian 2006; Peng et al. 2004).

Berberis buxifolia (Berberidaceae), is a native Patagonian species with a wide geographical distribution. In Argentina alone, it can be found from the Patagonian woods to Tierra del Fuego, the southernmost tip of the country. Historically, this plant was used by indigenous people for medicinal purposes and as a source of dyes. The extracts of *B. buxifolia* have multiple medicinal applications. They are employed mainly for their antibacterial (Villinski et al. 2003) and antidiarrheal properties. However, there are references that mention its use as antispasmodic (Caraballo and Caraballo 2004) and anti-inflammatory. These activities, particularly the antibacterial one, have been attributed in part to the alkaloid, berberine, although the evidence suggests that other chemical substances present in the extracts could be acting synergically (Pitta-Alvarez et al. 2008).

In particular, *B. buxifolia* can be propagated by seeds and rhizomes. However, plants that produce berberine have an extremely slow growth rate. For example, in Asia, berberine is extracted from 5-to-6-year-old plants of *Coptis* sp. With this in mind, the development of in vitro cultures for the production of this valuable compound becomes a relevant issue.

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B. buxifolia is particularly attractive because it produces berberine in large amounts (Arena et al. 2000). However, the calluses obtained from explants of this species are often subject to oxidation processes that become an obstacle in the establishment of submerged cultures. Therefore, the steps prior to the development of liquid cultures have to be optimized. Cell suspension cultures are not as susceptible as calluses to early oxidation processes and death. In this paper, we report the establishment of cell suspension cultures of *B. buxifolia* using two plant growth regulators: picloram and thiadiruzon. To the best of our knowledge, this is the first time these regulators have been employed successfully in obtaining cell suspension cultures of a berberine-producing species. Furthermore, we also explore the development of a two stage culture process to improve berberine productivity both in immersed cultures as in in vitro shoot cultures.

Materials and methods

Plant material

Rhizomes and stems from 8-to-10-y-old *B. buxifolia* plants were employed. The plants were collected in the National Parks of Tierra del Fuego, Ushuaia, Argentina (54°48′ SL, 68°15′ WL) in the years 1998 and 2005. The explants were obtained from shoots that originated in the rhizomes. The nodal segments were cut, sterilized and used as the initial explants for in vitro culture.

Sterilization and culture conditions

The nodal segments were submerged in 10% (w/v) NaOCl with the addition of two drops of Tween 20 for 20 min then washed three times with distilled water in axenic conditions in a laminar flow. One centimeter explants were placed in tubes (20 × 120 mm) with MS medium (Murashige and Skoog 1962). Multiplication of plant material was done in LS medium (Linsmaier and Skoog 1965) supplemented with MEC vitamins (George et al. 1987) and 4.4 μM 6-benzylaminopurine (BA) with the addition of citric acid (100 mg/l) and ascorbic acid (100 mg/l) to prevent oxidation.

Callus induction

Calluses were induced using the nodal segments of the multiplied plants as explants. The following treatments were tested: (1) half-strength MS medium (1/2MS) containing MS vitamins, inositol 100 mg l^{-1}, thiamine 0.4 mg l^{-1}, sucrose 30 g l^{-1} and agar 8 g l^{-1}, supplemented with 36 μM 2,4-dichlorophenoxy-acetic acid (2-4D) and 0.9 μM BA; (2) the same basic medium described in (1) but supplemented with N^6-(2-isopentenyl) adenine (2iP) at either 5, 24 or 50 μM.

Establishment of cell suspension cultures

Three weeks after callus induction, friable calluses, obtained from cultures with 2iP, were subcultured into liquid growth medium (GM) that is 1/2MS medium containing MS vitamins, inositol 100 mg l^{-1}, thiamine 0.4 mg l^{-1} and sucrose 30 g l^{-1}. The following plant growth regulators (PGRs) were tested: (1) picloram (PIC, 4, 21 and 40 μM) and (2) thidiazuron (TDZ, 4.5, 23 and 45 μM). The subcultures were carried out in 120 ml Erlenmeyer flasks with 25 ml culture media in gyratory shakers at 120 rpm. (PIC is a herbicide that is used as an auxin substitute, whereas TDZ is a phenylurea derivative with cytokinin-like activity.)

In vitro shoot culture

Shoots containing three nodes, including leaves, were transferred to 80 ml Erlenmeyer flasks containing 10 ml MS liquid medium (GM) supplemented with one of the following PGRs: (1) 40 μM PIC; (2) 45 μM TDZ; (3) 2.5 μM jasmonic acid (JA). JA was tested for its role in plant development processes, for acting as a signal in numerous abiotic and biotic stress responses (Van Pozo et al. 2005), and also for eliciting the isoquinolone alkaloid in the berberine-producing species *Thalictrum tuberosum* Frick and Kutchan (1999). The shoots were incubated on an orbital shaker (100 rpm) at 24 ± 1°C and in darkness and maintained in these media for 3 months. Afterwards, they were employed in the berberine-producing stage.

Berberine production

After 16 days culture, cell suspension cultures or shoot cultures were subcultured to the production medium

(PM) that is MS liquid medium supplemented with 4.4 μM BA, in the absence of any other PGR, and incubated with a 16 h photoperiod as described above. In the case of cell suspension cultures samples were taken every 5 days and for shoot cultures the first samples were taken 10 days after subculture into PM and afterwards, samples were taken every 5 days. In all cases growth and berberine content in the biomass and liquid medium were determined.

Analytical methods

The biomass obtained in each assay was estimated by dry weight (DW) (calluses and cell suspension cultures) and fresh weight (FW) (plants). Berberine was determined according to the method described by Nakagawa et al. (1986). Alkaloid content was analyzed by HPLC with a C18 column (Alliance Waters 2690) using, as mobile phase, methanol/water (28:72 v/v) adjusted to pH 3.2 with acetic acid. Berberine was detected at 360 nm.

Culture conditions

The cultures were all incubated at 24 ± 1°C, under a 16 h photoperiod of cool white fluorescent light (57 μE m^{-2} s^{-1}) from TLT 110W/54 RS Philips day-light tubes.

Chemicals

Berberine · HCl, all the PGRs and the media components were purchased from Sigma. The solvents used for HPLC analysis were purchased from Merck.

Statistical analysis

Significance of treatment effects were determined by using analysis of variance. Variations among treatments were analyzed by using Tukey's procedure ($P = 0.05$).

Results and discussion

Establishment of shoot cultures of *B. buxifolia*

The establishment of axenic cultures of plants of *B. buxifolia* had low rates of contamination (less than 20%). After 6 months of culture, shoot multiplication was carried out by culturing nodal segments on LS medium with BA as described in Material and methods. With BA the multiplication rate, shoot growth and the number of leaves and their expansion increased dramatically compared with the use of 2iP as PGR. However, a progressive browning of the shoots was observed, particularly in the basal section, which ultimately led to their death. This is in agreement with previous work done with the same species (Arena et al. 2000) and is probably due to oxidation and production of phenolic compounds. The reasons for this oxidation can be multiple. In vitro cultures itself produces stress and this could be one of the causes. The PGRs used or even the berberine produced and secreted into the medium could be involved. In this respect, Jayakumaran et al. (1992) proposed that in media with high PGR concentrations, the production and release of berberine into the culture media could result in browning. However, Figueiredo et al. (2000), who observed a similar phenomenon in cell suspension cultures of *Rollinia mucosa* with 2,4-D, suggested that the PGR itself could be involved in the formation of phenols. To overcome this problem, a combination of two antioxidants, ascorbic acid and citric acid, was added to the media. It was observed that this delayed the onset of browning.

Callus induction

Callus was induced using as explants internode segments derived from shoots in the multiplication stage before browning was observed. The medium composed of ½MS with the addition of 2,4-D and BA has been described in the literature for the induction of callus from *Coptis* sp. and *Thalictrum* sp. (Nakagawa et al. 1986). In our case, this combination resulted in the death of the explants. The ½MS media containing three different concentrations of 2iP induced the formation of callus after 4 weeks. However, the callus became compact and dark after 1 month culture. The browning process was similar to that observed in the multiplication step, and the same hypotheses discussed earlier could be applied.

Establishment of suspension cultures and production of berberine

Berberine biosynthesis in *T. minus* var. *hypoleucum* cell suspension cultures can be induced by cytokinins

(Hara et al. 1993, 1994, 1995). On the other hand, when cultured in growth media containing auxins, such as 2,4-D, berberine production in this species is strongly suppressed. With this in mind, a strategy was developed to try to circumvent the problems arising from the possible oxidation processes that took place in these cultures while at the same time allowing the production of berberine. To this end, the cells were subcultured, prior to their browning, to ½MS medium with the addition of BA (4.4 μM). Under these conditions, the cells produced berberine and secreted it into the medium, where the yellow pigmentation typical of this alkaloid was observed. Berberine in the biomass was found in traces (data not shown). However, after only 1 week culture the browning of the cultures was observed and eventually they died. This is probably due to the fact that, in *B. buxifolia*, BA is not favorable to growth as it is to berberine production. This is in contrast to the findings of Hara et al. (1994) who observed that in *T. minus* cell cultures BA induced the synthesis of berberine but was not detrimental to growth. In *B. buxifolia*, the results suggest that the cell cultures could be entering stationary phase at an early stage resulting in browning events and premature death. In addition, BA could not only be inducing berberine production but also other biosynthetic pathways leading to phenolic compounds. In this respect, Frick and Kutchan (1999) suggested that some biosynthetic enzymes could be common to both phenylpropanoid and alkaloid anabolism.

Finally, we performed a two-stage process, a first step for producing biomass and then cultures were transferred to berberine production media. In this case for establishing cell suspension cultures incipient friable and yellow calluses were used as inoculum in liquid culture medium with different concentrations of PIC and TDZ (Materials and methods). The immersed cultures were initiated prior to the browning process described above. Based on the GIs of the suspension cultures, six combinations of 2iP with either PIC or TDZ were chosen. Table 1 shows the GIs achieved by the different combinations of PGRs chosen. The maximum GI was obtained when the callus was induced by 24 μM of 2iP and then grown in fresh liquid medium with the addition of 40 μM PIC. On the contrary, the three treatments with TDZ displayed no major differences based on the previous treatment with 2iP.

For the production stage, suspension cell cultures were transferred to ½MS liquid medium supplemented with BA (4.4 μM) to induce the production of berberine. The cell lines obtained with all the concentrations of TDZ produced approximately the same amount of berberine (data not shown). However, when PIC was use at the maximum concentration, the levels of berberine were similar to the ones obtained with TDZ (Fig. 1). As shown in Fig. 1, the addition of BA induced the production of berberine immediately. However, at the same time, the cultures diminished their growth rate and entered the stationary phase. Browning was not observed until approx. day 30 (1 week after BA treatment), suggesting there was growth stagnation.

Berberine production was stimulated by this strategy in D3, D4, D5 and D6 medium and the synthesis of the metabolite did not cease even when growth of suspension cultures ceased. The highest berberine yield was 102 mg g^{-1} DW reached in medium D3 where 40 μM PIC was used as PGR in the biomass production step. Productivity attained its highest amounts when PIC (4 and 40 μM) and TDZ

Table 1 Influence on *B. buxifolie* cell suspension growth index (g $days^{-1}$) and berberine yield (mg l^{-1}) of different plant growth regulation used for calli induction and cell suspension biomass production

Media	Plant growth regulator			Growth index (g day^{-1})	Berberine yield (mg l^{-1})
	Calli induction step (μM)	Biomass production step (μM)			
	2iP	PIC	TDZ		
D1	5	4	0	0.003	0.21
D2	24	4	0	0.005	0.22
D3	24	40	0	0.006	0.21
D4	1	0	23	0.004	0.21
D5	1	0	45	0.004	0.21
D6	5	0	4.5	0.005	0.22

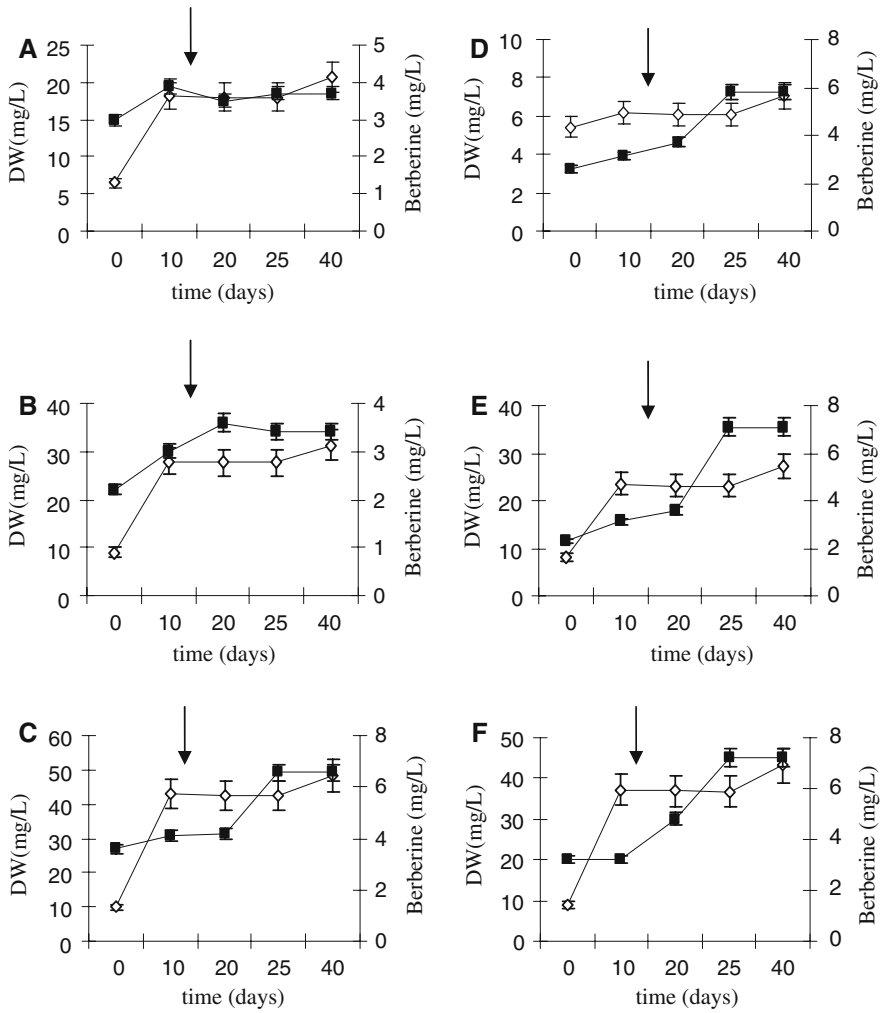

Fig. 1 Growth (■) and berberine production (◇) in cell suspension cultures of *Berberis buxifolia*. Cell suspension cultures were performed in two steps: the first with PIC (4, 21, 40 μM) or TDZ (4.5, 23, 45 μM) as PGR for biomass production. After 16 days, cultures were transferred to the second step for berberine production medium (*arrow*) with BA (4.4 μM) as PGR as was described in Materials and methods and Table 1. **a** Callus induction in 2iP 5 μM, biomass production step in PIC 4 μM. **b** Callus induction in 2iP 24 μM, biomass production step in PIC 4 μM. **c** Callus induction in 2iP 24 μM, biomass production step in PIC 40 μM. **d** Callus induction in 2iP 1 μM, biomass production step in TDZ 23 μM. **e** Callus induction in 2iP 1 μM, biomass production step in TDZ 45 μM. **f** Callus induction in 2iP 5 μM, biomass production step in TDZ 4.5 μM

(23 μM) were the PGR used for the biomass production step (Table 2). No tissue browning was shown in this strategy.

In vitro shoot cultures

Secondary plant metabolites usually reach higher amounts in organized tissues (Payne et al. 1991). We established in vitro shoot cultures for studying their performance for producing berberine. No significant differences in growth were found with the different PGR used. In all the cases, the maximal biomass attained before transferring to PM was of approximately 600 mg l^{-1} (Fig. 2a). On the other hand, when shoots were transferred to PM medium the onset of berberine production coincided with a progressive decrease of

Table 2 Growth and berberine production by *B. buxifolia* cell suspension cultures developed in culture media described in Table 1

Medium	TD (days)	Final biomass (g l^{-1})	Berberine concentration (mg l^{-1})	Berberine yield (mg g DW^{-1})	Productivity (mg l^{-1} day^{-1})
D1	4.88	0.27	7	26	0.18
D2	4.91	0.43	5	13	0.15
D3	4.78	0.07	7	102	0.18
D4	5.37	0.48	6	13	0.16
D5	6.73	0.20	3	17	0.09
D6	6.13	0.31	3	10	0.09

Period of culture: 30 days. Each value is a mean of three replicates. *TD* time of duplication

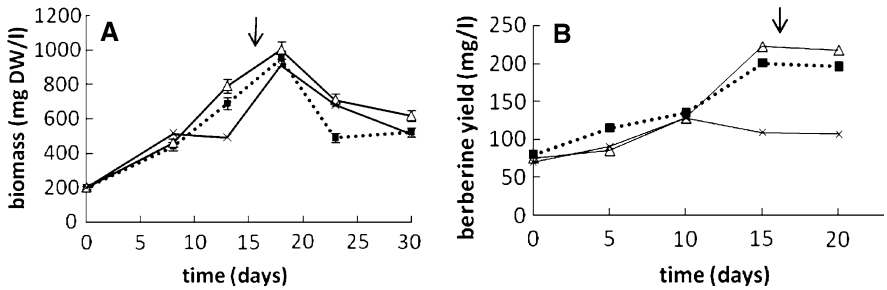

Fig. 2 **A** Growth of in vitro shoot cultures of *Berberis buxifolia*; **B** Berberine production. Cultures were performed in two steps: the first with TDZ (45 μM) (■), PIC (40 μM) (Δ) or jasmonic acid (2.5 μM) (×) as PGR for biomass production. After 16 days, cultures were transferred to berberine production medium with BA (4.4 μM) as PGR as was described in Materials and methods (*arrow*)

growth rate (Fig. 2a, b). Pretreatment with TDZ and PIC yielded the best results but JA was not as effective. The productivity attained in shoots at the end of the culture was 8.7 mg l^{-1} day^{-1} (TDZ), 9.7 mg l^{-1} day (PIC) and 4.7 mg l^{-1} day (JA). A possible explanation for the improved berberine yield in cultures pre-treated with TDZ is that this compound has cytokinin-like activity and could possibly begin to induce berberine production even before BA is added. The effects caused by pre-treatment with PIC and JA, however, are not entirely clear. In every case, practically all the berberine was in the culture medium and not in the biomass.

Conclusions

Cell suspension cultures and in vitro shoot cultures of *B. buxifolia* using PIC and TDZ as PGR for sustaining growth. To the best of our knowledge, this is the first time these PGRs have been used to this purpose in this species. In addition, the induction of berberine synthesis by BA was confirmed when *B. buxifolia* suspension cultures were transferred to PM medium. Yield values were significantly lower in cell suspension cultures (102 mg g^{-1} DW) and shoots (200 mg g^{-1} DW) regarding to that of the whole plant in the field (416 mg g^{-1} DW). Nevertheless, our results were valuable because of the possibility of establishing *B. buxifolia* cell suspension and shoot cultures capable of producing berberine. Furthermore, berberine is secreted into the medium practically in its entirety which is advantageous for conducting processes in bioreactors. Further studies must be conducted in order to analyze the possibility of optimizing and scaling up both kinds of in vitro cultures. On the other hand, we have already demonstrated the antimicrobial activity of the extracts of *B. buxifolia* in vitro cultures (Pitta-Alvarez et al. 2008). Since the agronomic culture of *B. buxifolia* has not been achieved our work appeared as a promising alternative for berberine production.

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