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Effect of the Medium pH on the Release of Secondary Metabolites from Roots of *Datura stramonium*, *Catharanthus roseus*, and *Tagetes patula* Cultured In Vitro

L. A. SÁENZ-CARBONELL, I. E. MALDONADO-MENDOZA, O. MORENO-VALENZUELA, R. CIAU-UITZ, M. LÓPEZ-MEYER, C. OROPEZA, AND V. M. LOYOLA-VARGAS*

Departamento de Biotecnologia, Div. de Biologia Vegetal, Centro de Investigación Cientifica de Yucatàn. Apdo. Postal 87, 97310, Cordemex, Yucatàn, Mèxico

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ABSTRACT

The release of alkaloids from root cultures *Datura stramonium* and *Catharanthus roseus*, and thiophenes from root cultures of *Tagetes patula* was found to increase when the pH of the culture media (ranging from 4.8 to 7.0) was reduced to 3.5. The extent of the effect was different in each type of culture. Increases ranged from 4- to 20-fold, which in some cases accounted for 75% of the total secondary metabolite pool produced per flask. When the release of individual metabolites was measured, even larger increases were observed (nearly 400-fold for ajmalicine). Increased release of alkaloids from *C. roseus* roots were also observed in cultures growing in a 14-L fermentor, when the medium pH was reduced. Reduction of the pH of the media did not affect growth of the root cultures in subsequent subcultures. The importance of this treatment as a stategy to improve the recovery of secondary metabolites from producing cultures is discussed.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: *Datura stramonium; Catharanthus roseus; Tagetes patula;* pH; root cultures; secondary metabolite release.

INTRODUCTION

Plants contain a large variety of secondary metabolites. Many of them are economically important and there is interest in trying to obtain in vitro tissue cultures of producing species able to accumulate the secondary metabolites of interest. There are many reports on cultures able to form secondary metabolites in vitro (*1,2*) but from a biotechnological point of view it is not only important to produce them in good yield but it would be very advantageous to recover them easily without destroying the culture. One way would be to recover the metabolites from the culture medium rather than from the cultured tissues or cells. Occurrence of secondary metabolites in the culture medium of producing species has been reported (*3,4*) but in most cases the largest proportion of the metabolites is in the cells and not in the culture medium. Therefore, efforts have been made to try to improve the release of secondary metabolites into the culture medium. Increased release has been obtained by using solvents (*5*), detergents (*6*), and resins (*7*). However, cell viability is usually affected with these treatments. The effect of reducing the pH of the medium has also been found to be effective on the release of alkaloids from *C. roseus* immobilized cell cultures (*8*), but it is not known whether this treatment affects the viability of the cultures. This paper reports the effect of acid pH on the release of secondary metabolites and culture viability of in vitro cultures of roots of two alkaloid-producing species and a thiophene-producing species.

MATERIALS AND METHODS

Plant Material

Hairy root cultures of *Datura stramonium* were obtained with *Agrobacterium rhizogenes* strain TR-105 (Maldonado-Mendoza et al., submitted). Transformed roots of *Tagetes patula* were obtained from *A. tumefaciens* LBA 4210 (Cit^-). Hairy root lines A2 and J1 of *C. roseus* were obtained with *A. rhizogenes* 1855 and 1855/pBin 19 GUS strains (kindly donated by H. Flores, and J. Hamill and F. Damiani) respectively.

The culture medium for *D. stramonium* normal and hairy root lines was B_5 (*9*). *C. roseus* transformed lines were cultured in B_5 medium at half strength. *T. patula* transformed roots were cultured in MS medium (*10*). All media were supplemented with 30 g/L sucrose, and the pH was adjusted prior to autoclaving of the medium to 5.7 with 0.1*N* KOH/HCl.

One hundred mL of medium were placed in a 250-mL Erlenmeyer flask and autoclaved for 20 min at 15 psi. Flasks were inoculated with 0.5 g (FW) of *D. stramonium* and *C. roseus* roots and 1 g of *T. patula* roots. Cultures were grown in darkness at 25°C and 100 rpm on a rotatory shaker. Normal roots of *D. stramonium* were grown in the same conditions except that the B5 medium was supplemented with 10 μM of indolbutyric acid (IBA).

Low pH Treatment

Root cultures were allow to reach the stationary or linear growth phase. This is indicated in each figure. At this point, the pH of the culture medium was adjusted to pH 3.5 using filter-sterilized (Millipore, Bedford, MA, 0.22 μm) 1*N* HCl.

Secondary Compounds Quantitation

Hyoscyamine and scopolamine in *D. stramonium*, total alkaloids, ajmalicine, and catharanthine in *C. roseus* were quantified as reported previously by Monforte-González et al (*11*). Total thiophenes were quantified as reported previously by Downum and Towers (*12*). Coumarines were quantified from the medium according to Davis and Hahlbrock (*13*).

Results

The effect of acidic external pH on the release of secondary metabolites was studied in root cultures of three different species: *C. roseus*, *D. stramonium*, and *T. patula*. The usual medium pH in control flasks of each of the three types of cultures at the time of treatment was always above 5.8 (Fig. 1A, B, and C) and was reduced to 3.5 during treatment. Growth of the cultures of the three species did not show any difference when subculturing after treatment (data not shown).

HAIRY ROOT FLASK CULTURES OF *C. roseus*

Two *C. roseus* hairy root lines (J1, A2) were used in these studies. The external pH of the cultures was reduced to 3.5 on day 15 and 21 respectively of the growth cycle, which corresponds to the linear phase. In response, the pH of the extracellular medium of the J1 cultures increased up to pH 3. Adjustment of the medium pH was required for these cultures every 12 h during the 5 d treatment. In contrast, A2 cultures did not show any response to the acidification of the medium and no further adjustment of the external pH was required.

As a result of acidification, alkaloid concentration in the medium of J1 cultures increased 16-fold after 4–5 d of treatment (Fig. 2A). The alkaloid

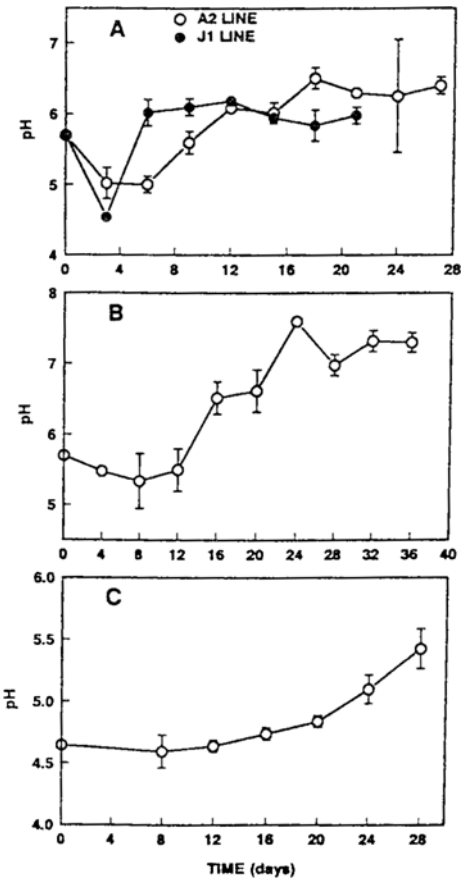

Fig. 1. Normal time course of pH of culture medium of each root culture studied in this work during a culture cycle. (A) hairy root cultures of *Catharanthus roseus;* (B) hairy root cultures of *Datura stramonium;* (C) transformed root cultures of *Tagetes patula.*

content in the tissues fell simultaneously (Fig. 2B). Total alkaloid production per flask showed very little change (Fig. 2C). Growth decreased as a result of treatment (Fig. 2D).

In A2 cultures acidification also increased alkaloid release about 9-fold (Fig. 3), but greater release occurred at the beginning of the treatment than at the end. Alkaloid content of the tissue fell at the beginning and returned to normal levels at the end of the treatment (Fig. 3B). In these cultures total alkaloid production per flask also increased with the treatment (Fig. 3C). Growth decreased in treated cultures (Fig. 3D).

The effect of acidification on the release of individual alkaloids was also studied. In J1 cultures, ajmalicine release increased 400-fold at the end of the treatment (Fig. 4A), whereas ajmalicine content in the tissues showed a comparatively smaller increase that took place at the beginning

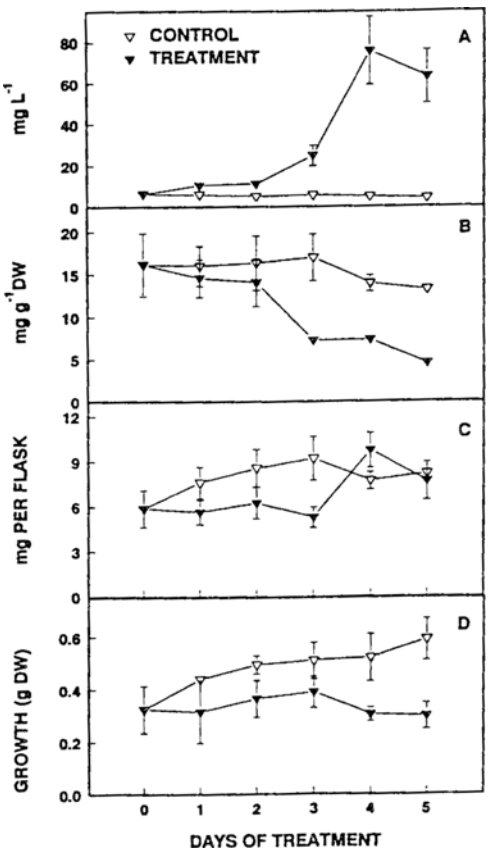

Fig. 2. Effect of pH in J1 line in hairy roots of C. *roseus*. (A) alkaloid release into the medium; (B) alkaloid in tissue; (C) total alkaloid production; (D) effect on growth. The treatment was applied at day 15 of the culture cycle.

of the treatment (Fig. 4B). Total ajmalicine content per flask also increased with the treatment (Fig. 4C). In A2 cultures ajmalicine release also increased (Table 1). In both J1 and A2 cultures the release of another alkaloid, catharanthine, was also found to increase with treatment (Table 1).

Hairy Root Fermentor Cultures of *C. roseus*

The effect of acid pH on alkaloid release was also studied in C. *roseus* J1 cultures grown in a 14-L fermentor. The medium pH was reduced to 3.5 on day 33 of culture and readjusted daily. As in the case of flask cultures, total alkaloid release and ajmalicine release increased several fold (Fig. 5A, B). The medium showed fluorescence owing to the presence of coumarines. The concentration of these secondary metabolites also increased as a result of acidification of the medium (Table 1).

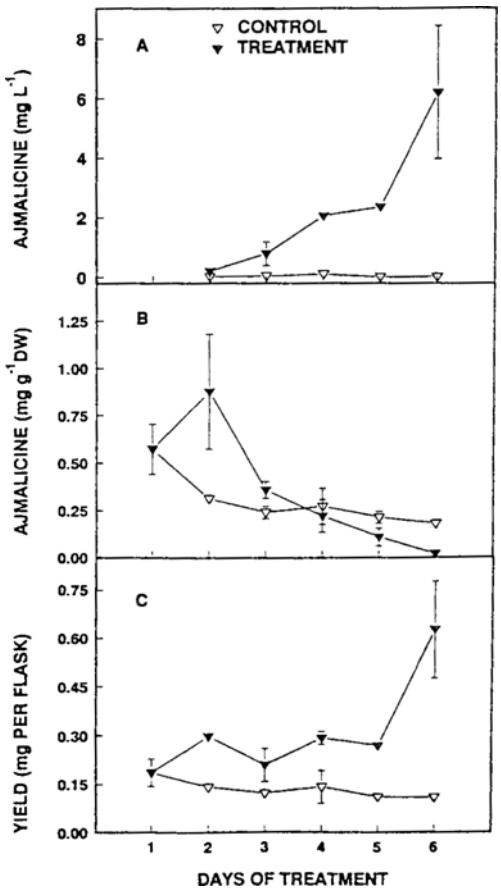

Fig. 3. Effect of pH treatment in A2 line of hairy roots of *C. roseus*. (A) alkaloid release into the culture medium; (B) alkaloid in tissue; (C) total alkaloid production; (D) effect on growth. The treatment was applied at day 20 of the culture cycle.

Root Cultures of *D. stramonium*

Two root culture lines were used; a transformed line and a normal line. The cultures were allowed to reach an early stationary phase of growth (24 d after inoculation) before the pH of the culture medium was lowered to 3.5. Since the cultures responded to increasing the medium pH (pH 1-2), it was readjusted to 3.5 every 12 h for 5 d. In both lines, acidification increased the release of alkaloids (Table 1). However, the increase in transformed cultures was greater than in normal cultures, 20 and 4.4 times respectively. Alkaloid yield was also increased about 4 times by the treatment. In regard to individual alkaloids, release of scopolamine increased more than hyoscyamine in transformed cultures whereas the opposite was

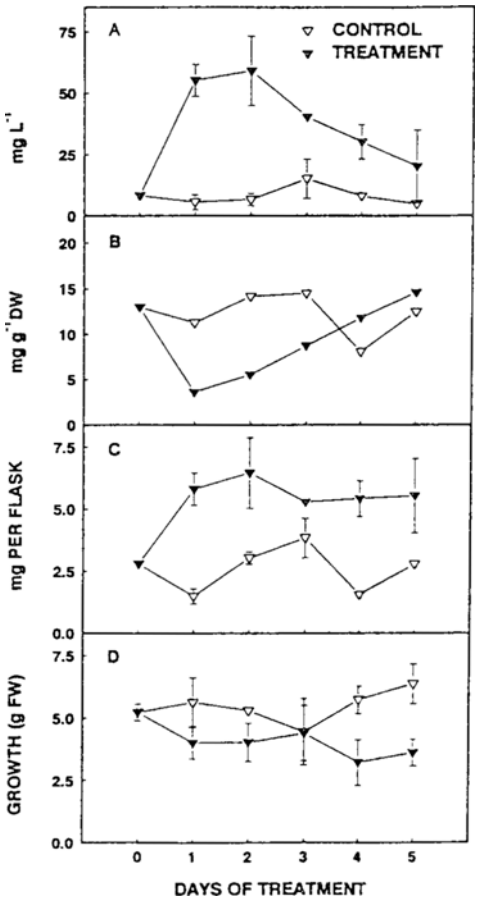

Fig. 4. Effect of pH treatment over ajmalicine release into the culture medium (A) ajmalicine content of tissue (B), and total ajmalicine production (C) in J1 line of hairy roots of *C. roseus*. The treatment was applied at day 15 of the culture cycle.

observed in normal cultures. In transformed cultures all the scopolamine was recovered from the medium during treatment, whereas in the case of hyoscyamine only half was recovered. In normal cultures similar patterns were observed for both alkaloids.

Root Cultures of *T. patula*

Transformed root cultures of *T. patula* were allowed to reach the stationary phase of growth before the pH of the medium was reduced to 3.5. The treatment promoted an eight-fold increase in the release of thiophenes (Table 1), which represents 0.5% of the total thiophense in the flasks.

Table 1
Excretion Levels of Different Groups
of Secondary Metabolites in Root Cultures of Different Species

			Control		Treatment		
Species	Line	Compound	mg/L	% of total	mg/L	% of total	Folds of increase
D. stramonium		Scop + hyosc	1.540	13	31.58	70	20.5
	TR	Scopolamine	0.49	11	14.87	100	30.3
		Hyosciamine	1.05	14	16.71	56.6	15.9
		Scop + hyosc	3.79	9.6	16.61	36.6	4.38
	Normal	Scopolamine	3.58	15	11.89	19	3.32
		Hyosciamine	0.21	1.65	4.72	15	22
C. roseus		Total alkaloids	4.82	6.24	75.76	75	15.7
	TR J1	Ajmalicine	0.016	0.77	6.18	98	386.2
		Catharanthine	0.064	5.55	1.94	100	30.3
		Total alkaloids	6.67	13.96	59.12	88.43	8.8
	TR A2	Ajmalicine	ND	*	9.7	100	*
		Catharanthine	ND	*	20.44	100	*
		Total alkaloids	9.65	*	30.14	*	3.12
	Ferm	Ajmalicine	ND	ND	1.06	100	*
		Catharanthine	6.99	*	22.7	*	3.24
T. patula	TR	Total Tiophenes	0.006	0.032	0.045	0.52	8.1

HR: hairy root line; NOR: normal root cultures. (T), (J1), (A2) are the lines analyzed in this work; Ferm: J1 line culture in a 14-L fermenter; ND: not detected; *calculations that could not be done. For *Tagetes patula* transformed root cultures, the pH treatment was applied at day 20 of the culture cycle.

DISCUSSION

Plant tissue cultures are able to accumulate secondary metabolites, however, these are often withheld within the cells. If plant tissue cultures are to be used routinely for the commercial production of secondary metabolites, they must secrete the targeted metabolites into the extracellular medium. Several approaches have been reported. In the particular case of indole alkaloids, reduction of the extracellular pH has been shown to increase their release in *C. roseus* immobilized cell cultures (*8*). In this study, this approach was tested for the release of secondary metabolites from root cultures of two alkaloid-producing species, *C. roseus* (indole alkaloids), and *D. stramonium* (tropane alkaloids), and the thiophene-producing species *T. patula*. The results showed that in all cases the release of secondary metabolites was increased when the medium pH was adjusted to 3.5 (without affecting growth in subsequent subcultures). Furthermore, the release of coumarines, another type of secondary metabolites present in *C. roseus* cultures, also increased with this treatment. In the case of tro-

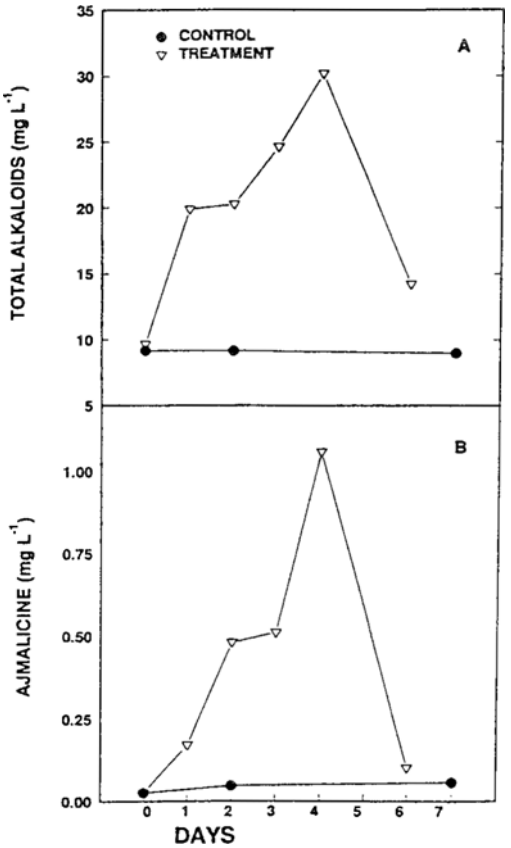

Fig. 5. Effect of pH treatment over total alkaloid (A) and ajmalicine (B) excretion in J1 line of hairy roots of *C. roseus* cultured in a 14-L fermentor. The treatment was applied at day 33 of the culture cycle.

pane alkaloids, the results contrast with those of Payne et al. (*14*) showing that acidification (pH 4.0) was found to have no effect on their release. This difference might be explained by the difference in medium pH, which was more acidic in the present study. There are no previous reports dealing with the effect of acidification on the release of alkaloids from *C. roseus* root cultures or on the release of thiophenes or coumarines.

The extent of the increase was not the same in all cultures. In *D. stramonium* cultures, alkaloid release was four times greater in the transformed root line than in the normal root line. In the *C. roseus* transformed root cultures, alkaloid release was two times greater in one line than in the other. The greatest increase was observed in the *D. stramonium* transformed root line (twenty-fold) and the lowest in the *T. patula* tranformed root line (eight-fold, which represents only 0.5% of the total). But even with these differences, the results show that medium acidification is promising as a general

approach for improving the recovery of secondary metabolites from in vitro cultures. Furthermore, when individual alkaloids were assessed, the treatment affected their release differently from the way it affected the release of total alkaloids. For instance, in the case of ajmalicine in *C. roseus* line J1, release increased nearly 400 times as opposed to only 16 times for total alkaloid production. In addition, acidification also promoted a several fold increase in alkaloid yield in two of the lines studied (*C. roseus* A2 and *D. stramonium* transformed line). The beneficial effect of acidification on secondary metabolites release was also confirmed in a 14-L fermentor.

Increased release of alkaloids after acidification of the medium has been explained by the formation of a pH gradient that favors ion trapping extracellularly (*15*). Alkaloids would be translocated through the plasma membrane in their uncharged lipophilic form and the charged form would be accumulated in the acidic medium. The results reported here on alkaloid release support this idea. However, these results also show an increased release of compounds that lack basicity, coumarines, and thiophenes. Although this does not necessarily preclude the ion trap mechanism for alkaloids, it suggests the occurrence of an alternative mechanism(s).

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