FUNGAL ELICITOR-MEDIATED CHANGES IN POLYAMINE CONTENT, PHENYLALANINE-AMMONIA LYASE AND PEROXIDASE ACTIVITIES IN BEAN CELL CULTURE

F. Broetto [1]*, J.A. Marchese [3], M. Leonardo [2], M. Regina [2]

[1]Institute of Biosciences, São Paulo State University, Botucatu 18618-000, Brazil; [2]Agronomic Sciences College, São Paulo State University, Botucatu 18603-970, Brazil; [3]Agronomy College, Paraná Federal University of Technology, Pato Branco, 85503-390, Brazil.

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Summary. Plant cells are able to shift their metabolism in response to aggressive environmental factors such as salinity, water stress, anoxia, flooding or biotic elicitors**.** In this work, cell suspensions of four bean (*Phaseolus vulgaris* L.) genotypes were stabilized and treated with elicitors isolated from fungal (*Fusarium oxysporum* f. sp. phaseoli) cell walls. The elicitation of the cells was effective in the induction of the activity of L-phenylalanine-ammonia lyase (PAL), a key enzyme related to defense reactions, as well as the peroxidase, an enzyme related to the antioxidative response system. The same effect was also verified in relation to the polyamine metabolism, mainly for the accumulation of the diamine putrescine. The obtained results give important information regarding the plant-pathogen interactions, mainly as subsidy for bean improvement programs seeking the adaptation to adverse environmental factors.

Key words**:** peroxidases, phenylalanine-ammonia lyase, polyamines, fungal elicitor, tissue culture

Abbreviations: E.C. - enzyme classification, MS - Murashige & Skoog medium, PA – Polyamines, PAL - phenylalanine ammonia-lyase, POD – peroxidases, TLC - thin layer chromatography, Phe – phenylalanine

* Corresponding author, e-mail: broetto@ibb.unesp.br

INTRODUCTION

Bean culture is susceptible to a large number of fungal diseases, among which is the wilt caused by *Fusarium oxysporum (*Nascimento et al*.,* 1995). In Brazil, the disease is found in the South, Southeast and Northeast of the country where it is considered as a limiting factor to the production (Ribeiro and Ferraz, 1984).

In general, plants have potential to mobilize biochemical response mechanisms against pathogenic attack including lignification (Köhle et al., 1985), suberization (Espelie et al., 1986), synthesis of phytoalexins (Kuc and Rush, 1985), induction of hydrolytic enzymes (Bollen, 1985; Broetto, 1995) and activation of the antioxidative response system (Broetto et al. 2002). The regulation of enzymes involved in the biosynthesis of metabolites produced in response to environmental stress has been studied in cell cultures of different plant species (Messner et al., 1991), aiming to accelerate *in vivo* studies. Schell & Parker (1990) suggested that the activaton to the phenypropanoid metabolism can be easily detected by the variation in the activity of its key enzyme, phenylalanine-ammonia lyase (PAL, E.C.4.3.1.5.). In another study, Lawton et al. (1983) treated a bean cell culture using a cell wall isolate of the fungus *Colletotrichum lindemuthianum*. As a response they observed a strong induction of PAL activity which was due to increased *de novo* synthesis of PAL-mRNA.

Bell et al. (1984) differentiated compatible and non-compatible bean cultivars according to their response to the pathogenic infection. The non-compatible cultivars (considered resistant) when infected accumulated high amounts of the phytoalexine phaseolin, accompanied by the increase of PAL activity and PAL-mRNA. A study of the induction of the activity of PAL and 4-coumarate-CoA-ligase after treatment of carrot cells with elicitors extracted from the fungus *Pythium aphanidermatum* showed similar correlations (Gleitz, 1989).

Campbell and Ellis (1992) treated cells of *Pinus banksiana* using ectomicorrhizal fungi as elicitors. They found that the activity of PAL was increased almost 10 times after 24-h treatment. The lignification of the elicited tissue was accompanied also by an increase of the activity of enzymes associated with the synthesis of lignin, such as caffeic acid *O*-methyl transferase, 4-coumarate-CoA-ligase and peroxidases. The peroxidases (EC 1.11.1.7) compose a group of enzymes stimulated in typical defense responses using H_2O_2 in several biological oxidation processes and they are involved in the pathogen enzyme inactivation by phenolic oxidation (Siegel, 1993; Broetto, 1995). The lignification of the infected plant tissues can be considered as a resistance mechanism (Vance et al. 1980). The final polymerization of lignin is due to the oxidation of the phenolic groups mediated by the enzyme peroxidase (Pascholati and Leite, 1995).

Another important subject of the study of plant-pathogen relationships is the amine metabolism, particularly the aliphatic di - and polyamines. Although less explored

this line of studies can contribute to the understanding of the adaptation strategies of plant cells in response to environmental stresses (Walters, 2000).

In plant tissues, di- and polyamines are detected in micromolar order and to above milimolar, depending to a great extent to the environmental conditions, especially stress (Flores and Filner, 1985). Some alterations in the polyamine metabolism in plants were reported by Slocum et al. (1984), as a response to fungal elicitation. Samborski and Rohringer (1970) mentioned that resistant wheat cultivars were elicited using several species of pathogens, accumulating putrescine conjugated to phenolic compounds, such as hydroxycinamic acid. This compound (*2*-hydroxyputrescine) possesses anti-microbe activity acting as phytoalexin. In leaves of barley infected by the fungus that causes the brown rust, Greenland and Lewis (1984) observed that chlorophyll was retained in sites of infection (called *green islands*), and in these tissues, the level of spermidine increased 6 - 7 times. Other amines conjugated with phenols, such as dicoumaroylagmatine, were found by Smith and Best (1978) after infection of barley seedlings by powdery mildew (*Erysiphe graminis*). The agmatine, coumaroylagmatine and dicoumaroylagmatine concentrations were detected in growth phases between 3 and 13 days after the inoculation. The authors observed that the infection induced an increase in the levels of hordantine (6 times) and agmatine (2 times) 13 days from inoculation of the seedlings. Walters & Wylie (1986) also observed that application of elicitors of fungus that causes powdery mildew in barley induced an increase in levels of polyamines in the young leaves as well as in the activity of the enzymes related to polyamine metabolism.

The aim of the present study was to examine some metabolic shift in cells of bean (*Phaseolus vulgaris* L.) treated with fungal elicitors with an emphasis on polyamine metabolism and enzymes related to resistance mechanisms to stress biological factors.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of four bean genotypes: IAC-carioca SH 80, IAPAR-14, JALO-EEP 558 and BAT-93 were used throughout the experiments. The callus cultures were established starting with the inoculation of embryo axes in a MS semi-solid medium (Murashige & Skoog, 1962) supplemented with organic components and plant regulators as described by Broetto (1995). After 28 days the bean calluses were disaggregated in stainless steel sieves (0.5 - 1.0 mm) and simultaneously washed with liquid MS medium. The cells were collected in a *Nylon* net (0.45 μm), suspended in liquid MS medium and transferred into flasks containing 50 mL of MS medium (4.0 mg of cells mL^{-1} of medium). The cell suspensions were maintained under agitation (110 rpm) in a culture room at 26 °C and light intensity of 2000 lux.

Fungal culture

An isolated culture of the *Fusarium oxysporum f.* sp. *phaseoli* was kindly provided by Dr. A.C. Maringoni, from the fungal collection of the Agronomy College at São Paulo State University, Botucatu, SP, Brazil. The fungi were initially cultivated on Petri dishes in a PDA medium (PDA; potato, dextrose, agar) maintained at 28 °C, until optimum mycelium growth (7 to 10 days).

The isolation of *Fusarium oxysporum* cell walls was done according to the protocol of Ayers et al. (1976) adapted by Broetto (1995). The determination of total soluble sugars (Dubois et al., 1956) was required as a dilution parameter (glucose equivalents).

The solution was centrifuged to isolate the particles in suspension and applied aseptically to the cultures of bean cells with a Millipore 0.2 μm membrane. The beans cell cultures were collected 48 h after elicitation and used further for biochemical determinations.

Suspensions of bean cells were established in triplicates (50 mL of medium, containing 4 mg of cells mL^{-1}) and received the elicitor solution as of the following treatments: 50, 100 and 200 (g mL^{-1} glucose equivalents). The control treatment received sterilized deionized water (1.0 mL) substituting the elicitor treatment.

The treatments denominated glucose equivalents represent the concentration of total soluble sugars after dilution with distilled water (to 1.0 mL). In this analysis, the hydrolyzed solutions presented concentrations of 200 μg glucose mL^{-1}. Samples of fresh cells (1 g) were macerated into 5 mL of potassium phosphate buffer, pH 6.7, 0.2 mol L^{-1}. After centrifugation at 12100 x *g* for 10 min at 4 °C, the supernatant was collected, distributed into glass vials and frozen at –20 °C.

Biochemical determinations

The concentration of soluble proteins present in the crude extract was determined in triplicate using the assay described by Bradford (1976) with bovine serum albumin as a standard.

Determination of peroxidase activity (POD; E.C 1.11.1.7) was done by the method of Allain et al. (1974). The enzyme activity was calculated using the molar extintion coefficient of 2.47 mM^{-1} cm^{-1}. The specific activity was expressed as μM H_2O_2 min^{-1} mg $^{-1}$protein.

The activity of L-phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5.) was determined by the method of Zucker (1965). Aliquots of 0.3 – 0.5 mL of the crude extract were mixed with 1.9 – 2.1 mL boric acid-borax buffer 0.2 mol L^{-1}, pH 8.8. The probes were thermostatized at 36 °C. The reaction started after the addition of 0.6 mL L-phenylalanine at a concentration of 0.1 mol L^{-1}. After 15 min of incubation at a constant temperature spectrophotometric reading began at 290 nm.

The enzyme activity was calculated based on the molar extinction coefficient of t-cinnamic acid at 290 mm = 10^4 mM^{-1} cm^{-1}. The specific activity was expressed as Kat Kg^{-1} prot. (mol s^{-1}).

The diamine putrescine and the polyamines spermine and spermidine were extracted, isolated and quantified according to the method of Flores and Galston (1982) based on direct dansylation, followed by separation of amines using TLC. The chromatographic plates were dried and the dansylpolyamine bands were quantified with an densitometer (Quick Scan – Flur-Vis; Helena Lab., USA). By activating the dansylated compounds at 365 nm, the fluorescence intensity was measured at 507 nm. Quantitative analyses were carried out by integration of peaks reffering to each amine, compared with those obtained of p.a. standards.

Statistical analysis

Identity of mean values was checked by *t*-test analyses after analyses of the identity of variances based on the *F*-test using the program Statistica for Windows v. 5.1 (StatSoft Inc., Tulsa, USA).

RESULTS AND DISCUSSION

The obtained results indicate that except for the control treatment, there were differences in PAL activity among the four bean cultivars studied. These differences demonstrated a strong dependence on the level of glucose equivalents, especially for BAT and JALO cultivars. PAL activity in the IAC cultivar showed a slight increase with increasing the concentrations of the eliciting solution in the culture medium, mainly for levels of 50 and 100 μg mL^{-1}. The cultivar IAPAR only respond to the highest levels of applied elicitor, 100 and 200 μg mL^{-1} (Fig. 1). In spite of the treatments with high glucose-equivalents have influenced more the activity of PAL, a decrease of this tendency was observed when the maximum concentration of elicitors (200 μg mL^{-1}) was applied, mainly for IAC, BAT and IAPAR. These results agree with the reported by Campbell and Ellis (1992); Moniz de Sá et al. (1992); Messner et al. (1991) for the same enzyme in other species.

Working with bean cells suspension, Dixon et al. (1981) observed a dose-response effect (elicitor concentration isolated from *Colletotrichum lindemuthianum*) in the activity of PAL. The authors observed two maxima of the enzyme activity in function to the application of smaller doses of elicitors (17.5 and 50 μg equivalent-glucose mL^{-1}). The activity reduced for the treatments with high concentration of glucose-equivalent. According to Albersheim and Valent (1978) the dose-response effect can be explained with the connection of the elicitors to specific sites at the plasmatic membrane of the host cells, when some competition can be established.

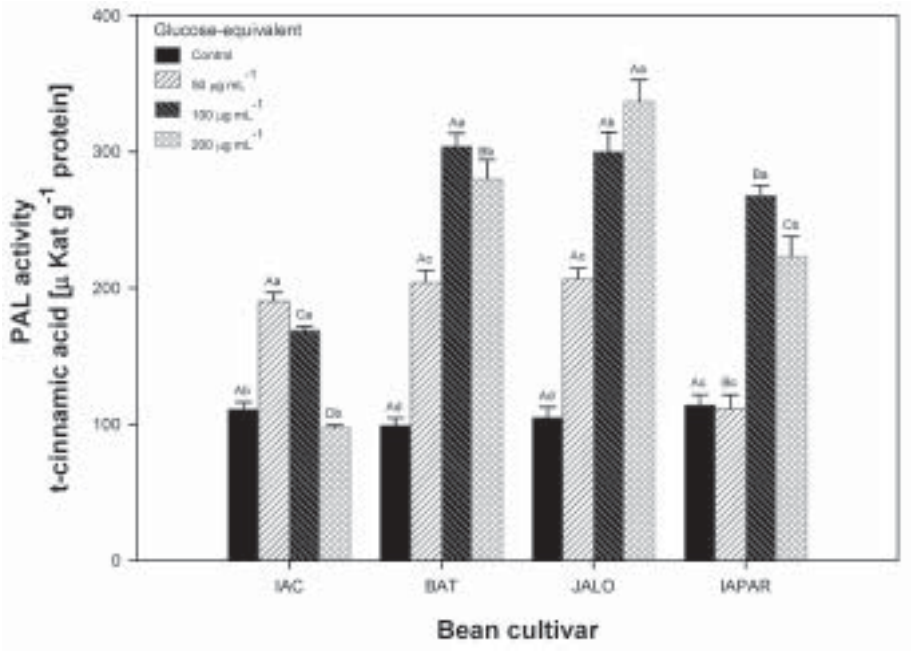

Fig. 1. Changes in the catalytic activity of phenylalanine ammonia-lyase in suspension-cultured *Phaseolus vulgaris* cells 48 h after treatment with an elicitor released from the wall of *Fusarium oxysporum*. Significant differences between means (extracts from 6 individual probes per treatment) of the different cultivars or different treatments within the genotypes are marked with different letter (Tukey test, 5%); Vertical bars indicate ± SE.

This competition can happen mainly due to the presence of low molecular weight carbohydrates among the components of PAL elicitors.

The dose-response effects for eliciting plant tissues can vary according to the studied species and the conditions of the treatments. Potato protoplasts presented hypersensitivity response, with log – linear model, when elicited with *P. infestans* (Doke and Tomiyama, 1980); Albersheim and Valent (1978) observed linear response for the glyceollin accumulation in soybean cells treated with *P. megasaperma* var. Sojae (PMS). A hyperbolic curve of the PAL activity was observed by Ebel et al. (1976), after elicitation of soybean cell suspensions with PMS. More complex relationships were observed by Dixon and Lamb (1979) and Lawton et al. (1980) for the induction of PAL and phaseollin accumulation, in bean cell suspensions treated with *Colletotrichum lindemuthianum*.

Some works (Lawton et al., 1983; Bell et al., 1984; Hahlbrock and Schell, 1989; Campbell and Ellis, 1992) point the enzyme PAL as the precursor of the lignin biosynthesis, phenols, flavonoids and phytoalexines by plant tissues, related to the plant response system against microorganisms, insects and other stress factors.

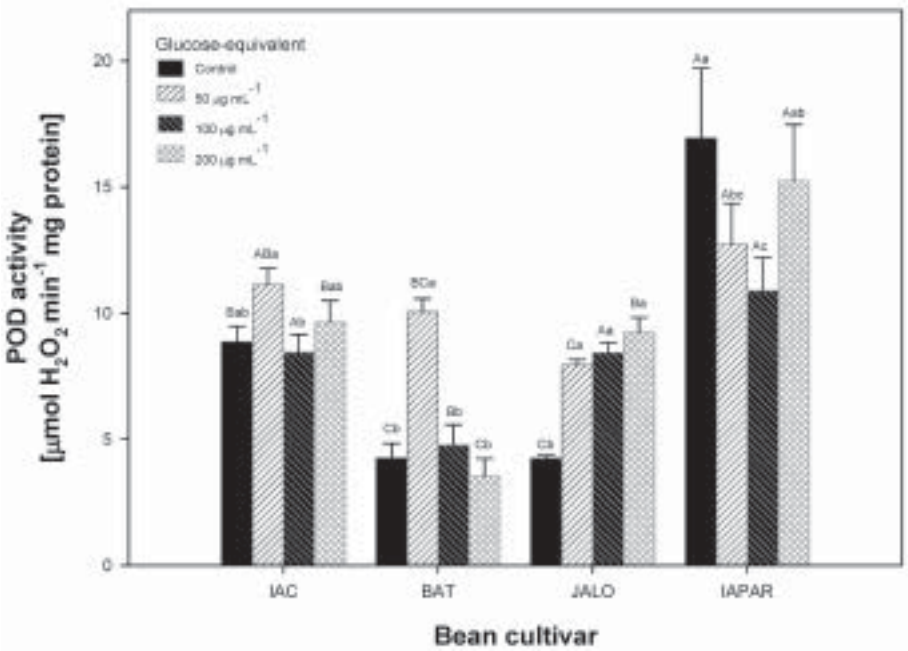

Fig. 2. Changes in the peroxidase activity in suspension-cultured *Phaseolus vulgaris* cells 8 h after treatment with an elicitor released from the wall of *Fusarium oxysporum*. Significant differences between means (extracts from 6 individual probes per treatment) of the different cultivars or different treatments within the genotypes are marked with different letter (Tukey test, 5%); Vertical bars indicate ± SE.

An induction was observed at the highest level of elicitors application in the cv. JALO. As for cv. BAT, with the exception of 50 µg mL^{-1}, there were not significant differences compared to the control. A decrease of enzyme activity due to all treatments was observed to the cv. IAPAR, compared to the control. The cells of cv. IAC didn't present differences among the treatments, compared to untreated cells (Figure 2).

The observed results with beans could reflect the cell response capacity due to the fungi cell wall hydrolisate applied. The potential of the cv. JALO is clear, presenting increase of activity of peroxidases in all applied treatments. Regarding the enzyme PAL, the cv. JALO also showed the highest activity among the tested materials, and it seems to be the most responsive genotype. The peroxidase activity, in general, increases under different stress conditions, like wounds, fungi infections, salinity, water stress and nutritional disorders, inducing also the lignin increment and production of ethylene (Van Huystee, 1987; Schallenberger, 1994). However, the shift in the activity of the referred enzyme can vary, motivating the discussion about peroxidase active role in the resistance (Moerschbacher, 1992). The peroxidase action could still happen in an indirect way, through the activity of sub-products, which

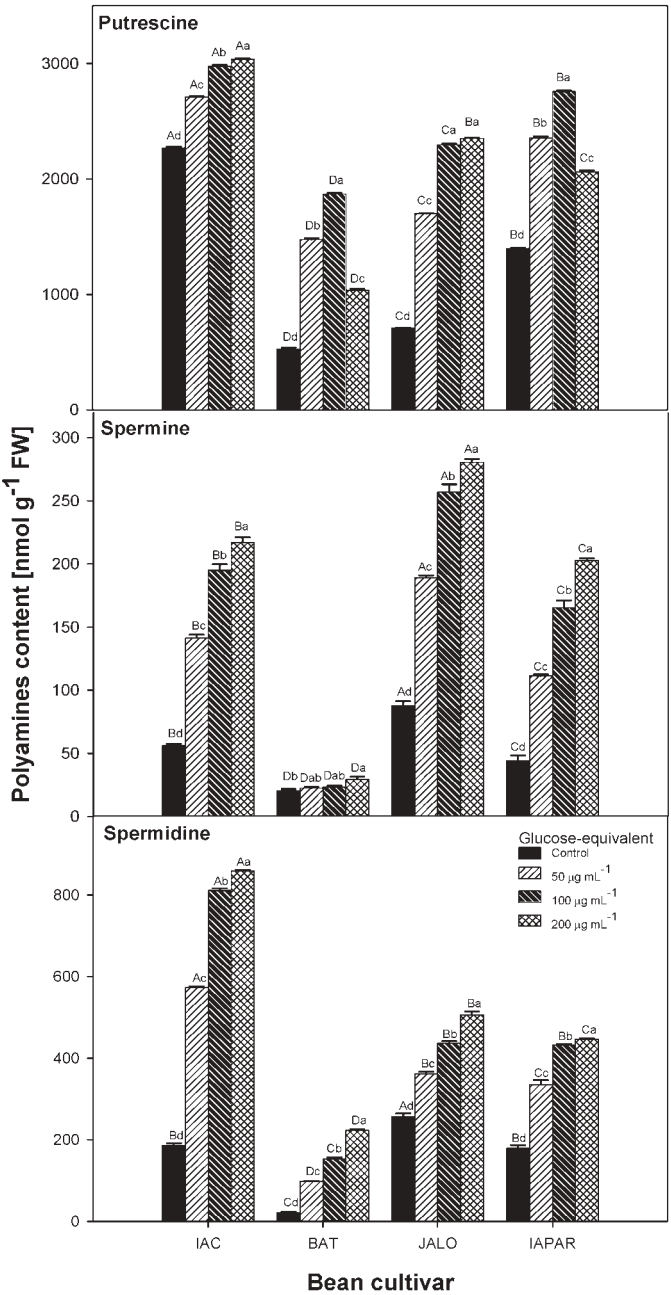

Fig. 3. Content of free polyamines in suspension-cultured *Phaseolus vulgaris* cells 48 h after treatment with an elicitor released from the wall of *Fusarium oxysporum*. Significant differences between means (extracts from 6 individual probes per treatment) of the different cultivars or different treatments within the genotypes are marked with different letter (Tukey test, 5%); Vertical bars indicate ± SE.

possessed an antimicrobial activity or by inducing the formation of structural barriers. Some mechanisms proposed to explain the peroxidase action and fenoloxidases during the host-pathogen interaction include that these enzymes induce the increase of the production of phenols oxidized at the cell wall (Retig, 1974). This activity, suggests a cell effort for the establishment of a physiochemical barrier, able to isolate the infected area (Urs and Dunleavy, 1975).

Changes of di- and polyamines contents can be expected to occur as a response to tissue infection. In this study, the incubation of bean cells with hydrolyzed *F. oxysporum* cell wall was effective to induce the biosynthesis of di- and polyamines for all studied genotypes (Figure 3). The accumulation of putrescine was clear, which was induced with levels higher than those of polyamines, mainly for the cv. IAC, JALO and IAPAR. Elicitation of cells with 100 μg mL^{-1} of glucose-equivalent was the most effective treatment for accumulation of putrescine. The polyamines spermine and spermidine accumulation presented only slight increases, especially in the highest glucose–equivalent concentration, with significant accumulation of these polyamines in cv. IAC and spermine in the case of cv. JALO. Plant polyamines (PA) biosynthesis in response to biotic stress agents has been an object of few studies, although some works about these interactions indicate that the metabolism of PA can play important role in the adaptation of plants to these agents (Walters, 2000).

Stoessl and Unwin (1978) demonstrated that the formation of cumaroyl-agmatine inhibited the fungi spores' germination in barley and this condition probably contributed to the resistance of barley plantlets to the fungi infection.

The formation of these compounds is catalyzed by the enzyme agmatine coumaroyl-transferase, which uses coumaroyl-CoA and agmatine (polyamine) as main substrate (Bird and Smith, 1983). In the present study, it was verified that the induction of the enzyme PAL and the formation of 4-coumaryl-CoA, probably favored the biosynthesis of substrate for the diamine formation and conjugated polyamines.

This hypothesis can be upheld by referring to the experiments conduced by Berlin & Forche (1981). Working with a *Nicotiana tabacum* cell culture, they observed that the increase of t-cinnamic acid synthesis (product of the PAL, utilizing L-*Phe* as substrate) was accompanied with increase of synthesis of cinnamyl-putrescine, which could exercise the function of phytoalexine.

In conclusion, the results of the present experiment suggest differences among the cultivars with respect to accumulation of di- and polyamines in elicited bean cells. This seems to indicate that the polyamines do, in fact, some function in the plants adaptation biotic stress.

The treatments induced the cells to accumulate di- and polyamines, principally for IAC and JALO. The accumulation of polyamines was greater in the treatments with higher concentration of elicitors.

The activity of the studied enzymes (PAL and peroxidases) presented different

levels of induction according to tested cultivar, with a strong dose-response relationship, mainly for IAPAR, BAT and IAC.

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