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Effect of Macerozyme on Secondary Metabolism Plant Product Production and Phospholipase C Activity in *Catharanthus roseus* Hairy Roots

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Summary

The effect of macerozyme on accumulation of indole alkaloid and coumarine and on the induction of tryptophan decarboxylase (TDC, E.C. 4.1.1.28), phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5) and phosphatydilinositol 4,5-bisphosphate phospholipase C (PLC, E.C. 3.1.4.3) activities on *Catharanthus roseus* hairy roots was investigated. Increasing concentrations of macerozyme induced an increase in indole alkaloid and coumarine accumulation. There was a 10-fold increase in TDC and a 4-fold increase in PAL activities in hairy roots treated with 1 % of macerozyme. In a dose-response experiment PLC activity decreased 38 % with 0.5 % of macerozyme and increased 40 % with 1 % of macerozyme when compared to untreated roots. The results demonstrate that macerozyme treatment can affect the production of secondary metabolites and PLC activity in *C. roseus* hairy roots.

Key words: Catharanthus roseus, macerozyme, phenylalanine ammonia-lyase, phospholipase C, tryptophan decarboxylase.

Abbreviations: EDTA = ethylenediaminetetraacetic acid; PIP_2 = phosphatidylinositol 4,5-bisphosphate; PLC = phospholipase C; PAL = phenylalanine ammonia-lyase; TDC = tryptophan decarboxylase.

Introduction

Plant cells have a sensitive perception system for a variety of external stress signals such as UV-light, ethylene and endogenous or exogenous elicitors (Darvill and Albersheim, 1984). After recognition of the stimuli, a multitude of plant resistance-associated reactions are initiated: ion flux across the plasma membrane, generation of highly reactive oxygen species (the oxidative burst), phosphorylation of specific proteins, activation of enzymes involved in the cell wall reinforcement, transcriptional activation of numerous defence genes, induction of phytoalexin synthesis, localised cell death at the infection sites and the induction of systemic acquired resistance in distal plant organs (Bent, 1996; Crute and Pink, 1996; Dangl et al., 1996; Hammond-Kosack and Jones, 1996; Ryals et al., 1996). There are compounds that stimulate a plant defence mechanism or elicitor. How elicitors are recognised by the plant is one of the major questions in modern plant biology. It has been shown that elicitors bind to a high affinity receptor which resides in the plasma membrane (Schmidt and Ebel, 1987; Cosio et al., 1996; Nürberger et al., 1994). The elicitor binding to the receptor in the plasma membrane is essential to induce a defensive response, and also to generate a second messenger that transduces intracellularly. It has been proposed that Ca^{2+} ions play a crucial role for signal transduction in the elicitation response (Kurosaki et

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al., 1987; Stab and Ebel, 1987). There is evidence suggesting that the products of the phosphoinositide metabolism may be involved as second messengers in the elicitation of a defence response in cell suspension cultures of soybean and carrot (Stab and Ebel, 1987; Kurosaki et al., 1987). However, contradictory results have been reported in cell suspension culture of parsley and soybean. In these systems, phosphoinositide metabolism was not involved in the defence response induced by elicitors (Strasser et al., 1986). Toyoda et al. (1992) demonstrated that a pathogenic fungus of pea, *Mycosphaerella pinodes,* secretes both polysaccharide elicitor and glycopeptide suppresor for the production of secondary metabolites. In this example the elicitor rapidly activated phosphatidylinositol and phosphatydilinositol-4-monophosphate kinases in plasma membranes isolated from pea epycotyls, although the supressor from the same fungus markedly inhibited these enzymes. These authors also showed that the *M. pinodes* elicitor induced a rapid and biphasic increase in levels of phosphatidylinositol-4,5-bisphosphate (PIP_2) in plasma membrane isolated from pea epycotils a few minutes after the treatment with the elicitor (Toyoda et al., 1993). Kamada and Muto (1994) demonstrated that an elicitor prepared from the cell wall of the pathogenic fungus *Phytophtora nicotinae* stimulated inositol phospholipid turnover in tobacco suspension cells followed by the induction of phenylalanine ammonia lyase (PAL) activity. These results suggest that rapid changes in phosphoinositide metabolism are important in the signal transduction that is related to the defence response in plants.

Plant tissue cultures are able to accumulate secondary metabolites as a response to elicitors (DiCosmo et al., 1987; Godoy-Hernández and Loyola-Vargas, 1991; Vázquez-Flota et al., 1994). The activity of some enzymes involved in the biosynthesis of secondary metabolites was also induced when cultures were treated with elicitors (Kombrink and Hahlbrock, 1986; Chappell and Nable, 1987; Seitz et al., 1989). In the past few years, the culture of transformed roots has emerged as an alternative to obtain different natural products (Signs and Flores, 1990). At the moment, hairy root cultures present several important characteristics such as fast growth and a high degree of genetic and biochemical stability (Rhodes et al., 1990) which can be used to study signal transduction and secondary metabolism pathways in plants. In our laboratory several parts of these two topics have been extensively studied using *C. roseus* hairy roots as a model (Sáenz-Carbonell et al., 1993; Ciau-Uitz et al., 1994; Vázquez-Flota et al., 1994; De Los Santos-Briones et al., 1997). This fact prompted us to investigate the effect of macerozyme on PLC activity and secondary metabolism in response to macerozyme as an elicitor in transformed roots of *C. roseus.*

Materials and Methods

Tissue culture

Hairy root line J1 of *C. roseus* was obtained by infection of leaves with *Agrobacterium rhizogenes* strain 1855 pBI 121.1 (Ciau-Uitz et al., 1994), and maintained in half strength B_5 medium supplemented with 3 % of sucrose (Gamborg et al., 1968) subcultured in 250 mL Erlenmeyer flasks with 100 mL of media, every 21 d. The initial inoculum was 0.5 g (F.W.). Flasks were incubated in the dark on a rotary shaker at 100 rpm at 25 °C.

Alkaloid content

Total alkaloids were extracted as described (Monforte-González et al., 1992) and their total content was determined spectrophotometrically at 280 nm. Individual alkaloid (ajmalicine) was separated using thin layer chromatography (TLC) plates on a solvent system of chloroform : acetone (8 : 2) and quantified in a Shimadzu scanner (Monforte-González et al., 1992).

Coumarin content

Total coumarins were extracted and quantified as described (Kombrink and Hahlbrock, 1986).

TDC assay

TDC activity was measured as described in Islas et al., 1994. Frozen transformed roots (1 g) were pulverised in a cold mortar to a fine powder and homogenised with 1.25 mL of 0.1 mol/L HEPES (pH 7.5), containing 3 mmol/L DTT, 5 mmol/L EDTA, 10.5 µmol/L leupeptine and 1 mmol/L PMSF. The brei was filtered through four layers of cheesecloth and centrifuged for 30 min at 18,000 g_n. The resulting supernatant was immediately used as a source of enzyme for the TDC activity assay. The assay mixture (total volume: 100 µL), which contained enzyme extract (10–25 µg of protein), L-[^{14}C methyle]-tryptophan (2.1×10^{-3} Mbq, 50 nmol), pyridoxal phosphate 41.4 µmol/L, and HEPES 5 µmol/L (pH 7.5), was incubated at 30 °C. After 1 h the reaction was stopped by the addition of 100 µL of 1 mol · L^{-1} KOH. Tryptamine was extracted with 500 µL of ethyl acetate and an aliquot of 300 µL tagged and added to a scintillation vial containing 5 mL of scintillation liquid Aquasol and the radioactivity measured.

Protein quantification

Protein concentration of the samples was measured according to Smith et al. (1985) using the BCA protein assay reagent. Bovine serum albumin was used as standard.

PLC assay

Hairy roots (1 g) were pulverized in a cold mortar to a fine powder and homogenized with 2.5 mL of 50 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.4, 250 mmol/L sucrose, 10 % glycerol, 1 mmol/L PMSF, 10 mmol/L sodium pyrophosphate and 0.2 mmol/L orthovanadate. The tissue debris was filtered through four layers of cheesecloth and centrifuged at 12,000 g_n for 30 min at 4 °C. The supernatant was further centrifuged at 100,000 g_n for 45 min. The supernatants (protein: 3.5–5 mg/mL) were recovered as soluble fractions. The pellet was resuspended in the extraction buffer (protein: 0.5–1.2 mg/mL), and was used as the membrane fraction. The hydrolysis of [^{3}H]PIP_2 was measured as described (Hernández-Sotomayor and Carpenter, 1993; De Los Santos-Briones et al., 1997) in a reaction mixture (50 µL) that contained 35 mmol/L NaH_2PO_4 (pH 6.8), 70 mmol/L KCl, 0.8 mmol/L $CaCl_2$ (final Ca^{2+} concentration 25 µmol/L), 200 µmol/L [^{3}H]-PIP_2 (20,000 cpm), 0.08 % deoxicholate. The reaction mixture was stopped with 100 µL of 1 % (w/v) bovine serum albumin and 250 µL of 10 % (w/v) TCA. Precipitates were removed by centrifugation (13,000 g_n for 10 min) and the supernatant collected for quantification of the release of [^{3}H]IP_3 by liquid scintillation counting Aquasol.

PAL assay

PAL activity was measured as described (Zimmerman and Hahlbrock, 1975) with slight modifications. Frozen transformed roots (1 g) were pulverized in a cold mortar to a fine powder and homogenized with 2 mL of 0.05 mol/L Tris-HCl (pH 8.8), containing 15 mmol/L β-mercaptoethanol, 1 mmol/L EDTA, 10.5 µmol/L leupeptine and 1 mmol/L PMSF. The brei was filtered through four layers of cheesecloth and centrifuged for 15 min at 10,000 g_n. The resulting supernatant was immediately used as a source of enzyme for the PAL activity assay. The assay mixture (total volume: 2 mL), which contained enzyme extract (protein: 2–20 mg) (0.5 mL), 10 mmol/L phenyalanine (0.5 mL), and Tris-HCl 50 mmol/L, pH 8.8 (1 mL) was incubated at 37 °C. After 1 h the reaction was stopped by the addition of 500 µL of 6 mol·L^{-1} HCl. The mixture was centrifuged at 10,000 g_n for 10 min and the supernatant was used for measured PAL activity in a spectrophotometer at 290 nm.

Materials

[^{3}H]PIP_2, [^{14}C]tryptophan, Aquasol were purchased by Dupont NEN Research. Unlabelled PIP_2 was purified from Folch extracts of brain lipid (Sigma) by a neomycin affinity column as previously described (Waldo et al., 1994). BCA protein assay reagent was supplied by Pierce Chemical Company. B_5 medium, neomycin, leupeptine and PMSF were obtained from Sigma Chemical Company.

Data presentation

All experiments were repeated at least three times and with three replicates each. Data were analysed using the t-student test. Results are the means (± S.E.).

Results

Induction of secondary metabolite accumulation by macerozyme addition

Previously, we showed that different hydrolytic enzymes induced a liberation of indol alkaloids into the culture medium along with a slight increase in the alkaloid content in the tissue (Vázquez-Flota et al., 1994). In this work we tested the effects of macerozyme (a hydrolytic enzyme that hydrolyses pectin in the cell wall) on the accumulation of secondary metabolites such as total indole alkaloids, ajmalicine and coumarines in *C. roseus* hairy roots. Increasing concentrations of macerozyme were added to the medium at day 30 of culture and hairy roots were harvested 72 h after the addition of the enzyme. Addition of macerozyme increased total alkaloid content and ajmalicine accumulation, as shown in Fig. 1 panel A and B. In the case of total alkaloids only a slight increase was observed with 0.05 % and 1 % macerozyme (Fig. 1 panel A); however, ajmalicine content increased 90 % with respect to control when the roots were treated with 0.05 % of macerozyme (Fig. 1 panel B).

After 72 h of macerozyme treatment, roots turned slightly brown, probably from the accumulation of coumarines and phenylpropanoid derivatives. These compounds were extracted with chloroform from the culture medium and their content measured spectrophotometrically as reported by Kombrink and Hahlbrock (1986). In untreated, roots the amount of coumarines in the culture medium was practically undetectable but their accumulation increased with the addition of macerozyme. Accumulation depended on the concentration of the elicitor used with a maximum increase at 0.5 % of macerozyme (Fig. 1 panel C).

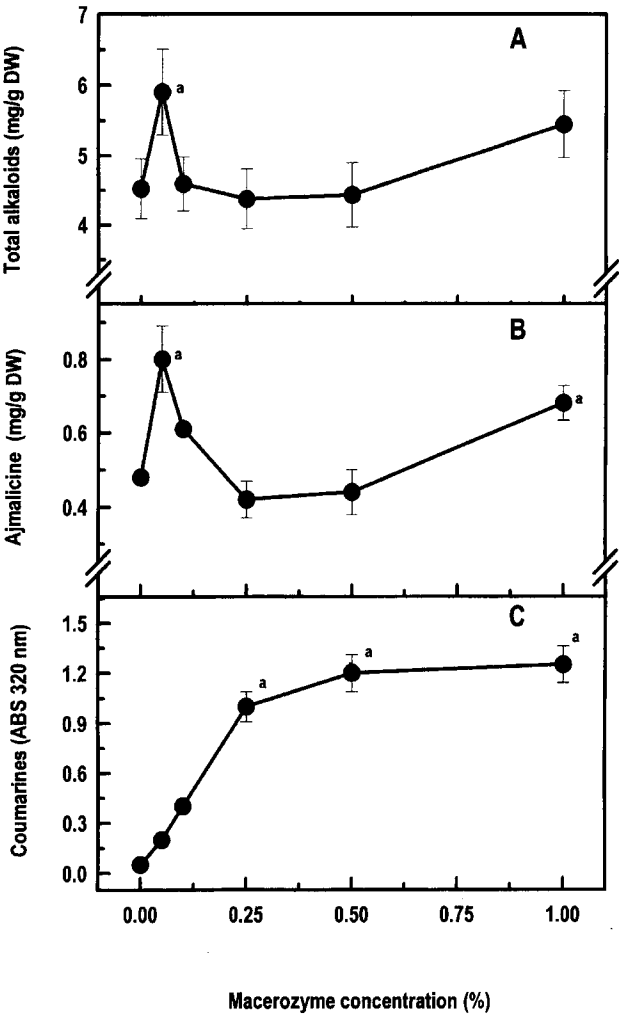

Fig. 1: Effect of increasing concentrations of macerozyme on secondary metabolite accumulation. Different concentrations of macerozyme were added to the culture media in the 30 day of culture and roots were harvested 72 h after macerozyme addition. (A) Total alkaloids; (B) individual alkaloid ajmalicine; (C) total coumarines. The results are the media of three different experiments with three replicates each one. A t-student test was applied. a, $p < 0.001$; respect to control without macerozyme.

Effect of macerozyme on TDC and PAL activity

Tryptophan decarboxylase (TDC) catalyses the conversion of tryptophan to tryptamine and it has been suggested that it may have a role as a possible site of regulation in indol alkaloid biosynthesis (Eilert et al., 1987; De Luca et al., 1988; Seitz et al., 1989). TDC activity was measured to explore its relationship with indol alkaloid accumulation in response to macerozyme. When hairy roots were treated with increasing concentrations of macerozyme, TDC activity increased in a

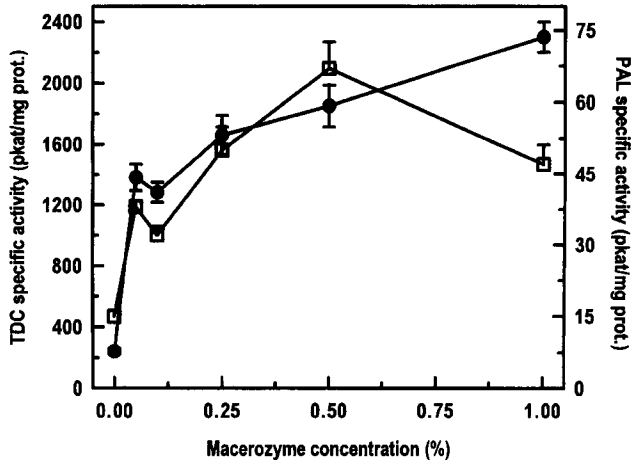

Fig. 2: Effect of a dose-response of macerozyme on the TDC and PAL activities. Different concentrations of macerozyme were added to the culture media in the 30 day of culture and roots were harvested 72 h after macerozyme addition. (●) TDC specific activity; (□) PAL specific activity. The results were the media of three different experiments with three replicates each one. All the points have $p < 0.001$ when compared to control without macerozyme.

dose-dependent manner. At the higher macerozyme concentration, TDC activity increased 10-fold when compared to untreated roots (Fig. 2).

Phenylalanine ammonia lyase (PAL) catalyses the first committed step in the phenyl propanoid pathway and this enzyme is highly regulated by elicitors. PAL undergoes a marked increase in its activity after treatment with macerozyme. The activity of this enzyme increased four-fold when compared to the control at 0.5 % of macerozyme (Fig. 2). The pattern of PAL activity in response to macerozyme correlates with the pattern of coumarin accumulation.

After the effect of different macerozyme concentrations was seen on TDC and PAL activities, it was important to determine the optimal treatment time. Hairy roots from the 30th day of culture were treated either with sterile water with 0.5 % of macerozyme. TDC and PAL activities were determined at time periods between 1 and 120 h after the elicitor addition. TDC and PAL activities from roots without treatment showed no significant changes during the time of the experiment whereas the enzyme activities were increased in elicitor-treated hairy roots (Fig. 3 panel A and B). TDC and PAL activities reached a maximum 72 h after the macerozyme addition; however, the activities of the two enzymes declined slightly after longer periods of treatment.

Regulation of PLC activity by macerozyme

As stated in the introduction, there is some evidence that correlates elicitor action and phosphoinositides as the signal transduction mechanism. To elucidate if PLC (a key enzyme in phosphoinositide metabolism) is involved in signal transduction mechanism response to elicitors in *C. roseus* hairy roots, we analysed the effect of the addition of macerozyme on PLC activity.

In the 30 th day of culture, 0.1 % and 1 % of macerozyme were added to the culture media. Hairy roots were harvested at different times and PLC activity was measured. PLC activity, as a function of incubation time after treatment, with two concentrations of macerozyme is given in Fig. 4. Non-treated roots show a constant PLC activity throughout the incubation period. Elicitor treatment leads to an increase in PLC activity, with 0.05 % macerozyme. PLC activity increased 1-fold after 1 h of the induction and rapidly declined to the level of untreated roots 2 h after addition of macerozyme. At 72 h after elicitor addition PLC activity was inhibited by approximately 30 % in relation to untreated roots (Fig. 4). With 1 % macerozyme, PLC activity increased 4-fold at 1 h after elicitor addition followed by a decrease (Fig. 4).

Discussion

Elicitor treatment has proved to be a powerful tool for increasing the levels and the excretion of secondary metabolites in cell cultures that produce only trace amounts and to study the mechanisms that regulate the biosynthesis of these compounds (Kombrink and Hahlbrock, 1986; Eilert et al., 1987; Funk et al., 1987; Seitz et al., 1989; Nef et al., 1991; Moreno et al., 1993; Vázquez-Flota et al., 1994; Garnier et al., 1996). As shown in our previous work, hydrolytic enzymes such as chitinase, macerozyme and cellulase increased the excretion of alkaloids into the culture media in hairy roots of *C. roseus*

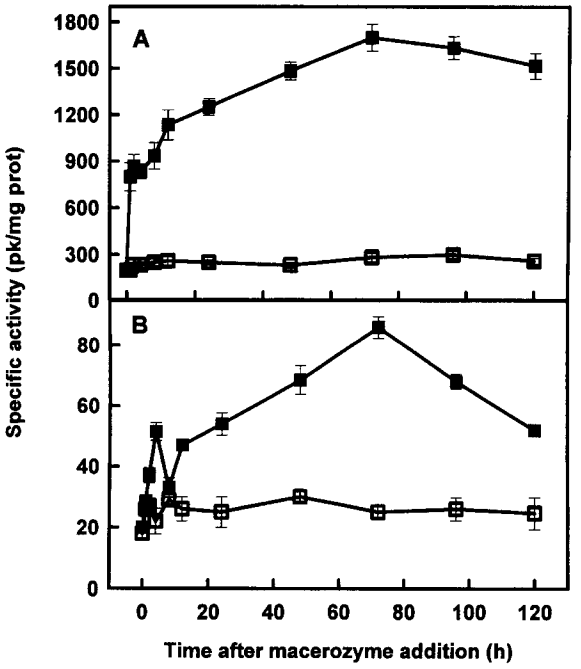

Fig. 3: Time course for TDC and PAL elicitation in *C. roseus* hairy roots. 0.05 % of macerozyme was added on the 30 day of culture and the roots were harvested at different periods of time. (A) TDC activity, in the presence (■) or absence of 0.05 % macerozyme (□). (B) PAL activity, in the presence (■) or absence of 0.05 % macerozyme (□). The results were the media of three different experiments with three replicates each one.

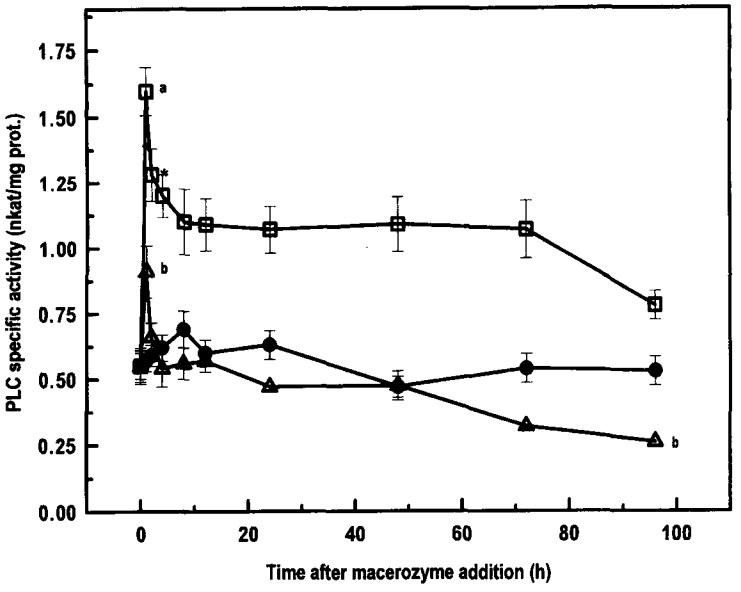

Fig. 4: Time course for PLC elicitation in *C. roseus* hairy roots of 0.1 % and 1 % of macerozyme was added on the 30 day of culture and the roots were harvested at different periods of time. (●) Control; (△) 0.1 % of macerozyme; (□) 1 % of macerozyme. The results were the media of three different experiments with three replicates each one.

(Vázquez-Flota et al., 1994). Macerozyme is a commercial preparation of pectinase that hydrolyses the cell wall and generates endogenous elicitors. In the present work we showed that induced an increase in the total indol alkaloid content (33 % when compared to control roots) and the individual indol alkaloid, ajmalicine (90 % when compared to control) (Fig. 1), in contrast to the results obtained by Garnier et al. (1996). They found that the addition of macerozyme to the culture medium provoked an ajmalicine decrease but induced a tryptamine increase in cell suspension culture of *C. roseus*. The cell wall of hairy roots and cell suspension culture of *C. roseus* could be different and the endogenous elicitors that liberate the macerozyme could induce different responses in the cultures.

Elicitors, by definition, induce the synthesis of phytoalexins, such as coumarins and phenyl propanoid derivatives. Increasing the concentrations of macerozyme induced an accumulation of coumarines and this accumulation was dependent on the amount of elicitor used (Fig. 1), as reported by Kombrink and Hahlbrock (1986) and Conrath et al. (1989). As we can see in Fig. 1 panels A, B and C, macerozyme can induce the accumulation of secondary metabolite derivatives from different, but related, indol alkaloids and phenyl propanoids in *C. roseus* hairy roots.

We also demonstrated that macerozyme can regulate two key enzymes in the secondary metabolism, TDC and PAL (Figs. 2 and 3). Seitz et al., 1989, using the cell line 615 from *C. roseus*, observed transient increases in PAL and TDC enzyme activities with elicitor treatment. The elicitor mediated induction of TDC enzyme activity lagged behind in contrast to that of PAL and phenol accumulation, lagged behind and only occurred after indol alkaloid accumulation had peaked. In the present work (Figs. 1, 3), PAL and TDC activities peaked 72 h after the elicitor addition. Total alkaloids, ajmalicine and coumarines were measured and also found to be the highest.

The questions now are, how will the signal generated by the endogenous elicitors provoked by the action of macerozyme modify these enzymes and which transduction pathway is involved. As we show in figure 4, macerozyme can also regulate PLC activity. When macerozyme was added and PLC activity measured after 72 h, (the time taken to see the effect on alkaloid accumulation) a biphasic behaviour was observed, as shown in Fig. 4. This type of behaviour has been observed in response to other elicitors. For example, in pea (Toyoda et al., 1993) epicotyls, a fungal elicitor caused a biphasic increase in levels of PIP_2 and IP_3.

In summary, our results demonstrate that macerozyme treatment can dramatically affect the production of secondary metabolites and PLC in *C. roseus* hairy roots.

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