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ORIGINAL RESEARCH PAPER

Yeast extract induction of sanguinarine biosynthesis is partially dependent on the octadecanoic acid pathway in cell cultures of *Argemone mexicana* L., the Mexican poppy

Cecilia Guízar-González · Miriam Monforte-González · Felipe Vázquez-Flota

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Abstract
Objective To analyze the involvement of the octadecanoic (OCDA) pathway in the accumulation of sanguinarine induced by yeast extract (YE) in cell suspension cultures of *Argemone mexicana* (Papaveraceae).
Results Exposure to YE promoted sanguinarine accumulation. This was not observed when they were exposed to methyl jasmonate (MeJa). Use of diethyldithiocarbamic acid (DIECA), an inhibitor of the OCDA pathway, resulted in partial impairment of this response. Exogenous application of MeJa did not reverse this effect in DIECA-exposed cultures. qRT-PCR revealed that the accumulation of transcripts corresponding to the berberine bridge enzyme gene, which was induced by YE exposure, was blocked by OCDA pathway and reversed by exogenous MeJa. Interestingly, this response pattern could not be observed on dihydrobenzophenanthridine oxidase enzyme activity, which was promoted by YE, but unaffected by either OCDA or MeJa.
Conclusion Results suggest partial involvement of OCDA pathway in this response.

Keywords *Argemone mexicana* · Benzophenanthridines · OCDA pathway · Sanguinarine

Introduction

Argemone mexicana (Papaveraceae) produces different benzylisoquinoline alkaloids (BIA's), derived from tyrosine. The main *Argemone* alkaloids are the benzophenanthridines sanguinarine, dihydrosanguinarine (DHSA) and the berberine, a protoberberine. In mature plants sanguinarine and DHSA are restricted to the roots and mature seeds, whereas berberine is distributed throughout the plant tissues (Rubio-Piña and Vázquez-Flota 2013). In Mexico, this plant is collected from the wild for the elaboration of herbicides and pesticides to be used in organic agriculture. However, wide variations in alkaloid contents often occur (Rubio-Piña and Vázquez-Flota 2013). Although these differences could be accounted for the genetic composition, the effects of environmental conditions

Cecilia Guízar-González and Miriam Monforte-González have contributed equally to this work.

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C. Guízar-González · M. Monforte-González · F. Vázquez-Flota (✉)
Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43, No. 130 Chuburna, 97205 Mérida, Yucatán, Mexico
e-mail: felipe@cicy.mx

C. Guízar-González
e-mail: sshecy@hotmail.com

M. Monforte-González
e-mail: mmg@cicy.mx

on the expression of genes involved in secondary metabolism are well known (Hartmann 2007). In this way, understanding the mechanisms underlying such effects may lead to the development of strategies to control them. The use of cell culture approaches has been proved to be a valuable tool in that direction (Vázquez-Flota et al. 2012). Over the years, a number of in vitro cell lines derived from different tissues have been generated in our laboratory. In all cases, the ability to produce berberine was lost in the undifferentiated cultures, but they were able to accumulate sanguinarine and DHSA (Trujillo-Villanueva et al. 2010, 2012; Vázquez-Flota et al. 2012). One of these cell lines, named AmMiF, accumulates significant amounts of sanguinarine without any induction treatment (Guízar-González et al. 2012). In fact, sanguinarine amounts in this cell line are comparable to those in the whole plant on a dry matter basis. This trait differs from other sanguinarine producing in vitro cultures, such as *Eschscholzia californica*, *Papaver somniferum* and *Sanguinaria canadensis* (Archambault et al. 1996; Ignatov et al. 1999; Weiss et al. 2006), which require exposure to inducers of secondary metabolism in order to accumulate significant amounts of sanguinarine. However, upon exposure to chemical elicitors, such as methyl jasmonate (MeJa) or yeast extract (YE), AmMiF only displayed a limited response. Although such response increased when these same elicitors were sequentially applied, its magnitude remained lower than the one observed in other sanguinarine producing species (one *vs* two or three magnitude orders; Trujillo-Villanueva et al. 2010; 2012). DHSA corresponds to the reduced form of sanguinarine and, interestingly, the AmMiF cell line interconverted both forms during a 21-day culture cycle in a developmentally related fashion (Guízar-González et al. 2012). This conversion of sanguinarine into DHSA also occurred in elicited cultures, since an increase in the accumulation of one occurred concomitantly with a decrease in the other (Trujillo-Villanueva et al. 2010, 2012). DHSA was detected at the cell periphery in contrast to sanguinarine, which remained around the nucleus (Trujillo-Villanueva et al. 2012). Such feature is part of a detoxification mechanism, which involves the reduction of sanguinarine to the less toxic dihydro form, in association to a vesicle-mediated excretion system that eliminates alkaloid excess (Weiss et al. 2006).

In here, we show that blocking the octadecanoic (OCDA) pathway, through the use of biosynthetic inhibitors, did not totally annul the response of *A. mexicana* AmMiF cell line to YE. Moreover, the exogenous supply of MeJa to these cultures did not restore the full response, suggesting the operation of two different signalling routes for the synthesis of sanguinarine in this cell line, one of them independent from OCDA products.

Materials and methods

Biological material and elicitation treatments

Argemone mexicana AmMiF cell line was obtained from leaves and has been maintained by biweekly subcultures in PC-BN media (i.e. Phillips and Collins inorganic salts and vitamins, supplemented with 0.5 and 1.5 mg l^{-1} BAP and NAA, respectively and 20 sucrose g l^{-1} at pH 5.8; Vázquez-Flota et al. 2012). Five ml of a 14-day old suspension (representing between 0.20 and 0.22 g FW ml^{-1}) were transferred to 250 ml Erlenmeyer flasks, containing 50 ml of fresh media and kept under continuous illumination and shaking for 10 days. Cultures were induced by exposure to methyl jasmonate (MeJa) or yeast extract (YE). Doses for the time course experiments were set after assaying different concentrations for each elicitor at 24 and 48 h (Table 1). Once the optimal doses were selected, elicitors were assayed during a 72 h time course, collecting samples at the times shown in the Figures. Elicitors were diluted in 10 % ethanol (MeJa) or water (YE) and sterilized by filtration prior to application to the cultures. Control cultures were mock induced with either water or ethanol.

Inhibition of jasmonate formation

The formation of endogenous jasmonate by the OCDA pathway was inhibited by preincubating 10-day old cultures with 100 µM diethyldithiocarbamic acid (DIECA) (Menke et al. 1999) before induction. Controls received equivalent volumes of water, mocking either the induction or inhibition process.

Analytical procedures

Alkaloids were extracted from freeze-dried tissues and spent media with 0.5 % HCl in methanol and

Table 1 Sanguinarine contents (mg g^{-1} DW) in *A. mexicana* AmMiF cell line exposed to methyl jasmonate (MeJa) or yeast extract (YE)

MeJa (μM)	Sanguinarine (mg g^{-1} DW) Time (h)		YE (mg l^{-1})	Sanguinarine (mg g^{-1} DW) Time (h)	
	24	48		24	48
0*	1.06 ± 0.13	0.97 ± 0.12	0*	1.12 ± 0.14	1.13 ± 0.1
50	1.10 ± 0.09	1.04 ± 0.08	50	1.43 ± 0.12	1.32 ± 0.22
100	1.87 ± 0.11	2.05 ± 0.14	100	1.66 ± 0.09	1.89 ± 0.17
150	1.84 ± 0.08	0.59 ± 0.13	200	3.05 ± 0.21	5.39 ± 0.36
200	0.53 ± 0.12	ND	300	2 ± 0.32	ND

Average of triplicates with standard deviation. *ND* not determined

* Cultures were mock-induced with either 200 μl 10 % ethanol (MeJa) or deionized water (YE)

quantified by in situ fluorescence after TLC separation (Monforte-González et al. 2012) in a Camag TLC Scanner 4, controlled by the winCATS Planar Chromatography Manager (1.4.10). Relative abundance of *BBE* transcripts was estimated by qRT-PCR, using 5′-CCGTATCGATTCTCGGTGGT-3′ and 5′-ACCC TTACGATGAGGAAACGG-3′ as forward and re verse primers, respectively and according to standard procedures (Simon 2003). Dihydrobenzophenanthridine oxidase enzyme (DBO) activity was determined as described before (Ignatov et al. 1999). Briefly, desalted crude protein extracts (ca. 15 mg of total soluble protein) were incubated in 100 mM phosphate buffer, pH 7.0 at 37 °C. The reaction was initiated by adding 20 μM DHSA and stopped after incubation of 20 min with 1 ml of 80 % ethanol. Sanguinarine formed during the reaction was measured spectrofluorometrically at 324 and 408 nm excitation and emission wavelengths, respectively (Ignatov et al. 1999).

Results and discussion

Sanguinarine accumulation in response to elicitors in *A. mexicana* AmMiF cell line

Argemone mexicana AmMiF cell line accumulated significant amounts of sanguinarine, even without exposure to elicitors of secondary metabolism. Values between 1 and 2 mg g^{-1} DW, comparable to those found in roots from field-collected plants, were recorded in the controls (Table 1). This trait has remained stable since AmMiF cell line was generated in 2008 (Trujillo-Villanueva et al. 2010). Responsiveness to MeJa and YE has also been maintained through time (Trujillo-Villanueva et al. 2010, 2012; Guízar-González et al. 2012) and it is periodically assayed to confirm it. In this way, and in order to set the elicitor optimal doses, cultures were exposed to MeJa and YE in concentrations up to 200 μM and 300 mg l^{-1}, respectively. Controls (10 % ethanol or water) were also included (Table 1).

In MeJa exposed cultures, no significant response in sanguinarine accumulation was recorded at lower doses than 100 μM for up to 48 h (Table 1). Higher doses (150 μM and above) resulted harmful, since cultures turned brownish within the first 24 h (Supplementary Fig. 1) and a significant reduction in the packed cell volume (PCV) occurred (from 68 ± 7 to 41 ± 6 %; 1 h of free decantation in conical tubes). Maximal sanguinarine accumulation in cultures exposed to YE was detected at the 200 mg l^{-1} dose, although an important increase in alkaloid production also occurred at 100 mg l^{-1} (Table 1). As observed in MeJa treated cultures, those exposed to higher doses of YE (300 mg l^{-1}) turned brown (Fig. S1) and notoriously reduced their growth (PCV decreased from 73 ± 9 to 38 ± 8 %).

Participation of the OCDA pathway in the sanguinarine induced accumulation in AmMiF cell line

Based on the previous result, time course experiments were performed setting MeJa and YE doses at 100 μM and 200 mg l^{-1}, respectively. At such doses, sanguinarine reached maximal accumulation, without

compromising the culture's viability. In comparison to control, exposure to YE almost tripled sanguinarine accumulation after 24 h and increased more than eight-fold at 48 h (Fig. 1). Such values were maintained for up to 72 h. MeJa exposure also increased sanguinarine production, but in a less marked fashion than with YE. These effects were more evident after 48 h (Fig. 1). Maximal response to MeJa treatment was about half of that obtained with YE (Fig. 1). Regardless of the duration of YE treatment, DIECA pre-incubation reduced the magnitude of the response, even when it did not completely annul it. YE plus DIECA cultures still presented ca. 50 % of the response of the YE-elicited cultures (Fig. 1). Interestingly, MeJa exposure of these DIECA-treated cultures did not restore the original response to YE, although they resembled the response to MeJa alone (Fig. 1). MS-GC revealed the absence of jasmonate in DIECA exposed YE-elicited cultures confirming that, as previously reported, DIECA effectively blocks the OCDA biosynthetic pathway (Menke et al. 1999). It should be mentioned that alkaloid contents in the mock induced, DIECA pre-incubated cultures (either with water or 10 % ethanol), remained at similar values as in controls throughout the experiment (ca. 1 mg g^{-1} DW).

Berberine bridge enzyme (BBE) catalyses the formation of scoulerine from reticuline, which is a critical step in the synthesis of all groups of BIA's. This enzyme is transcriptionally activated in response to elicitation in different in vitro cultures (Hagel and Facchini 2013), including AmMiF cell line (Trujillo-Villanueva et al. 2012). As expected, *BBE* transcript levels increased in response to YE after 24 h and, even when they decreased afterwards, they always remained above control values (Fig. 2a). Exposure to MeJa produced a similar positive effect in *BBE* transcript levels (Fig. 2a). However, in contrast to what was observed for sanguinarine accumulation, DIECA pre-incubation completely reversed the YE-induced *BBE* transcript accumulation (Fig. 2a). Moreover, the addition of MeJa to the DIECA exposed YE-elicited cultures restored the original response (Fig. 2a).

DBO is the enzyme catalysing the last reaction of sanguinarine formation and its activity increased in

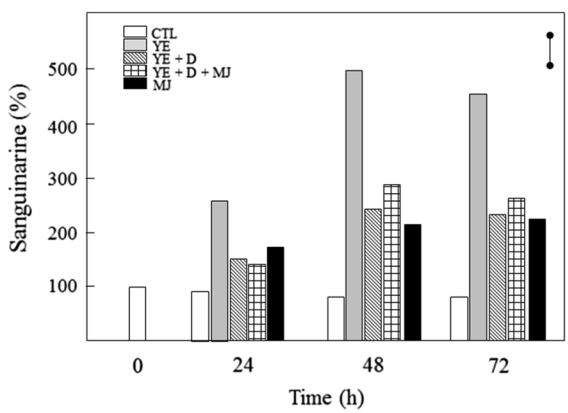

Fig. 1 Involvement of the OCDA pathway in YE-induced sanguinarine accumulation in *A. mexicana* AmMiF line. *YE* yeast extract; *D* diethyldithiocarbamic acid (DIECA); *MJ* methyl jasmonate. See the "Materials and methods" section for further detail. 100 % correspond to the value of sanguinarine found in unexposed control cultures (1.1 mg g^{-1} DW). Average of triplicates in each case. Maximal SD represented as the *bar* on the *upper left corner*

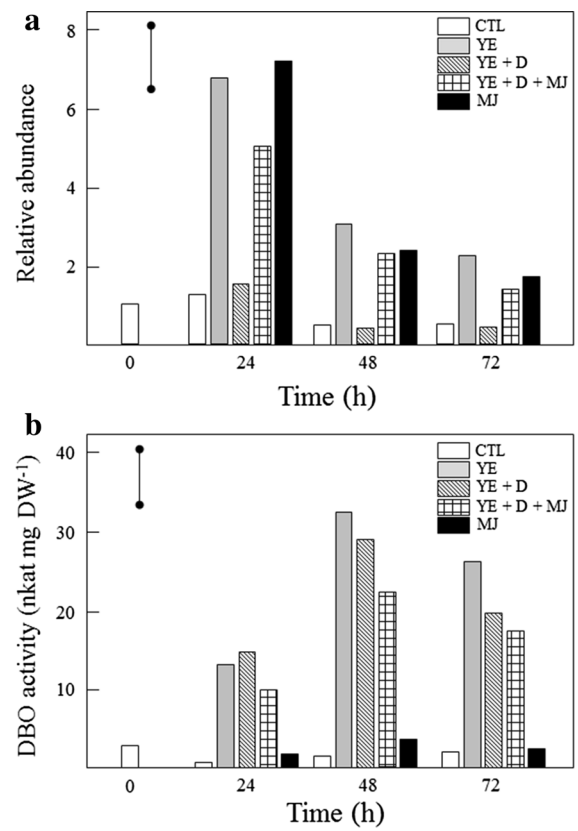

Fig. 2 Relative abundance of *BBE* transcripts (**a**) and DBO enzyme activity (**b**) in *A. mexicana* AmMiF cell line exposed to YE. Abbreviations as in Fig. 1. In **a** one unit correspond to *BBE* transcript relative abundance to actin in unexposed control cultures (0 h). Average of triplicates in both panels. Maximal SD represented as the *bar* on the *upper left corners*

response elicitation in other plant species (Ignatov et al. 1999). At the conditions assayed, DBO activity increased in the YE-, but not in the MeJa-exposed cultures (Fig. 2b). Noteworthy, and in contrast to what was observed for *BBE* transcript accumulation (Fig. 2a), DBO response to YE (Fig. 2b) followed a similar pattern to the one determined for sanguinarine accumulation (Fig. 1). There was a slight increase in activity during the first 24 h, whereas maximal response occurred at 48 h and it was maintained for up 72 h (Fig. 2b). Moreover, DBO activity in the YE-elicited cultures was not sensitive to DIECA, which remained at comparable values in all the assayed treatments, including those with MeJa (Fig. 2b).

Taken together, these data suggest that sanguinarine synthesis induced in *A. mexicana* AmMiF cell line in response to YE (Fig. 1) is partially dependent on the OCDA pathway (Fig. 3). Moreover, these results suggest that for the early biosynthetic reactions, including up to the BBE step (which resulted sensitive to DIECA; Fig. 2a), OCDA products would be mediating the response to YE (Fig. 3). However, since DIECA did not completely annul YE-induced sanguinarine accumulation (Fig. 1), other signalling mediators may be involved and operating after the BBE reaction. In that sense, the last biosynthetic reaction required for sanguinarine formation (involving DBO) was not sensitive to DIECA (Fig. 2b). Moreover, DBO basal activity appears to be sufficient to support an increase in sanguinarine synthesis and might also explain the intrinsic capacity of AmMiF cell line to produce significant sanguinarine amounts under common culture conditions (Guízar-González et al. 2012). This contrasted with results for BBE, which required to be induced to subsidise alkaloid synthesis (Fig. 2).

Sanguinarine accumulation in in vitro cultures in response to elicitors has been described for different species, including *E. californica*, *P. somniferum*, *P. bracteatum*, and *S. canadensis*, among others (Archambault et al. 1996; Cho et al. 2007; Cline and Coscia 1988; Ignatov et al. 1999; Weiss et al. 2006). The *A. mexicana* AmMiF cell line differs from most of them since it accumulates significant amounts of this alkaloid without any induction treatment. However, when exposed to elicitors, a limited response was obtained (Guízar-González et al. 2012; Trujillo-Villanueva et al. 2010, 2012). This suggests that regulatory mechanisms in this cell line are only partially

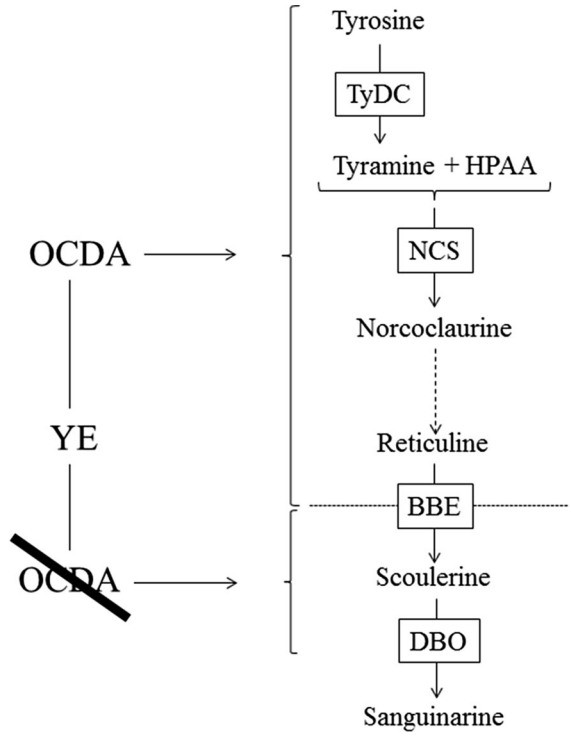

Fig. 3 Proposed model for YE-induced sanguinarine accumulation in *A. mexicana* AmMiF cell line. *TYDC* tyrosine decarboxylase; *NCS* norcoclaurine synthase; *BBE* berberine bridge enzyme; *DBO* dihydrobenzophenanthridine oxidase; *HPAA* 4-hydroxyphenylacetaldehyde. YE promotes sanguinarine accumulation activating the early biosynthetic reactions through OCDA participation, which is not required for the activation of the late biosynthetic steps in the pathway

shared with other species. Moreover, dissimilarities among cell cultures from the different species in response to elicitors are frequently found. For instance, in *E. californica* MeJa induced sanguinarine accumulation within 24 h of exposure, whereas in *S. canadensis*, higher doses were required to obtain a lower response. In contrast, salicylic acid produced a better response in *S. canadensis* than in *E. californica* (Ignatov et al. 1999; Cho et al. 2008a, b). Interestingly, the combination of salicylic acid and MeJa produced a synergistic effect in *E. californica* and this was related to transcriptional activation of the early biosynthetic pathway. However, the activation of the late steps in the pathway, observed as the appearance of DBO antigens, only occurred after YE addition (Cho et al. 2008a, b). *A. mexicana* AmMiF cell line did not respond to salicylic acid, neither as a single elicitor nor in combination with MeJa and YE (Trujillo-Villanueva et al. 2012).

In plant cells, a single stimulus can trigger two or more different signalling pathways serving diverse purposes. For instance, it can act as a mechanism to modulate the intensity of the final response, to lead to parallel events related to a common final response, or to activate partial events required at different times of an overall final response (Derksen et al. 2013). The pathway leading to sanguinarine synthesis involves more than 12 enzymes, with the initial part being common for the formation of sanguinarine and other BIA's types, such as the protoberberine-type berberine (Hagel and Facchini 2013). Although the latter is not found in AmMiF cell line, it is abundant in *Argemone* tissues (Guízar-González et al. 2012). On the other hand, an excess of sanguinarine accumulation can lead to cell toxicity and necrosis (Weiss et al. 2006; Trujillo-Villanueva et al. 2012). In this way, the act of splitting the control of early and late reactions would allow to furnish biosynthetic intermediaries to support an increase in the formation of alkaloids in response to a stimulus invoking chemical defence (such as YE), while modulating its amplitude would lead to avoidance of cell toxicity. Besides, such mechanism would allow sparing higher amounts of common intermediaries, which later on would be used to supply different metabolic pathways.

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Supporting information Supplementary Figure 1. Aspect of the *A. mexicana* AmMiF cell suspensions exposed to different doses of methyl jasmonate (MeJa) or yeast extract (YE) for 24 h. Cultures were treated as described in the Materials and methods section. Controls were exposed to water (for YE) or 10 % ethanol (for MeJa). Figures on top of photographs correspond to the concentration applied in μM for MeJa or mg l^{-1} for YE.

References

Archambault J, Williams RD, Bédard C, Chavarie C (1996) Production of sanguinarine by elicited plant cell culture I. Shake flask suspension cultures. J Biotechnol 46:95–105

Cho HY, Lee-Parsons CWT, Yoon S-YH, Rhee HS, Park JM (2007) Enhanced benzophenanthridine alkaloid production and protein expression with combined elicitor in *Eschscholtzia californica* suspension cultures. Biotechnol Lett 29:2001–2005

Cho HY, Rhee HS, Yoon SY, Park JM (2008a) Differential induction of protein expression and benzophenanthridine alkaloid accumulation in *Eschscholzia californica* suspension cultures by methyl jasmonate and yeast extract. J Microbiol Biotechnol 18:255–262

Cho HY, Son SY, Rhee HS, Yoon SYH, Lee-Parsons CW, Park JM (2008b) Synergistic effects of sequential treatment with methyl jasmonate, salicylic acid and yeast extract on benzophenanthridine alkaloid accumulation and protein expression in *Eschscholzia californica* suspension cultures. J Biotechnol 135:117–122

Derksen H, Rampitsch C, Daayf F (2013) Signaling cross-talk in plant disease resistance. Plant Sci 207:79–87

Guízar-González C, Trujillo-Villanueva K, Monforte-González M, Vázquez-Flota F (2012) Sanguinarine and dihydrosanguinarine accumulation in *Argemone mexicana* (L.) cell suspension cultures exposed to yeast extract. J Mex Chem Soc 56:19–22

Hagel JM, Facchini PJ (2013) Benzylisoquinoline alkaloid metabolism—a century of discovery and a brave new world. Plant Cell Physiol 54:647–672

Hartmann T (2007) From waste products to ecochemicals: fifty years research of plant secondary metabolism. Phytochemistry 68:2831–2846

Ignatov A, Clark WG, Cline SD, Psenak M, Krueger J, Coscia CJ (1999) Elicitation of dihydrobenzophenanthridine oxidase in *Sanguinaria canadensis* cell cultures. Phytochemistry 46:1141–1144

Menke FLH, Parchmann S, Mueller MJ, Kijne JW, Memelink J (1999) Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. Plant Physiol 119:1289–1296

Monforte-González M, Guízar-González C, Rubio-Piña J, Carrillo-Pech M, Vázquez-Flota F (2012) Berberine and sanguinarine quantitation in *Argemone mexicana* L. (Papaveraceae) tissues by TLC-in situ fluorography. J Planar Chromatogr—Mod TLC 25:358–360

Rubio-Piña J, Vazquez-Flota F (2013) Pharmaceutical applications of the benzylisoquinoline alkaloids from *Argemone mexicana* L. Curr Top Med Chem 13:2200–2207

Simon P (2003) Q-Gene: processing quantitative real-time RT–PCR data. Bioinformatics 19:1439–1440

Trujillo-Villanueva K, Rubio-Piña J, Monforte-González M, Vázquez-Flota F (2010) *Fusarium oxysporum* homogenates and jasmonate induce a limited sanguinarine accumulation in *Argemone mexicana* cell cultures. Biotechnol Lett 32:1005–1009

Trujillo-Villanueva KA, Rubio-Piña J, Monforte-González M, Ramírez-Benítez E, Vázquez-Flota F (2012) The sequential exposure to jasmonate, salicylic acid and yeast extract promotes sanguinarine accumulation in *Argemone mexicana* cell cultures. Biotechnol Lett 34:379–385

Vázquez-Flota F, Monforte-González M, Guízar-González C, Rubio-Piña J, Trujillo-Villanueva K, Coello-Coello J (2012) Establishment of a sanguinarine producing cell suspension culture of *Argemone mexicana* L. (Papaveraceae). Induction of alkaloid accumulation. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Plant cell culture protocols, 3rd edn. The Humana Press/Springer, New York, pp 271–276

Weiss D, Baumert A, Vogel M, Roos W (2006) Sanguinarine reductase, a key enzyme of benzophenanthridine detoxification. Plant, Cell Environ 29:291–302