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Effect of biotic elicitors on polyphenol production in *Cayratia trifolia* cell suspension cultures analyzed by HPLC

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Abstract

Cayratia trifolia Linn. Domin Syn. *Vitis trifolia* (family: *Vitaceae*) contains polyphenols as major bioactive compounds. Plant polyphenolics, such as stilbenes, possess a broad spectrum of pharmacological and therapeutic effects, including antioxidative, anticancerous, antiatherosclerotic, cardioprotective, hepatoprotective, and neuroprotective effects. Elicitation is one of the important strategies to enhance secondary metabolite production in cell suspension cultures. In the present study, the effect of two biotic elicitors, namely one fungal (*Fusarium* sp., and *Helminthosporium* sp.) extract and another higher plant-originated gum (*Anogeissus latifolia*), was evaluated for enhanced production of stilbenes in cell suspension culture of *C. trifolia*. For the fungal elicitor, the cell suspension cultures of *C. trifolia* were treated with varying concentrations of *Fusarium* extract (FE) and *Helminthosporium* extract (HE) on the 7th day of culture. Increasing concentration of FE in the medium resulted in slightly decreased cell growth but increased the production of stilbenes, with the optimal concentration at 1000 mg/l. This increase in the yield of stilbenes was ~5 times (9.30 mg/l) higher than the control value. HE was less effective than FE in increasing stilbene production at optimal concentrations. The addition of 50 mg/l *A. latifolia* plant gum sieved through a 150 mesh (0.1 mm) to *C. trifolia* cell culture enhanced stilbene accumulation and yield by 8- to 9-fold over the control value.

Key words: *Cayratia trifolia*, fungal elicitors, gum, suspension culture

Introduction

The term phytoalexin (from the Greek word *phyton* meaning "plant" and *alexein* meaning "to defend") was first proposed by Müller and Börger (1940) in their hypothesis that defense responses in plants (potato) could be activated after infection with fungi (*Phytophthora infestans*). The NATO Advanced Study Institute on "Active Defense Mechanisms in Plants" (Paxton, 1981) defined phytoalexin as "low molecular weight antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms." Phytoalexins have received much attention over the past 70 years, and much of this work has provided new insights on gene regulation and phytochemical diversity specially related to secondary metabolites. Elicitors are physical or chemical factors that trigger morphological and physiological responses such as phytoalexin accumulation when they come in contact with living plant cell systems. Elicitors are either biotic or abiotic in origin. Abiotic elicitors are substances of non biological origin such as organic salts and physical factors (Angelova et al., 2006). Biotic elicitors are substances with biological origin, e.g., extracts of pathogenic microorganisms (chitins and glucans), enzymes such as cellulase or pectinase, and plant gums and glycoproteins,whose functions are coupled to receptors and act by activating or inactivating a number of enzymes or ion channels (Goyal et al., 2012). The interaction of any of these components with the plant cells induces numerous defense responses, which include the production of phytoalexins, pathogenesis-related proteins, protease inhibitors, and a variety of other defense compounds (Arora et al., 2012).Recent studies have shown that appropriate biochemical treatment and manipulation (e.g., elicitors and precursors) can result

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in an increase in the production of some secondary compounds and accumulation of chemicals that would not normally be synthesized in the source plants (Giri and Zaheer, 2016; Naik and Al-Khayri, 2016). Responses induced by plant oligosaccharides include stem elongation, stimulation of ethylene production, auxin antagonization, and elicitation of various defensive actions. The structurally defined fragments of plant cell wall polysaccharides can also function in plant cell/tissue culture as chemical messages with specific regulatory properties (Winkler et al., 2017; Xu et al., 2017). Biotic elicitors such as chitosan and its oligomers obtained from fungal cell wall (Katiyar et al., 2015) and plant gums (Dass and Ramawat, 2009) have shown strong elicitation effect in cell cultures for the production of secondary metabolites. Previous studies have reported increased production of indole alkaloids in cell suspension cultures of *Catharanthus roseus* (Liang et al., 2018; Sharma et al., 2019) and taxol and related taxanes in suspension cultures of *Taxus* sp. (Vidal-Limon et al., 2016; Sarmadi et al., 2018). Among abiotic elicitors, salicylic acid (SA) and methyl jasmonate (MeJa) induced high accumulation of isoflavonoids in cell cultures of *Pueraria tuberosa* (Goyal and Ramawat, 2008), and stilbenes in cell cultures of *C. trifolia* (Roat and Ramawat, 2009a). According to Ramirez-Estrada et al. (2016), elicitors have different mechanisms of elicitation, and when used in combination, they synergistically enhance metabolite production in cultured plant cells. Enhancement of secondary metabolites by elicitation is one of the few strategies that has recently found commercial applications such as production of pharmaceuticals, insecticides, flavoring agents, drugs (morphine, codeine, cocaine, quinine, etc.), and many other important biochemicals (Silpa et al., 2018). We have previously reported the production of stilbenes stimulated by elicitors (Roat and Ramawat, 2009a) and by plant growth regulators in cell cultures (Roat and Ramawat, 2009b) and root cultures (Arora et al., 2009) of *C. trifolia*. In the present study, we report enhanced production of stilbenes in *C. trifolia* cell cultures through the use of two biotic elicitors (fungal and gum).

Materials and methods

Cell cultures and experimental setup

Cell suspension culture were initiated from 4-week-old callus (grown from *C. trifolia* leaf explants) by transferring 2 g friable cream colored callus into 250 ml Erlenmeyer flask containing 50 ml of liquid MS basal medium (Murashige and Skoog, 1962) supplemented with 2,4-D at 0.25 mg/l, NAA at 0.2 mg/l, kinetin at 0.2 mg/l, and casein hydrolysate at 250 mg/l with the addition of 3% w/v sucrose. Subsequently, 2,4-D was removed from the medium, and the cultures were maintained in MS basal medium supplemented with NAA at 0.25 mg/l and kinetin at 0.2 mg/l (growth medium). Subcultures were performed at every 15th day by transferring 15 ml (~125 mg dry mass (DM)) of the culture into 85 ml of fresh medium. The cultures were grown in dark at 25°C on an orbital shaker at 100 rpm.

Effect of fungal elicitor

Cell cultures were treated with dried powder of fungal cell wall obtained from autoclaved cultures of two fungal species (*Fusarium* species and *Helminthosporium* species). Different concentrations (50, 100, 200, 500, 1000, and 2000 mg/l) of autoclaved aqueous suspensions were added to the medium on the 7th day of culture. These suspensions were *Fusarium* extract (FE) and *Helminthosporium* extract (HE). Time course study was performed for cell growth and stilbene production.

Effect of gum polysaccharides as elicitor

Gum samples obtained from *Anogeissu slatifolia* (gum ghatti) were grinded in a mortar and strained through two different sized sieves (0.4 mm, 40 mesh and 0.1 mm, 150 mesh; Sigma Co., USA). Fatty acids and pigments were extracted overnight from the gum by using chloroform. Chloroform was then removed by filtration. Polyphenol and low-molecular-weight compounds were extracted from the residues with methanol several times until a colorless extract was obtained. After filtration, the residue was washed thrice in ice-cold water. The gum powder (50, 150, and 300 mg/l) thus obtained was added to the growth medium on the 7th day of culture, and the cells were harvested on the 15th day of culture.

Growth determination

The cells were harvested after 15 days by vacuum filtration, rapidly washed with cold distilled water, and weighed to obtain fresh mass per 100 ml medium. DM was then determined by drying the cells at 60°C in an oven until a constant weight was obtained.

Determination of Stilbene content by HPLC

The cell cultures were harvested, washed with distilled water, and filtered under mild vacuum. Dried homogenized cells (50 mg) were extracted in acetone-water (3 : 2 (v/v)) for 12 h (ambient temperature) on a test tube rotator and centrifuged at 2000 rpm for 15 min, and the supernatant was then concentrated under vacuum at 40°C until the complete removal of acetone. The aqueous extract was then partitioned twice with an equal amount of ethyl acetate. Finally, the ethyl acetate phase was concentrated under vacuum till dryness. This extract was re-dissolved in HPLC-grade methanol, filtered, and analyzed by HPLC (pump L2130, autosampler L-2200, FL detector L-2485, Merck-Hitachi). Separation was accomplished on a (LichroCART)® 250 × 4 mm Purospher® (5 mm) RP-18 column protected by a guard column of the same material. The solvent system used was as follows: Solvent A- 0.0025% TFA in water; solvent B- 80% ACN (E. Merck, India) in solvent A. The mobile phase consisted of solvent A and solvent B. The step gradient program of solvent B was as follows: 0–3 min, 14%–18%; 3–12 min, 18%–18%; 12–25 min, 18%–22%; 25–30 min, 22%–22%; 30–38 min, 22%–40%; 38–43 min, 40%–40%; 43–46 min, 40%–60%; 46–48 min, 60%–70%; 48–50 min, 70%–70%; 50–52 min, 70%–80%; 52–54 min, 80%–80%; 54–56 min, 80%–85%; 56–58 min, 85%–100%; 58–60 min: 100%–100%; 60–62 min, 100%–14%; 62–65 min, 14%–14%. Separation+ was performed at a flow rate of 1.0 ml/min, and chromatographic peaks were monitored at λ exc 300 nm and λ em 390 nm (Krisa et al., 1999). Standard compound trans-resveratrol (3,5,4′-trihydroxystilbene) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and trans-piceid (3,3,4,5′-tetrahydro-xystilbene), trans-ε-viniferin, and ampelopsin A were kindly provided by Prof. J.M. Merillon, France. Standard compounds were dissolved in methanol to yield a final concentration of 1.0 mg/l, and a standard curve was prepared using resveratrol with concentrations ranging from 50 to 500 ng/ml. The amount of the other compounds was calculated on the basis of trans-resveratrol. The results of stilbenes are expressed as μg/g cell DM.

Statistical analysis

All results were averaged over two separate analyses for stilbene estimation, and two consecutive laboratory experiments with six replicate flasks for each treatment were performed for growth value determination. For growth and individual stilbene concentration, the data were analyzed by ANOVA followed by Dunnett's multiple comparison test (comparing all versus control) using Prism statistical software. Mean values were considered statistically significantly at $P \leq 0.05$ when compared with control.

Results

Effect of fungal elicitors

Time course for stilbene induction

The results for the time course experiments for the induction of stilbenes in cultures treated with 200 mg/l of FE and HE are presented in Table 1. In the cultures treated with 200 mg/l FE added on the 7th day of culture, maximum concentrations of piceid (67.8 μg/g), resveratrol (76.0 μg/g), and ampelopsin (433.1 μg/g) were recorded, while viniferin (82.8 μg/g) concentration was decreased when compared with control conditions. The concentration of stilbenes decreased when FE was added at the 10th and 12th day of culture; however, except for viniferin, it remained higher than the control value. The cultures accumulated maximum total stilbenes (659.8 μg/g) on the 7th day of treatment with a yield of 5.2 mg/l (Table 1). On the basis of these results, different concentrations (50, 100, 200, 500, 1000, and 2000 mg/l) of FE were added on the 7th day of culture to obtain effective concentration of FE for increased accumulation of stilbenes. In the cultures treated with 200 mg/l HE, added on the 7th day of culture, maximum concentrations of piceid (16.6 μg/g), viniferin (169.0 μg/g), and ampelopsin (326.3 μg/g) were recorded as compared to control conditions, while the maximum concentration of resveratrol (95.8 μg/g) was recorded when HE was added on the 10th day of culture. The concentrations of all stilbenes decreased when HE was added on the 10thand the 12th day of culture as compared to that when added on the 7th day; however, except for resveratrol, it remained higher than the control value. The cultures accumulated maximum total stilbenes (540.3 μg/g) on the 7th day of treatment with a yield of 4.2 mg/l (Table 1). On the basis of these results, different concentrations of HE were added on the 7th day of culture to obtain effective concentration of HE for increased accumulation of stilbenes. An authentic

Table 1. Time course of cell growth and stilbene production in *C. trifolia* cell culture grown in 250 ml flasks in growth medium elicited with fungal elicitor

Fungal elicitor [200 mg/l]	Day of addition	DM [g/l]	Stilbene concentration µg/g DM ± SD					Yield [mg/l]
			piceid	resveratrol	viniferin	ampelopsin	total	
	control	8.1	12 ± 1.1	26.1 ± 1.8	159.0 ± 9.0	50.0 ± 4.8	248.4	2.0
Fusarium extract	7th day	7.9	67.8 ± 4.5	76.0 ± 4.8	82.8 ± 4.9	433.1 ± 22.5	659.8	5.2
	10th day	8.0	51.2 ± 3.5	45.9 ± 3.5	50.2 ± 2.5	357.6 ± 18.9	504.9	4.0
	12th day	8.0	48.2 ± 2.5	37.1 ± 2.8	91.1 ± 4.6	161.7 ± 7.6	373.9	2.9
Helminthosporium extract	7th day	7.8	16.6 ± 1.4	28.4 ± 1.6	169.0 ± 6.2	326.3 ± 27.3	540.3	4.2
	10th day	7.9	10.4 ± 1.2	95.8 ± 3.2	104.1 ± 5.1	240.3 ± 20.5	450.6	3.6
	12th day	8.0	9.8 ± 1.3	53.1 ± 2.3	90.6 ± 4.5	262.7 ± 12.1	416.2	3.3

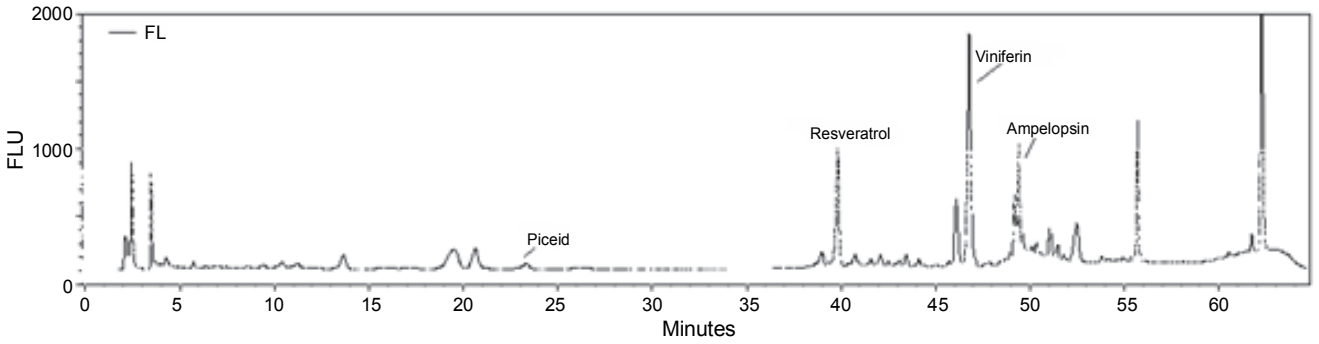

Fig. 1. HPLC chromatogram showing four important stilbenes, namely piceid, resveratrol, viniferin, and ampelopsin, for reference

chromatogram showing four important stilbenes, namely piceid, resveratrol, viniferin, and ampelopsin, is given for reference (Fig. 1).

Effect on stilbenes production by different concentrations of FE and HE

The cell suspension cultures of *C. trifolia* were treated with varying concentrations of FE and HE on the 7th day of culture (Table 2 and Table 3). Increasing concentration of FE in the medium resulted in slightly decreased growth of the cells (Table 2), while the increasing concentration of elicitor effectively increased stilbene productionin the medium, with the optimal concentration at 1000 mg/l. This increase in the yield of stilbenes was ~5 times (9.30 mg/l) higher than the control value (2.01 mg/l). With further increase in the concentration to 2000 mg/l, the total yield of stilbenes was 7 mg/l, which was also significantly higher than the control value. Therefore, it was concluded that FE can be used as a triggering agent for stilbene induction at the onset of stationary phase cultures. The concentrations of piceid (147.2 µg/g), resveratrol (137.5 µg/g), and ampelopsin (843.7 µg/g) showed a marked increase as compared to the control value with increasing concentration of FE up to 1000 mg/l, while viniferin concentration showed a gradual decrease with increasing concentration of FE as compared to the control value. Because viniferin is known to be a dimmer of resveratrol (Pezet et al., 2003), its decrease is correlated with the increase in resveratrol concentration. When HE was added to cell cultures in increasing concentration, a trend similar to that of FE was observed in terms of cell growth and stilbene production, but overall HE was less effective than FE at the optimal concentration (Table 3). Resveratrol (138.5 µg/g) and viniferin (820.6 µg/g) concentrations increased with an increase in HE concentration up to 2000 mg/l, while the maximum concentrations of piceid (23.3 µg/g) and ampelopsin (326.6 µg/g) were obtained at 500 and 200 mg/l HE concentrations, respectively.

Table 2. Effect of fungal elicitor (*Fusarium* extract, added on the 7th day) on cell growth and stilbene accumulation in *C. trifolia* cell cultures grown in 250 ml flasks in growth medium; the data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (comparing all versus control)

Fungal elicitor [mg/l]	DM [g/l]	Stilbene concentration μg/g DM ± SD					Yield [mg/l]
		piceid	resveratrol	viniferin	ampelopsin	total	
Control	8.1	12 ± 1.1	26.1 ± 1.8	159.0 ± 9.0	50.0 ± 4.8	248.4	2.01
50	8.1	22.9 [d] ± 1.4	32.5 [c] ± 1.8	143.8 [c] ± 6.1	163.1 [d] ± 7.1	362.3 [c]	2.93
100	7.9 [d]	35.1 [d] ± 2.3	35.43 [c] ± 5.7	133.7 [d] ± 5.8	399.0 [d] ± 22.8	603.3 [d]	4.77
200	7.9 [d]	67.8 [d] ± 4.3	76.0 [d] ± 6.2	122.0 [d] ± 5.4	433.2 [d] ± 22.1	699.0 [d]	5.52
500	7.8 [d]	120.7 [d] ± 2.3	101.5 [d] ± 5.3	112.0 [d] ± 5.1	444.4 [d] ± 23.2	778.9 [d]	6.08
1000	7.7 [d]	147.2 [d] ± 6.2	137.5 [d] ± 4.2	80.1 [d] ± 2.3	843.7 [d] ± 38.2	1208.4 [d]	9.30
2000	7.6 [d]	24.7 [d] ± 1.4	100.2 [d] ± 4.1	70.8 [d] ± 5.6	726.5 [d] ± 25.4	922.2 [d]	7.00
F-value	26.9 [b]	813 [b]	274 [b]	90.5 [b]	512 [b]	229.3 [b]	

a – non significant ($P > 0.05$), b – significant ($P < 0.01$), c or d – mean values are significantly different when compared with control at ($P < 0.05$) or ($P < 0.01$), according to Dunnett's multiple comparison test; mean data without footnote label are non significantly different from the control value

Table 3. Effect of fungal elicitor (*Helminthosporium* extract, added 7th day) on cell growth and stilbene accumulation in *C. trifolia* cell cultures grown in 250 ml flasks in growth medium; the data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (comparing all versus control)

Fungal elicitor [mg/l]	DM [g/l]	Stilbene concentration μg/g DM ± SD					Yield [mg/l]
		piceid	resveratrol	viniferin	ampelopsin	total	
Control	8.1	12.0 ± 1.2	26.1 ± 1.9	159.0 ± 7.1	50.0 ± 4.8	248.4	2.01
200	8.0	16.6 [d] ± 1.4	28.4 ± 1.6	169.0 ± 6.2	326.3 [d] ± 27.3	540.3 [d]	4.32
500	7.9 [d]	23.3 [d] ± 1.8	102.9 [d] ± 5.1	410.8 [d] ± 25.5	164.4 [d] ± 6.3	701.5 [d]	5.54
1000	7.8 [d]	17.3 [d] ± 1.6	109.9 [d] ± 5.8	661.5 [d] ± 35.6	92.1 [d] ± 4.2	834.8 [d]	6.51
2000	7.7 [d]	13.9 ± 1.1	138.5 [d] ± 6.3	820.6 [d] ± 43.3	30.9 ± 2.1	1003.9 [d]	7.73
F-value	39.5 [b]	26.6 [b]	477 [b]	335 [b]	259 [b]	206 [b]	

a – non significant ($P > 0.05$), b – significant ($P < 0.01$), c or d – mean values are significantly different when compared with control at ($P < 0.05$) or ($P < 0.01$), according to Dunnett's multiple comparison test; mean data without footnote label are non significantly different from the control value

Discussion

Elicitors of fungal origin have been widely used to increase natural product formation in plant cell culture, and this strategy has been effective in stimulating the production of many chemical classes of secondary metabolites, such as terpenoids, coumarin derivatives, alkaloids, and flavonoids (Espinosa-Leal et al., 2018; Salehi et al., 2018). When grapevine experiences biotic stress, stilbenes act as phytoalexins. Grey mold (*Botrytis cinerea* Pers.), downy mildew (*Plasmoparaviticola* Berk.), berry rot (*Rhizopusstolonifer* Ehrenb. Fr. Lind), and some *Aspergillus* spp. are some of the main fungi that attack the grapevine and lead to the production of *trans*-resveratrol, *ε*-viniferin, *α*-viniferin, *δ*-viniferin, pterostilbene, and piceatannol, in both grape berries and leaves (Saigne-Soulard et al., 2015). In *Linum album* hairy roots fungal elicitation causedan increase in the levels of amino acids, phenolic compounds, and lignin (Tashackori et al., 2019). Chitosan and salicylic acid enhanced biomass accumulation and polyphenols in callus cultures of *Fagoniaindica* (Khan et al., 2019). The endophytic fungal elicitor has also been shown to increase the expression of specific genes in medicinal plants, which can result in enhanced accumulation of bioactive molecules by

the activation of a series of specific secondary metabolic pathways (Zhai et al., 2017). Various strategies using fungal elicitors for enhancing the production of secondary metabolites have been briefly described by Shasmita et al. (2018) and Baldi et al. (2009). The fungal elicitor *Aspergillus flavus* enhanced the total indole alkaloid production of *Catharanthusroseus* cambial meristematic cell cultures. Transcriptome analysis of such cultures showed increased expression of various genes of the biosynthetic pathway of indole alkaloids (Liang et al., 2018). Various signaling pathways and their receptors such as avirulent determinants and corresponding plant R proteins; heterotrimeric and small GTP binding proteins; ion fluxes, especially Ca^{2+} influx, and Ca^{2+} signaling are regulated by elicitors for enhanced production of secondary metabolites (Zhao et al., 2005).

In the present study, a 5-fold increase in total stilbene production by cell suspension culture in the presence of fungal elicitors was significant and can be used in combination with other PGRs, sucrose, and precursors, to further enhance stilbene production. Attempts were made to grow cells with all elicitors in shake flasks and bioreactor (Goyal and Ramawat, 2008a). Significant effects of fungal elicitors on stilbene production and accumulation in plant cell cultures were demonstrated in this work. This effect of biotic elicitors can be further investigated for the high yield of secondary metabolites in a short time in bioreactors. Reduction in time and increase in production have significant effects on cost effectiveness.

Gum polysaccharides as elicitors

The results obtained with gum ghatti are presented in Figure 2. In the treatment, the following two parameters were measured: 1) effect of gum polysaccharides (50–300 mg/l) as elicitor and, 2) effect of particle size of gum (strained through two different sized sieves: 0.4 mm, 40 mesh and 0.1 mm, 150 mesh) on stilbene production. All three concentrations of the elicitor did not alter the growth of *C. trifolia* cells; however, stilbene accumulation and yield was enhanced by 8- to 9-fold over the control. Gum ghatti at 50 mg/l concentration was most effective in enhancing stilbene accumulation (7.2 mg/l), followed by the concentrations of 150 (3.44 mg/l stilbene) and 300 mg/l (2.77 mg/l stilbene) in the medium in both particle sizes. Gum resin production is a stress-induced phenomenon, and high yields are obtained during pathogenesis abiotic and biotic stress. Plant gums, especially gum ghatti, is a complex mixture of polysaccharides and arabinogalactan proteins (Pal, 2008; Kora et al., 2012). Several types of gelling agents including plant gums have been used in plant tissue culture (Jain et al., 2005), but their role as elicitors has been demonstrated only in *Commiphora wightii* (Das and Ramawat, 2009). Failure of higher concentrations of the used gum to elicit higherstilbene accumulation may be explained on the basis of oxidative stress due to H_2O_2 production, leading to cell death (Yu et al., 2002). When the gum was strained through 150 mesh metal sieves and added to the cell culture, it was more effective in stimulating stilbene production than gum strained through 40 mesh metal sieves (Fig. 2) as particle with smaller sizes have more contact with cell surface. The problem of size effect on cell uptake of nanoparticles is currently an important issue in the field of nanobiology, and it has been shown in various drug delivery systems that particle size, shape, and surface charge affect the cellular uptake of particular compounds (He et al., 2010; Kulkarni and Feng, 2013; Zheng and Yu, 2016)

It has been found that particle size can affect the efficiency and pathway of cellular uptake by influencing the adhesion of the particles and their interaction with cells. However, many factors, such as surface chemistry, ion charge, and size of the elicitor compound affect its uptake (Lu et al., 2009; He et al., 2010). The use of gum as an elicitor is a new concept (Dass and Ramawat, 2009). In *C. trifolia* cell cultures, gum ghatti stimulated higher stilbene accumulation at lower concentrations; hence, it could be used in combination with precursors (Phenyl ammonia lyase, cinnamic acid etc.) and different abiotic elicitors for further enhanced accumulation of stilbenes as a cost-effective system. Because gum is effective at small particle sizes, it can be used at nano sizes, which could open new possibilities in secondary metabolite production through cell suspension cultures, as the concept can be used with other biotic and abiotic elicitors.Treatment with biotic and abiotic elicitors can be an effective strategy to increase the yield of specific metabolites obtained from *C. trifolia* cell cultures. Understanding how plant cells perceive these elicitors and how this perception leads to the accumulation of secondary metabolites may help us to improve the production of stilbenes in terms of quantity and quality of the compounds. The knowledge gathered in the past

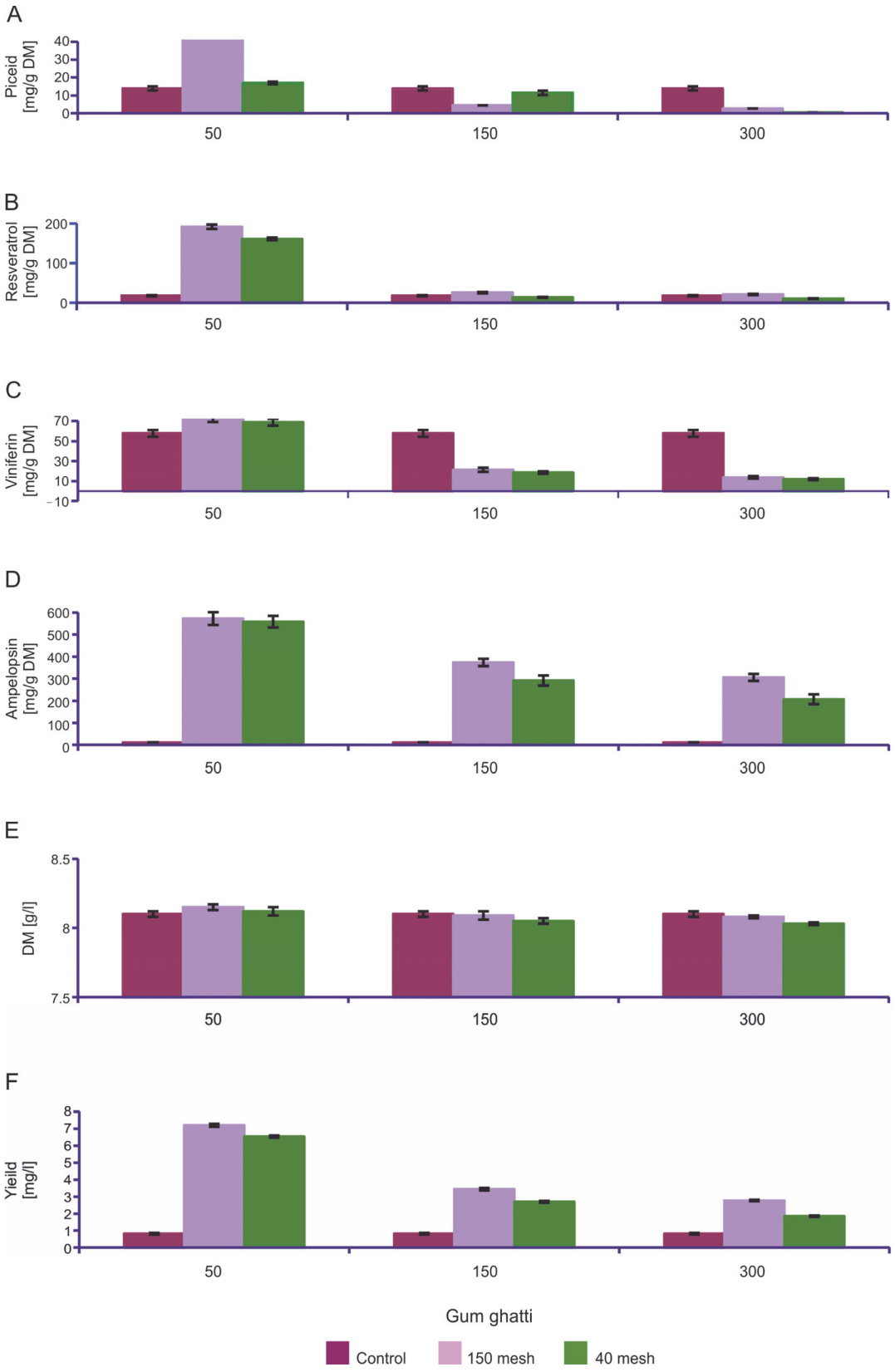

Fig. 2. Effect of varying concentration of gum ghatti when sieved through two different mesh sizes (0.4 mm, 40 mesh and 0.1 mm, 150 mesh) on stilbene production of A) piceid, B) resveratrol, C) viniferin, D) ampelopsin, E) total cell growth and F) total yield in *C. trifolia* cell cultures grown in 250 ml flask, in growth medium

decades on elicitor perception and transduction is now being combined with high-throughput methodologies such as transcriptomics and metabolomics to engineer plant cells that can produce compounds of interest at the industrial scale (Salehi et al., 2019). Thus, we conclude that diverse elicitors can promote higher accumulation of secondary metabolites in *C. trifolia* cell cultures.

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