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ORIGINAL ARTICLE

Elicitor enhanced production of protoberberine alkaloids from in vitro cell suspension cultures of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms

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Abstract The present research investigates the effect of *Piriformospora indica*, an endophytic fungus, on production of protoberberine alkaloids in in vitro cell suspension cultures of *Tinospora cordifolia*. Although *T. cordifolia* produces a number of protoberberine alkaloids, the simultaneous production of jatrorrhizine and palmatine in cell suspension cultures of *T. cordifolia* was observed for the first time with the use of *P. indica* as biotic elicitor. The cells in suspension cultures were elicitated with *P. indica* on 14th day of culture initiation and the production of the alkaloids on 16th day was monitored. The autoclaved as well as filter sterilized cultures of *P. indica* were used in addition to the use of fungal cell extract. The elicitor effect of *P. indica* was analyzed and compared with other abiotic elicitor (methyl jasmonate) and biotic elicitors (chitin and chitosan). The culture filtrate of *P. indica* in the filter sterilized (5.0% v/v) form gave better response with enhanced 4.2-fold production of jatrorrhizine (10.72 mg/g DW) and 4.0-fold production of palmatine (4.39 mg/g DW). The production of these compounds was at par with that achieved in methyl jasmonate (at 250 µM) treated cell suspension cultures.

Keywords Abiotic elicitor · Biotic elicitor · Jatrorrhizine · Palmatine · *Piriformospora indica*

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Introduction

Tinospora cordifolia (Willd.) Miers ex Hook. F. & Thoms., commonly known as Giloy, Guduchi and Amrita in India, belongs to the family of Menispermaceae. This woody creeper is deciduous in nature with succulent stem bearing long filliform, fresh aerial roots and heart-shaped membranous chordate leaves. It produces a plethora of diverse categories of bioactive compounds, such as diterpenoid lactones, alkaloids, sesquiterpenoids, phenolics, steroids, glycosides, polysaccharides and other aliphatic compounds. *Tinospora* is an ancient Ayurvedic medicinal plant, known to possess anti-pyretic, anti-diabetic, anti-inflammatory, anti-neoplastic, immunomodulatory and hepatoprotective activity, which is clinically proven (Singh et al. 2003). Alkaloid fraction from stems of *T. cordifolia* have been reported to contain protoberberine alkaloids, such as jatrorrhizine, magnoflorine and palmatine, that exhibited hypoglycemic effect in rats (Patel and Mishra 2011). Berberine and other protoberberine alkaloids are potential adjuvant antibiotic drugs (Yu et al. 2005; Wang et al. 2009), possess antimalarial property (Vennerstrom and Klayman 1988) and also have toxic effects on insects (Philogene et al. 1984; Shields et al. 2008). The production of these alkaloids increases in the plant as a result of defense mechanism.

A root colonizing endosymbiont fungus, *Piriformospora indica* of the class Basidiomycota and order Sebacinales, was used in this study to elevate the production of alkaloids in plant cell suspension cultures. In nature, this fungus forms symbiotic interaction with the host plants like arbuscular mycorrhizal fungus, but can also be grown on several synthetic media as pure culture (Singh et al. 2013). Phytopromotional outcomes of *P. indica* include increased growth and development of the plant, nutrient uptake, abiotic along with biotic stress tolerance, biotization and

stimulation of secondary metabolite accumulation (Varma et al. 1999; Franken 2012). There are a few reports which mention the use of this fungus in stimulating the production of bioactive metabolites in several plants, such as spilanthol in *Spilanthes calva* (Rai et al. 2004), artemisinin in shoots of *Artemisia annua* (Sharma and Agrawal 2013), curcumin and volatile oil in *Curcuma longa* (Bajaj et al. 2014) and ursolic acid (UA), oleanolic acid (OA) and betulinic acid (BA) in *Lantana camara* (Kumar et al. 2016). The fungus-induced enhanced production of medicinal compounds is not only limited to field grown plants but also observed in in vitro grown plant cell cultures and hairy root cultures (Baldi et al. 2010).

The purpose of the present study, therefore, was to investigate if *P. indica* had a positive effect on enhanced production of protoberberine alkaloids in in vitro cell suspension cultures of *T. cordifolia* also, and to compare its effect with that of other biotic elicitors (chitin and chitosan) and abiotic elicitor (methyl jasmonate). Accordingly, different types and concentrations of the elicitors were used to assess their effect on growth of *T. cordifolia* and on production of jatrorrhizine and palmatine.

Materials and methods

Establishment of in vitro cell culture and culture conditions

For the establishment of callus cultures of *T. cordifolia* (Accession Number GUBH 79866, Gauhati University, Guwahati, India), leaf-disc explants of 5 mm size were surface sterilized and inoculated in Murashige and Skoog (1962; MS) medium supplemented with 11 µM benzyl aminopurine (BAP; Sigma-Aldrich, St. Louis, MO) and 5 µM 1-naphthaleneacetic acid (NAA; Sigma-Aldrich, St. Louis, MO) in glass test tubes (25 × 150 mm, O.D. × length, Borosil Glass Works Ltd, Gujarat, India). The medium was enriched with 3% sucrose, pH was adjusted to 5.8 with 1 N NaOH/HCl, solidified with 0.8% agar and autoclaved at 121 °C for 15 min. Callus initiation was observed after 6 weeks from the margins of the leaf-disc explants. It was further multiplied on MS semi-solid medium enriched with BAP (2.2 µM) and NAA (19.8 µM) (Rao et al. 2008). The 3-weeks old, friable, healthy callus with inoculum size of 3.0 g/l [dry weight (DW) basis] was transferred to liquid MS medium enriched with BAP (2.2 µM) and NAA (19.8 µM) in 250 ml Erlenmeyer flasks (85 × 145 mm, O.D. × height, Borosil Glass Works Ltd, Gujarat, India) The cultures were incubated at 25 ± 2 °C on a gyratory shaker at 120 rpm under a photoperiod of 16 h light and 8 h dark (Rao et al. 2008). The cells from the flasks were collected at every 2 days interval to analyze dry cell weight and to quantify the two alkaloids content, jatrorrhizine and palmatine. The values are represented as mean value ± SD of triplicate readings.

Preparation of cell extracts of *P. indica* as a biotic elicitor

Piriformospora indica (DSM 11827, Deutsche Sammlung-für Mikroorganismen und Zellkulturen, Braunschweig, Germany) was obtained from Prof. Ajit Varma, Amity University, Noida, India. The fungal cultures were maintained on Kaefer medium at pH 6.5 (Käfer 1977), agitation speed of 200 rpm and temperature of 30 ± 1 °C. According to Baldi et al. (2010), for preparation of biotic elicitors, fungal cultures were harvested on the 6th day (late exponential phase). The biotic elicitor preparations of *P. indica* (that is, autoclaved culture filtrate, filter-sterilized culture filtrate and fungal cell extract) were added to the cell suspension cultures of *T. cordifolia* on 14th day (in the late log phase). On 16th day, dry weight of the cells and the alkaloid content (jatrorrhizine and palmatine) were determined. Experiments were conducted in triplicates and the values were reported as average values ± SD of triplicates.

Application of chitosan and chitin as biotic elicitors and methyl jasmonate as abiotic elicitor

The cell suspension cultures were also treated with abiotic elicitor, such as methyl jasmonate (MJ) [95% pure from Sigma-Aldrich (St. Louis, MO)]. It was dissolved in 96% ethanol and filter sterilized before use. The cell cultures were treated with MJ to obtain 25, 62.5, 125, 250, 625 and 1250 µM of absolute concentrations. The biotic elicitor, chitin from shrimp shells (≥95% acetylation, Sigma-Aldrich) was prepared in 1 M acetic acid and was autoclaved. It was added to the cell culture to have final concentrations of 25, 50, 100, 250 and 500 mg/l. Chitosan, isolated from crab shells (>75% deacetylated; Sigma-Aldrich) was prepared in 5% (v/v) HCl and was stirred overnight (Wiktorowska et al. 2010). Chitosan was also added to the medium in the similar way as chitin.

Estimation of cell dry weight (DW)

The cells were harvested at designated time intervals, drenched with distilled water and filtered under vacuum using a Buchner funnel. They were further dried at 60 ± 2 °C until a constant weight was achieved. The weight of the cells was recorded as g/l DW (Godoy-Hernández and Vázquez-Flota 2012). Elicitor-treated cell cultures were also assessed similarly.

Extraction and quantification of alkaloids from cell cultures

Extraction of desired alkaloids from both treated as well as non-treated cell cultures was performed in methanol according to Zhao et al. (2001a, b) and the compounds were further quantified according to Patil et al. (2010) but in a modified version, in which the alkaloids were separated in a shorter run time of 25 min. Identification and quantification of compounds was performed on an Agilent 1200 HPLC system and the retention time of the compounds were compared with the retention time of the standards of jatrorrhizine (>95% pure) and palmatine with purity of >97% (Sigma-Aldrich, St. Louis, MO). The alkaloids were purified with the aid of Hypersil Gold™ column (250×4.6 mm; 5 µm; Thermo Scientific, USA) with a Hypersil Gold drop-in guard column (5 µm; 10×4 mm). The solvent system consisted of 20 mM ammonium acetate in Milli-Q (formic acid being used for pH adjustment to 4.8) as component A while component B was acetonitrile and was run at a flow rate of 0.6 ml/min. The linear gradient was optimized and began with 20–40% of B for 0–5 min, 40–45% of B for 5–15 min and 45–20% of B for 15–20 min, followed by equilibration time of 5 min. The temperature of the column was 25 °C and the target alkaloids were successfully detected at wavelength of 345 nm. Injection volume of 20 µl of each sample and the standards were used for the HPLC analysis and were filtered through 0.2 µm PTFE syringe filters (Merck-Millipore, Germany). The alkaloids were further verified by ESI-QTOF-MS analysis.

Confirmation of alkaloids by ESI-QTOF-MS

The confirmation of the alkaloids was successfully performed in ESI-QTOF-MS (Waters, Milford, PA). The electrospray source was operated in positive ion mode. The standards and the samples were run in ESI-MS, quadrupole time-of-flight premier mass spectrometer with microchannel plate detector, operated on desolvation temperature at 250 °C, desolvation gas flow rate at 450 l/h, cone gas flow rate at 50 l/h and capillary voltage at 2500 V. Mass-to-charge ratio was obtained in full scan mode from 100 to 1000 m/z values. MassLynx software (4.1) was used for procurement and analysis of MS data.

Statistical analysis

Every experiment was performed thrice and the values are represented as mean ± SD. All average observations were computed via one-way analysis of variance (ANOVA) and IBM SPSS Statistics software was used for data analysis. The Tukey post-hoc test was done on ANOVA to give multiple comparison table with significance level set to 0.05.

Results

Establishment of cell culture of *T. cordifolia*

In the present study, establishment of callus cultures of *T. cordifolia* was performed using 5 mm size leaf-disc explants from field grown parent plant (Fig. 1a, b). Callus initiation was observed from the margins of the leaf-discs within 6 weeks of culture on MS medium enriched with BAP (11 µM) and NAA (5 µM) (Fig. 1c). Callus cultures were further multiplied and maintained when transferred to MS medium containing BAP (2.2 µM) and NAA (19.8 µM) where the calli turned friable and light brown in color (Fig. 1d). They were sub-cultured into fresh medium every 3 weeks. Initiation of cell suspension cultures was obtained by sub-culturing fresh, friable and proliferative callus into the liquid medium of the same composition. Three weeks old cell suspension culture of *T. cordifolia* on the same medium is shown in Fig. 1e.

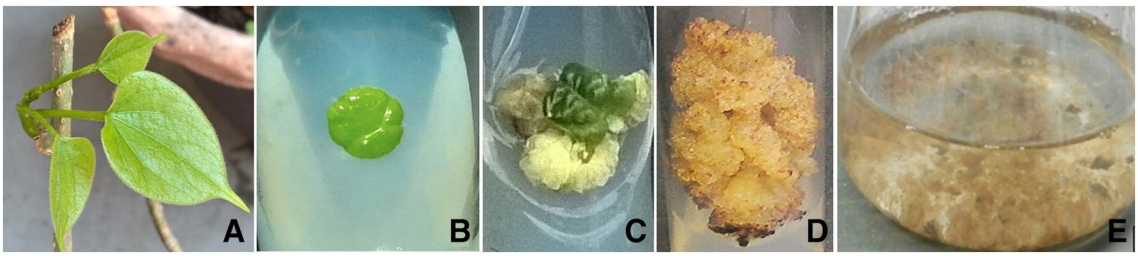

Fig. 1 Establishment of in vitro leaf cultures from *Tinospora cordifolia*. **a** Intact leaves, **b** 2-days-old leaf-disc explant at culture on MS + BAP (11 µM) + NAA (5 µM) medium, **c** same as (**b**), after 6 weeks, showing initiation of callus from the margins of the disc, **d** 3-week-old culture calli grown on (**c**) were subcultured on MS + BAP (2.2 µM) + NAA (19.8 µM) medium. Note the presence of light brown and soft callus multiplied on this medium, **e** 3-week-old cell suspension culture in the medium as in (**d**)

Growth profiling study and quantification of alkaloids

The growth profile of cell suspension culture of *T. cordifolia* was studied. It consisted of 4 days of lag phase followed by log phase of 13 days beginning from 4th day to 16th day of inoculation. Stationary phase of suspension culture started from 16th day and continued till 22nd day (Fig. 2).The highest concentration of biomass (12.1 g/l) was obtained on 16th day of inoculation. The alkaloids, jatrorrhizine and palmatine, were identified in the cell culture of *T. cordifolia* at retention times of 11.876 and 13.381 min, respectively (Fig. 3a), which were nearly the same for standard jatrorrhizine (11.893 min) and palmatine (13.251 min) (Fig. 3b). The confirmation of the presence of these alkaloids in the cell culture extract was done by ESI-QTOF-MS. Jatrorrhizine ($C_{20}H_{20}NO_4$) with monoisotopic mass of 338.12 m/z value (Fig. 4) and palmatine ($C_{21}H_{22}NO_4$) with monoisotopic mass of 352.14 m/z value (Fig. 5) was clearly seen at 338.17 and 352.20 m/z values, respectively, in methanolic cell extract (Fig. 6). There were no traces of these alkaloids in the culture medium, therefore, we inferred that the alkaloids get accumulated at intracellular compartment of the cell. The production profile indicated a maximum jatrorrhizine content of 2.55 mg/g DW (the corresponding concentration was 30.8 mg/l) and palmatine content of 1.08 mg/g DW (the corresponding concentration was 13.07 mg/l) on 16th day of cultivation. Therefore, 16th day was chosen as the best time for harvesting the suspension cultures of *T. cordifolia* to achieve maximum alkaloid concentration.

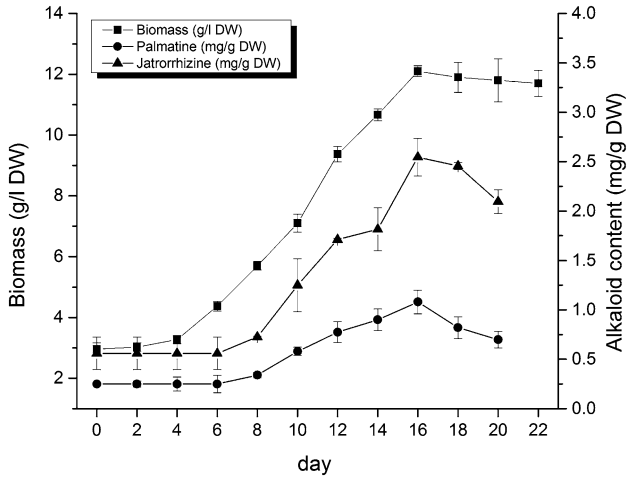

Fig. 2 Growth and alkaloids production profile in cell suspension culture of *T. cordifolia*. Data represents mean values ± SD of three replicates. Biomass, *filled squares* (g/l DW) on the *left axis* and alkaloids content (mg/g DW) on the *right axis, filled circle* palmatine, *filled triangle* jatrorrhizine

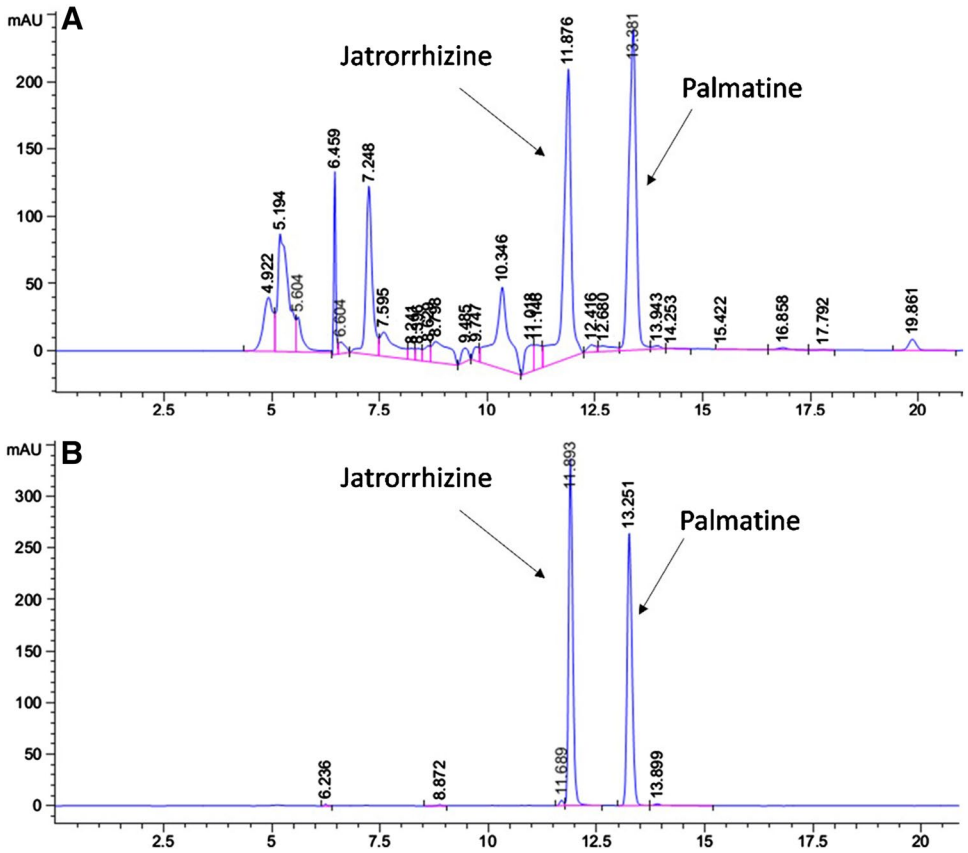

Fig. 3 LC-DAD at 345 nm showing chromatogram of **a** cell extract of callus culture of *T. cordifolia*, **b** mixture of standards of jatrorrhizine and palmatine

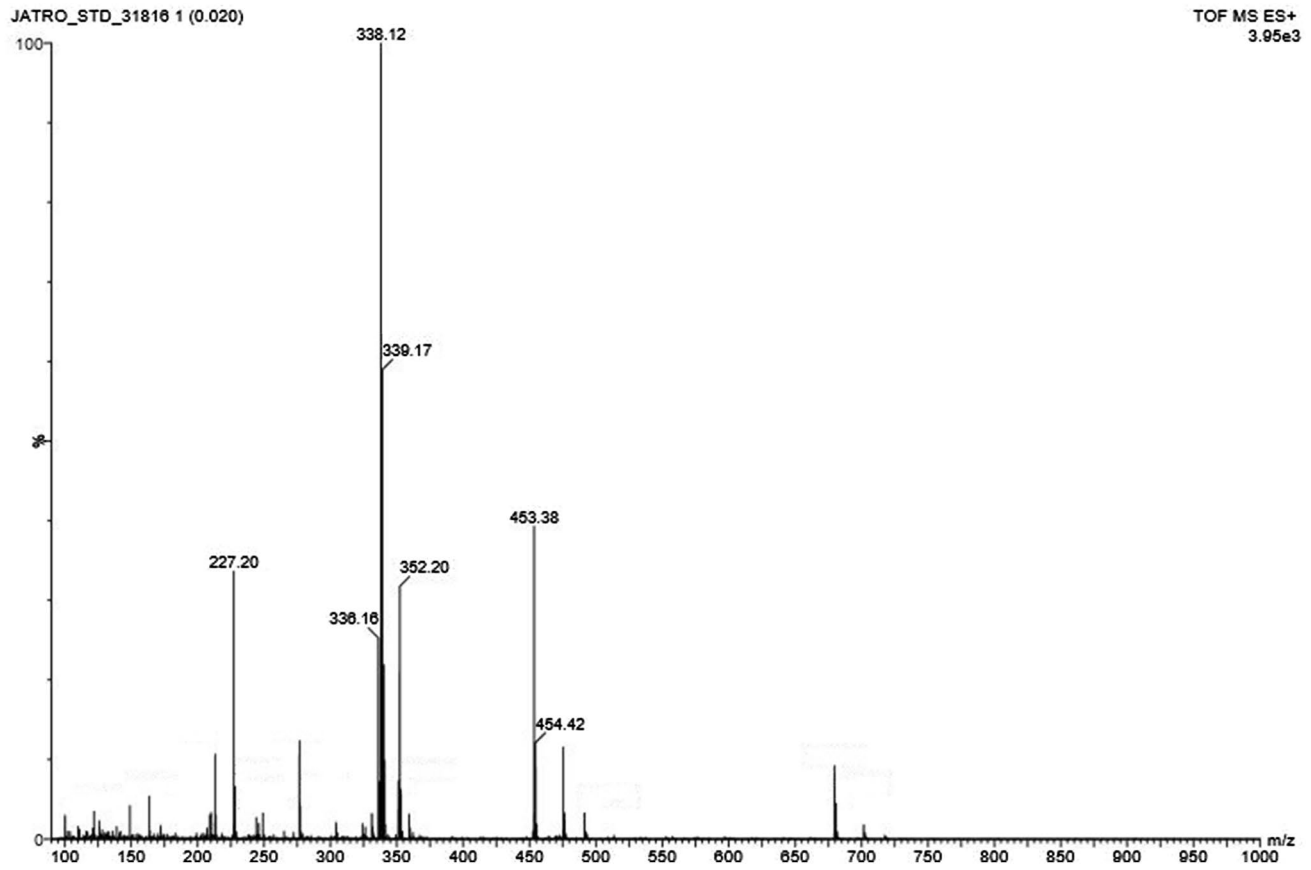

Fig. 4 Mass spectrum of standard sample of jatrorrhizine with 95% purity showing $[M]^+$ as 338.12 m/z value

Impact of biotic elicitors of *P. indica* on growth and alkaloids production in suspension cultures of *T. cordifolia*

All biotic elicitor preparations (autoclaved culture filtrate, filter-sterilized culture filtrate and fungal cell extract) of *P. indica* had suppressing effect on the biomass accumulation in the suspension cultures of *T. cordifolia*. A dose-dependent decrease in biomass accumulation was, in general, observed with the addition of all these biotic elicitor preparations. The autoclaved culture filtrate of *P. indica* at any of the tested concentration did not have any effect on the alkaloids production in the cell suspension cultures of *T. cordifolia*, as the production of both jatrorrhizine and palmatine was the same at all elicitors' concentrations (Fig. 7). On the other hand, the filter-sterilized culture filtrates at 5% concentration enhanced the jatrorrhizine content by 4.2-fold to 10.72 mg/g DW and palmatine content by fourfold to 4.39 mg/g DW (Fig. 7). This indicated that the elicitor moieties in the culture filtrate of *P. indica* were most probably heat-labile and may have lost their elicitation capability on autoclaving. Addition of fungal cell extract of *P. indica* at a concentration of 2.5% (v/v) enhanced the palmatine content maximally by 2.8-fold to 3.10 mg/g DW but jatrorrhizine content was maximally increased by 2.3-fold to 6.07 mg/g DW at 5.0% (v/v) concentration of the fungal extract (Fig. 7). A dose-dependent response on enhanced production of these alkaloids was thus observed with the filter-sterilized culture filtrate and with the cell extract of *P. indica*.

Impact of chitosan, chitin and methyl jasmonate as elicitors

All these elicitors had a suppressing effect on the biomass accumulation in the suspension cultures of *T. cordifolia* (Fig. 8). Methyl jasmonate is a wide spectrum elicitor. In the present study, methyl jasmonate at an optimum concentration of 250 µM increased the palmatine content by 4.1-fold to 4.43 mg/g DW and jatrorrhizine content by 2.9-fold to 7.34 mg/g DW (Fig. 8). Variation of chitin concentration from 25 to 500 mg/l had the same effect on production of either palmatine or jatrorrhizine in suspension cultures of *T. cordifolia*; at 100 mg/l concentration, it increased palmatine content by 1.7-fold to 1.81 mg/g DW and jatrorrhizine content by 1.2-fold to 2.94 mg/g DW compared to

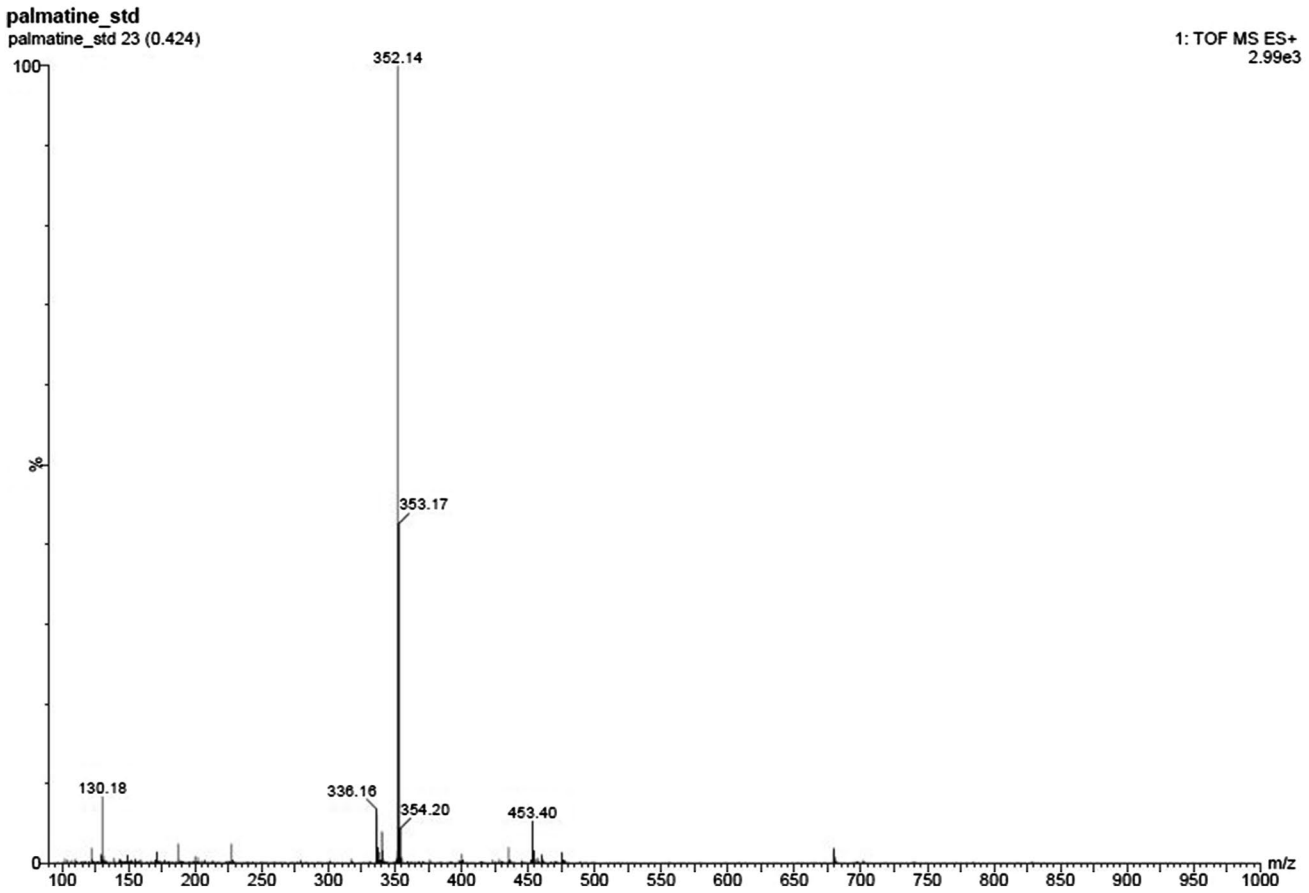

Fig. 5 Mass spectrum of standard sample of palmatine with 97% purity showing $[M]^+$ as 352.14 m/z value

the control cultures (Fig. 8). Chitosan resulted in maximum 3.5-fold increase in palmatine content to 3.77 mg/g DW at 50 mg/l but the maximum 1.9-fold increase in jatrorrhizine content to 4.91 mg/g DW was obtained at 100 mg/l of chitosan (Fig. 8). Thus, a dose dependent effect was observed on alkaloid production with the use of biotic (chitosan) and abiotic (methyl jasmonate) elicitors. However, none of these three elicitors were as competitive as the filter sterilized culture filtrate of *P. indica*.

Discussion

The current study embodies to be an important piece of work on enhanced production of palmatine and jatrorrhizine simultaneously in cell suspension cultures raised from the leaf explants of *T. cordifolia*. The quantification of palmatine and jatrorrhizine was performed through optimized less time-consuming methods using HPLC, which required only 25 min compared to the reported method which needed 55 min (Patil et al. 2010) The alkaloids were further confirmed through MS analysis which is more precise, sensitive and reproducible (Patil et al. 2010). In this study, maximum palmatine content of 4.39 mg/g DW and jatrorrhizine content of 10.72 mg/g DW was obtained on addition of 5.0% filter-sterilized culture filtrate of *P. indica* to cell suspension cultures of *T. cordifolia*. It is worth mentioning here that the autoclaved culture filtrate of *P. indica* was not effective in enhancing the synthesis of these alkaloids. This indicated the presence of a heat labile compound in the culture filtrate which was responsible for the observed effect. In the reports of Chintalwar et al. (2003), 4.27 mg/g DW of jatrorrhizine and 1.92 mg/g DW of berberine was obtained with no detection of palmatine in the cell suspension cultures of *T. cordifolia*. Rao et al. (2008) reported a maximum berberine content of 5.5 mg/g DW in the suspension cultures of *T. cordifolia*. Breuling et al. (1985) optimized the cultivation of cell cultures of *Berberis wilsonae* in a 20 l airlift bioreactor with total alkaloids concentration of 3 g/l containing jatrorrhizine (101 mg/g DW) and palmatine (less than 2 mg/g DW). Thus, the simultaneous production of jatrorrhizine and palmatine in cell suspension cultures of *T. cordifolia* has been observed for the first time.

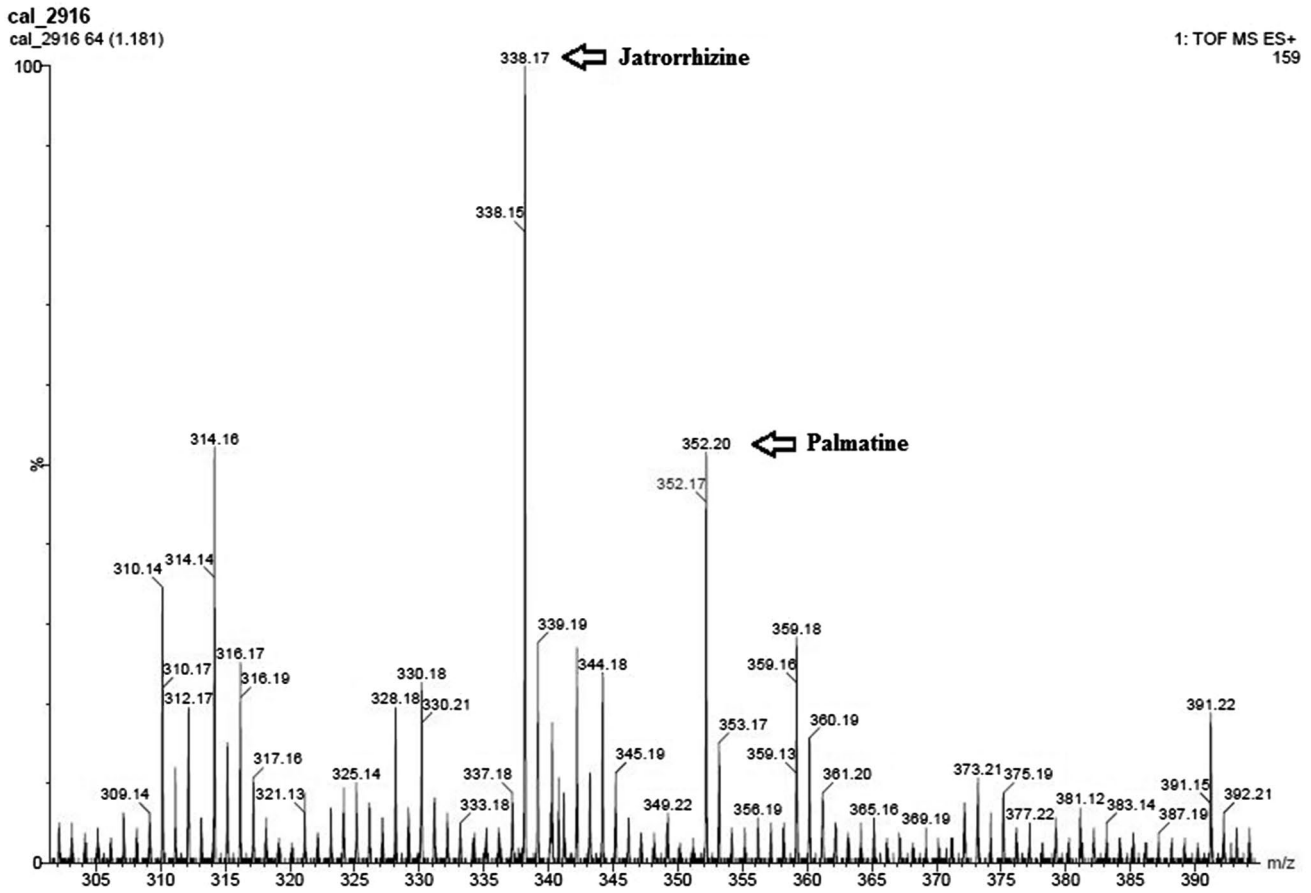

Fig. 6 Mass spectrum of methanolic cell extract of *T. cordifolia* showing jatrorrhizine at 338.17 m/z and palmatine at 352.20 m/z values, respectively

Elicitation of target alkaloids using elicitors, like methyl jasmonate, chitosan and *P. indica*, was highly significant. Among all *P. indica* showed the best elicitation effect. *P. indica*, an endophytic fungus, could be an effective substitute of biotic elicitors. Though its action is non-specific, but studies have shown that it down regulates the genes of phenyl propanoid pathway (Schäfer et al. 2009). *P. indica* has been supplemented to the cell culture in different modes, to evaluate the most effective treatment method with least detrimental effect on biomass production (Kumar et al. 2016). Culture filtrate in filter-sterilized form at 5% (v/v) resulted in utmost increment in the alkaloids production in the cell cultures of *T. cordifolia* ($P < 0.05$) whereas the autoclaved culture filtrate did not have any effect ($P > 0.05$). Similar observation was noticed in *L. camara*, where filter-sterilized culture filtrate (2.5% v/v) of *P. indica* resulted in enhanced production of oleanolic acid and ursolic acid compared to autoclaved culture filtrate (Kumar et al. 2016). On the contrary, Baldi et al. (2010) reported that the autoclaved culture filtrate of *P. indica* (2.5% v/v) led to an increase in podophyllotoxin and 6-methoxypodophyllotoxin content in cell cultures of *Linum album*. However, Bagde et al. (2013) also reported that the growth of *Aristolochia elegans* Mast. was augmented when treated with the filter-sterilized culture filtrate of *P. indica*. In comparison to untreated control plants, there was an upsurge of 136% in total biomass and also in the production of aristolochic acid by 7.6–28.8%. This cell growth enhancing and elicitation potency of culture filtrate of *P. indica* might be due to the cumulative effect of multiple mechanisms owing to the presence of several known as well as unknown constituents (Sirrenberg et al. 2007; Adya et al. 2013).

Chitin and chitosan usually elicitate the production of phenolics via phenyl propanoid pathway (Ahmed and Kim 2011). However, the experimental results showed chitosan as promising elicitor in stimulation of benzylisoquinoline pathway, which was indicated due its effect on the accumulation of jatrorrhizine in the cell suspension cultures of *T. cordifolia*. However, chitosan at 50 mg/l was also influential in enhancing the yield of palmatine by 3.5-fold. Chitin was found to be least effective as a biotic elicitor, at all the tested concentrations.

In the present study, methyl jasmonate clearly indicated its effect on the yield of the protoberberine alkaloids where

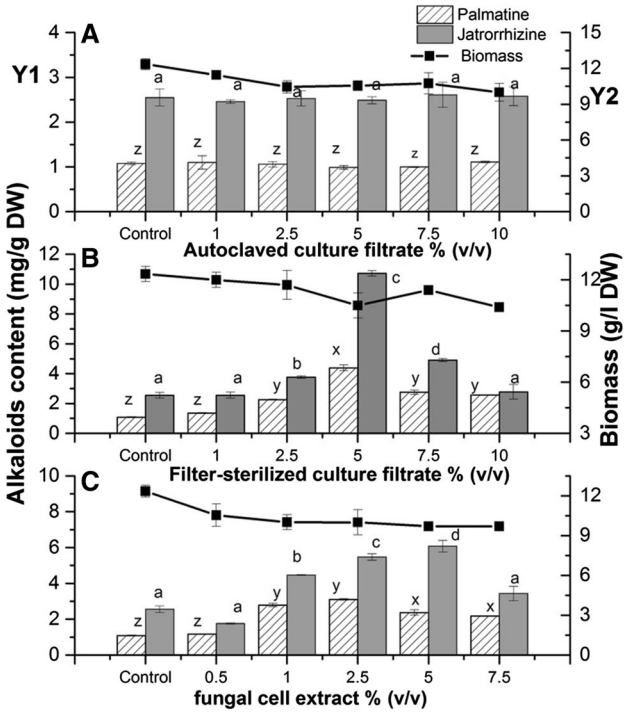

Fig. 7 Growth and alkaloids production in cell suspension cultures of *T. cordifolia* treated with autoclaved culture filtrate (**a**), filter-sterilized culture filtrate (**b**) and fungal cell extract of *P. indica* (**c**). Data represents mean values ± SD of three replicates. Palmatine (*dashed bar*) and jatrorrhizine (*grey bar*) contents (mg/g DW) are represented on the *Y1 axis* and biomass, *filled squares*, (g/l DW) is represented on the *Y2 axis*. Within each set of experiments, *bars with different letters* are significantly different ($P < 0.05$)

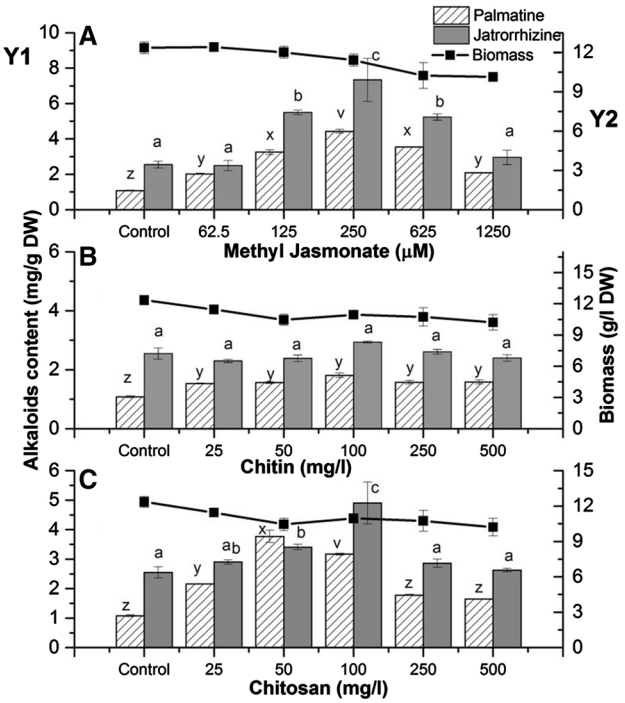

Fig. 8 Effect of methyl jasmonate (**a**), chitin (**b**) and chitosan (**c**) on growth and alkaloids accumulation in the cell suspension cultures of *T. cordifolia*. Data represents mean values ± SD of three replicates. Palmatine (*dashed bar*) and jatrorrhizine (*grey bar*) contents (mg/g DW) represented on *Y1 axis* and biomass accumulation, *filled squares*, (g/l DW) on *Y2 axis*. Within each set of experiments, *bars with different letters* are significantly different ($P < 0.05$)

250 μM concentration was very significant for increased alkaloid production of jatrorrhizine and palmatine among the tested concentrations. MJ has been reported to stimulate benzophenanthridine alkaloid accumulation in cell suspension cultures of *Eschscholtzia californica* (Cho et al. 2008).

The elicitors used in the present investigation exhibited a negative effect on the growth of *T. cordifolia*; higher the concentration of the elicitor, higher was the decrease in biomass concentration, in general. Cai et al. (2012) speculated that the effect of elicitors on the growth profile of the plant depends upon the plant species, type and concentration of elicitor, stage of treatment and the experimental conditions. This could be possibly due to cross talk between the elicitor and the suspension cells, competition of nutrient uptake between them, and the hypersensitive reaction of the cells in response to the elicitor that induced stress and thereby resulted in reduced biomass (Kumar et al. 2013). The earlier reports showed contradictory effect of *P. indica*; the culture filtrate of *P. indica* had an inhibitory effect on growth of *L. album* in cell suspension cultures (Baldi et al. 2010) but a positive effect on growth of hairy roots of *L. album* (Kumar et al. 2013). It has also been reported by van der Plas et al. (1995) that secondary metabolism often accompanied with slow growth rate of plant cells due to competition of precursors for synthesis of metabolites. Thus, physiological state of the culture seems to be responsible for the elicitation effect of *P. indica* on growth of *T. cordifolia* cell cultures. It is possible under conditions employed in the present study that greater energy expenditure may be occurring in the production of secondary metabolites (jatrorrhizine and palmatine) in detriment to the production of biomass in *T. cordifolia* cultures.

Another critical aspect of the study is the production of jatrorrhizine and palmatine simultaneously from the callus cultures raised from leaf explants of *T. cordifolia*. None of the reports published so far showed the existence of these alkaloids in leaf since the alkaloids accumulates mostly in stem portion of the plant (Patel and Mishra 2011). This implies the effect of culture conditions on the production of target alkaloids. Medium composition containing BAP and NAA seems to have stimulative effect on the production of palmatine and jatrorrhizine in detectable amounts in *T. cordifolia*. There have been reports in which BAP and NAA stimulated the biosynthetic pathway for berberine synthesis (Nair et al. 1992; Hara et al. 1993).

It is interesting to note that *P. indica* has been found to have beneficial effect on synthesis of lignans (podophyllotoxin) in cell cultures (Baldi et al. 2010) and hairy roots of *L. album* (Kumar et al. 2013) and of triterpenoids (ursolic acid, oleanolic acid and betulinic acid) in cell cultures of *L. camara* (Verbenaceae) (Kumar et al. 2016). In this study, it has enhanced the synthesis of protoberberine alkaloids (jatrorrhizine and palmatine) in cell cultures of *T. cordifolia*. Thus, it appears that the fungus may have the potential of acting as a generalized elicitor in enhancing the production of secondary metabolites in these and many more plant cell cultures.

Conclusion

In the present study, cell suspension culture was successfully developed from leaf explants of *T. cordifolia*, which produces a number of protoberberine alkaloids. It also reported a less time-consuming method for quantitative estimation of jatrorrhizine and palmatine in cell suspension cultures of *T. cordifolia*. Chitosan and methyl jasmonate resulted in increased production of jatrorrhizine and palmatine but the filter-sterilized culture filtrate of *P. indica* at 5.0% (v/v) level was most effective for maximizing the production of both jatrorrhizine (4.2-fold) and palmatine (4.0-fold) to 10.72 and 4.39 mg/g DW, respectively, in suspension cultures. Moreover, simultaneous production of jatrorrhizine and palmatine in cell suspension cultures of *T. cordifolia* was observed for the first time. Thus, the present study provides an effective approach by which *P. indica* could be used as a source of elicitors for increased synthesis of secondary metabolites in plant cell cultures.

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