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Nastaran Hemmati, Majid Azizi, Rosella Spina, François Dupire, Hossein Arouei, et al.. Accumulation of ajmalicine and vinblastine in cell cultures is enhanced by endophytic fungi of *Catharanthus roseus* cv. Icy Pink. *Industrial Crops and Products*, 2020, 158, pp.112776. 10.1016/j.indcrop.2020.112776 . hal-02977149

HAL Id: hal-02977149

<https://hal.univ-lorraine.fr/hal-02977149>

Submitted on 17 Oct 2022

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Accumulation of ajmalicine and vinblastine in cell cultures is enhanced by endophytic fungi of *Catharanthus roseus* cv. Icy Pink

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Accumulation of ajmalicine and vinblastine in cell cultures is enhanced by endophytic fungi of *Catharanthus roseus* cv. Icy Pink

Abstract

Catharanthus roseus belonging to the Apocynaceae family synthesizes indole alkaloids like vinblastine and ajmalicine which are used in cancer treatment and for decrease high blood pressure, respectively. However, these compounds with a high value added are accumulated at low level in the plants. For the first time, endophytic fungi isolated from leaves (*Stemphylium amaranthi*) and roots (*Gliomastix masseei*) of *C. roseus* cv. Icy Pink were used as fungal elicitors to improve the biomass of *C. roseus* callus culture as well as the accumulation of indole alkaloids in the calli. Polyphenols and the antioxidant activity of endophytes extracts were examined. The ethyl acetate extract of *S. amaranthi* showed the maximum scavenging activities in 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays. The maximum amount of total flavonoid and total phenol content were found in *S. amaranthi* (18.29 ± 0.03 mg gallic acid equivalents.g⁻¹ and 0.117 ± 0.00001 mg quercetin equivalents.g⁻¹, respectively). Also 5% cell extract and 5% culture filtrate extract of this endophytic fungus during 7 days of calli culture led to the highest contents of ajmalicine ($0.735 \mu\text{g.g}^{-1}$ fresh weight) and vinblastine ($0.059 \mu\text{g.g}^{-1}$ FW) respectively. Thus, endophytic fungi isolated from *C. roseus* could be used as fungal elicitor to improve the indole alkaloid production.

Keywords: Endophyte; Vinblastine; Ajmalicine; GC-MS; LCMS TQ

1. Introduction

The periwinkle (*Catharanthus roseus* (L.) G. Don) is a perennial tropical plant which belongs to Apocynaceae family. Periwinkle is capable of synthesizing valuable alkaloids, including vincristine, vinblastine, vindoline, ajmalicine, catharanthine and serpentine, which some of them like vinblastine and vincristine are commonly used in treating cancer. Also ajmalicine is an antihypertensive compound which can decrease high blood pressure (Ramezani et al., 2018). The yield of some indole terpenoid alkaloid compounds such as vinblastine and vincristine are relatively low in the aerial part of this plant (approx. 0.0005%) (Tonk et al., 2016). Due to the high price, importance and trace amounts of these alkaloids in *C. roseus*, attempts to enhance the production of these compounds have led to comprehensive researches on this plant as a model for plant specialized metabolism (Anjum and Chandra, 2019). The *in*

vitro culture technique is one of the promising methods to give the opportunity to regulate metabolism, cell growth and to direct the culture to produce the specialized metabolites (Zhang et al., 2011). In the study of Mekky et al. (2018), they announced that the biosynthesis of vincristine and vinblastine showed a 3.39-fold increase in callus culture compared with the wild *C. roseus* plant. It has been recognized that a large number of biotic compounds are effective to enhance specialized metabolites biosynthesis when they were added in plant tissue cultures. Arbuscular mycorrhizal fungal induction increased levels of hypericin and pseudohypericin in *Hypericum perforatum* root culture (Zubek et al., 2012). Various fungi like *Colletotrichum lindemuthianum*, *Trichoderma harzianum*, and *Fusarium oxysporum* enhanced asiaticoside accumulation and biomass in shoot culture of *Centella asiatica* (Prasad et al., 2013). About *C. roseus*, *Aspergillus flavus*, as a fungal elicitor, improved biomass of callus culture and alkaloid yield (Tonk et al., 2016). Also *in vitro* shoot cultures of *C. roseus* indicated the highest biosynthesis of vindoline and catharanthine after 16 h of *Pythium aphanidermatum* treatment (Shukla et al., 2010). Endophytic fungi, that typically coexist with their hosts asymptotically, represent a wide source of uncharacterized and unexplored microorganisms capable of producing novel and different metabolites (Parthasarathy et al., 2020). Some of fungal endophytes synthesized compounds such as phenols, alkaloids and flavonoids that have biological functions (Hughes, 2016). So far, less than 16% of the identified fungal species have been studied for their biosynthesis capacity and fewer than 5% of the total fungal species have been characterized, they present an important source of natural compounds (Bedi et al., 2018). In cell suspension culture of *C. roseus*, endophytic fungi could increase yield of total alkaloids (Tang et al., 2011). It seems that fungal endophytes produce some compounds that can play elicitor role and this fungal elicitor may increase the alkaloid content in the *C. roseus*. So this research was done in order to investigate both the phytochemical characteristics of two endophytic fungi isolated from *C. roseus* cv. Icy Pink plant and the possibility of improving the biomass of *C. roseus* callus culture as well as the accumulation of indole terpenoid alkaloids in the calli through the application of these two new endophytes used as fungal elicitors. Also it should be mentioned that this study has been performed on icy pink cultivar for the first time.

2. Material and methods

2.1 Isolation of endophytic fungi

Healthy tissues including roots and stems were collected from *Catharanthus roseus* (L.) G. DON cv. Icy Pink grown in May 2018 in Gorgan, Iran and were stored at 4°C (Waksman et al., 1916). The stems and roots of the plant were cut into 5 cm pieces and rinsed with running water for 10 minutes to remove soil and other particles. The samples were surface sterilized with 70% alcohol for one minute and 1.5% sodium hypochlorite for 10 minutes and washed with sterilized water 3 times. Samples were dried under the laminar on sterile blotters and then the sterilized small pieces were put on the Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) petri dish (100×15 mm) combined with tetracycline (50 mg. L⁻¹) to prevent the bacterial growth and incubated for up to 6 to 8 weeks at 25°C with 12 h light and dark cycles to enable the endophytic fungi growth. By the hyphal tip culture, the pure fungal cultures of endophytic isolates were acquired. To identification of colonies that emerged from different tissue parts, colonies were transferred to antibiotic-free PDA (Hatamzadeh et al., 2020).

2.2 Molecular Studies

Fungal endophytes were grown for 7 days in 200 mL of PDB (Potato Dextrose Broth) at 28 °C. The fungal mycelia were freeze-dried. The DNA was extracted as described by Saar et al. (2001). Strains were sequenced with ITS primer (Table 1). The PCR amplifications were carried out on a GeneAmp PCR System 9600 (Perkin Elmer, USA) with a total volume of 12.5 µL solution containing 10–20 ng of template DNA, 0.7 µL DMSO (99.9%), 1 × PCR buffer, 2 mM MgCl₂, 0.5 µM of each primer, 1.0 U Taq DNA polymerase (NEB) and 25 µM of each dNTP. The amplification procedure was started with pre-heating at 95°C for 1 min, accompanied by 40 denaturation cycles at 95°C for 30 s, extension at 72°C for 10 s and a final extension at 72°C for 5 minutes. Electrophoresis was used to examine the products of the PCR reaction using 1% (w/v) agarose gel. BLAST analysis and sequences were documented in the GenBank Database at NCBI.

2.3 Endophytic fungi extraction

Each of the pure cultures of endophytic fungi was re-cultivated on PDA at 25°C for 30 days. Afterward, solid fungal culture was crushed and then soaked in ethyl acetate (EtOAc) at room temperature for 24 h according to Khiralla et al. (2015). Then the mixture of solvent and PDA was filtered to separate mycelia and crushed pieces of PDA from extract. The filtrate extracts were dried in a rotary evaporator (Buchi Rotavapor R-210). The extract residue was dissolved

in methanol (1mg. mL⁻¹), stored at 4°C as stock solution for phytochemical analysis and for measurement of biological activities.

2.4 Determination of antioxidant activities

ABTS and DPPH free radical-scavenging were used to investigate the antioxidant activity of endophytic fungi extracts. DPPH test was done according to the improved method previously described by Zhou et al. (2013). Trolox (Tx) and vitamin C (Vc) were used as references. The extract's ability to scavenge ABTS radical was performed according to Zhou et al. (2013). Tx and Vc were used as references.

2.5 Determination of total flavonoids

The total content of flavonoid in endophytic extract was measured using a colorimetric assay with rutin as standard (Zheng et al., 2011). Results were represented in mg of rutin equivalents per g.

2.6 Determination of total polyphenols

The Folin–Ciocalteu assay was used to determine the total polyphenol content of the endophytic extract (Sabir and Rocha, 2008). The results were represented in mg of gallic acid equivalents per g.

2.7 *Catharanthus roseus* cv. Icy Pink callus cultures

Seeds of *C. roseus* (L.) G. DON cv. Icy pink were provided by Syngenta seeds Co, Enkhuizen, Netherlands. Sterilized seeds were placed in glassy jar containing 50 ml of solid MS medium without any plant growth regulator (Murashige and Skoog, 1962). The germinated seedlings were maintained in *in vitro* conditions as long as seedlings grow and produce leaves. Young leaves parts were used as explants to produce callus. For callus induction, the MS medium was supplemented with 1 mg.L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg.L⁻¹ 6-benzylaminopurine (BA). The medium was solidified with 0.8% agar. Each petri dish contained 15 mL of medium. The medium pH was set to 5.7 before autoclaving. All cultures were incubated at 25 ± 2°C under 16/8 (light/dark) photoperiod.

2.8 Endophytic fungi elicitor preparation and elicitation conditions

The fungal endophyte elicitors were prepared with some modifications in accordance to the process defined by Baldi et al. (2009). Two fungal endophytes cells (agar plugs containing mycelia) were put into a 250 mL Erlenmeyer flask containing 50 mL of PDB media. The flasks were incubated on an incubator shaker at $25 \pm 2^{\circ}\text{C}$ and rotating at 200 rpm to allow the fungal endophytes cells to grow until the end of the log phase (6 days). Then the culture broth was collected by passing the fungal culture through a Whatman N°1 filter paper and fungal mycelia were kept. Afterwards the culture broth was centrifuged at 5000 rpm for 15 min to eliminate fungal cells. The centrifuged culture broth was filtered by passing through 0.22 μm cellulose acetate syringe filters and labeled as Culture Filtrate (CF). The fungal mycelia were separated from the broth by filtration and was rinsed 3 times with sterile distilled water and dried at 40°C and crushed. The fungal powders were immersed in sterile distilled water (3 mg.mL^{-1}) and were autoclaved. Then the fungal suspensions were centrifuged and the supernatant was collected and designated as Cell Extract (CE).

Two fungal endophyte elicitors were added separately in 4 concentrations (0, 2.5%, 5%, 10% v/v) in solid MS medium of *C. roseus* cv. Icy pink callus culture containing 4 callus in each petri dish and sampling was done at the end of 7th day and 14th day of culture.

2.9 GC-MS identification

The ethyl acetate extracts of endophytic fungi were analyzed using GC-MS QP2010-Shimadzu equipment operating in the EI mode at 70 eV. An agilent DB5MS column (30 m \times 0.25 mm \times 0.25 μm) was used with a 26 min temperature program of 60-320 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C}/\text{min}$. The temperature for the injector was 250 $^{\circ}\text{C}$, the carrier gas (helium) flow rate was 1 mL/min, and the split ratio was 1:5. The identification of the compounds was done by comparing the measured data with literature data as indicated in the text.

2.10 LC-MS analysis

In *in-vitro* culture experiments, 200 mg of fresh callus were macerated in methanol (1:10 w/v) for 24 h and then were sonicated at room temperature for 30 min in an ultrasonic bath (Transsonic 460/H Elma). After 10 minute centrifugation at 4000 rpm, the total methanolic extracts were purified by SPE procedure as reported by Saliba et al. (2016).

The analytical column used was an ODS 250 \times 2.1 column (250 mm \times 2.1 mm). For elution at 200 $\mu\text{L}/\text{min}$ flow rate, solvent A consisted of 10 mM ammonium formate in H_2O at pH 6.8

and solvent B consisted of pure methanol. The linear gradient solvent system used was: 0 min, 10% of B, 0-2 min, 10% of B; 2-5 min 10% to 50% of B; 5-15 min, 50-99% of B, 15-20 min, 99% of B, 20-22 min, 99-10% of B, 22-25 min, 10% of B. The LC system was coupled to a Bruker Elite TQ MS mass spectrometer (LCMS TQ) with an atmospheric pressure electrospray interface. Bruker MS Workstation version 8.2.1 was used for LC-MS system control and data analysis. Alkaloid identification was done by multiple reaction monitoring (MRM) transition and retention time. The parameters used for the mass spectrometer under the ESI⁺ mode were as follows: spray voltage 4500 V, cone temperature 300°C, cone gas flow 20 psi, heated probe temperature 250°C, probe gas flow 35 psi, nebulizer gas (nitrogen) flow 40 psi. Parameters for the cone energy and collision energy are listed in Table 2. All parameters were automatically optimized by Workstation.

2.11 Data Analysis

Treatments with 3 replications were arranged in a completely randomized factorial design. The data are reported as means \pm standard deviation (SD). Variance analysis was carried out by using the SAS software (9.1 version) and means were measured by using LSD's test ($p \leq 0.05$).

3. Results and discussion

After the isolation and the identification of endophytic fungi from *C. roseus* cv. Icy Pink leaf and root, phytochemical analysis of ethyl acetate crude extracts of these endophytes were determined then the stimulation effect of these endophytes on the growth of *C. roseus* callus and biosynthesis of vinblastine and ajmalicine were studied.

3.1. Endophytic fungi

3.1.1. Identification of fungal endophytes

The first fungus (Fig.1a) separated from *C. roseus* cv. Icy Pink leaf was identified as *Stemphylium amaranthi* by analysis of the sequences of ITS4-ITS5 region (Fig.2a). This fungus showed pale brown to dark brown colonies on PDA and has branched hyphae. Hatamzadeh et al. (2020) isolated and identified *S. amaranthi* from stem of *Achillea millefolium* and other species from *Stemphylium* genus such as *S. vesicarium*, *S. botryosum* and *S. globuliferum* from *Achillea millefolium*, *Anthemis altissima* and *Cichorium intybus*

plants respectively. In leaf of *C. roseus*, *Stemphylium herbarum* was previously identified (Abdel-Rahman et al., 2010).

Separating the second fungal isolate (Fig.1b) was from the root of *C. roseus* cv. Icy Pink and identified as *Gliomastix masseei* by the sequences of ITS4-ITS5 region analysis (Fig.2b). These fungal endophyte colonies are velvety black and their mycelium showed solitary, funicular envelope. There are few publications about this endophytic fungus. However *G. masseei* endophyte was reported in bamboo (Bhilabutra et al., 2010) and in *Manglietia garrettii* (Kodsueb et al., 2008). It is the first time that *G. masseei* is reported as a fungal endophyte from *C. roseus* plant.

3.1.2. Identification of natural compounds

To investigate compounds present in the ethyl acetate crude extracts of *S. amaranthi* and *G. masseei* endophytic fungi, GC-MS and LC-MS were used. For GC-MS analysis, the identification of the compounds was performed by comparing the measured data with literature data. Thirteen and thirty-five compounds were identified in extracts of *S. amaranthi* (Table 3) and *G. masseei* (Table 4) respectively. Long-chain unsaturated or saturated fatty acids, such as oleic acid and octadecanoic acid were found in the both endophytic fungi while eicosanoic acid, 3-heptenoic acid, 2-nonenoic acid, butyric acid and oxalic acid were identified only in *G. masseei* extract. Gamma-sitosterol was reported only in the extract of *S. amaranthi*.

Ethyl acetate extracts were analyzed using LC system coupled with MS (LCMS TQ) in view to detect the presence of indole alkaloids. No alkaloid was detected in the endophytic crude extract of the both endophytic fungi.

3.1.3. Antioxidant activity

Antioxidant activities of ethyl acetate crude extracts from the two endophytic fungi are presented in Table 5. In both ABTS and DPPH tests, *S. amaranthi* extract exhibited the maximum antioxidant activity with 24.15 ± 0.44 and 454.02 ± 3.60 $\mu\text{g/mL}$ IC_{50} value respectively. In addition, the ABTS radicals scavenging ability of *S. amaranthi* extract was higher than vitamin C, and this extract showed ABTS radicals scavenging activity close to the control Trolox. As reported by Arnao (2000), the DPPH and the ABTS radicals reveal some important variations in their response to antioxidants in relation with the interferences

induced by plant-derived materials at different wavelengths. It was reported that the crude supernatant extract of the endophytic fungus *Nigrospora sphaerica*, isolated from *C. roseus*, showed antioxidant activity (Ayob et al., 2019). Also Khiralla et al. (2015) reported that the endophytic extracts of *C. roseus* had extremely wide range of antioxidant activity (IC₅₀ values), from $18.0 \pm 0.1 \mu\text{g/mL}$ for *Curvularia* sp. to $2686.0 \pm 51.7 \mu\text{g/mL}$ for *Phoma* sp. in DPPH assay. Other researchers confirmed that fungal endophyte extracts isolated from *C. roseus* such as *Chaetomium nigricolor* (Dhayanithy et al., 2019), *Mucor* sp. (Shukla et al., 2018) and *Curvularia geniculata* (Chowdhury et al., 2018) had a great potential as antioxidant agent.

3.1.4. Determination of total phenol and total flavonoid contents

Total phenol and flavonoid contents of ethyl acetate extracts of *S. amaranthi* and *G. masseei* endophytic fungi were evaluated (Table 6). *S. amaranthi* extract contained both more total phenols ($18.29 \pm 0.03 \text{ mg gallic acid equivalents.g}^{-1}$) and total flavonoids ($0.12 \pm 0.00001 \text{ mg quercetin equivalents.g}^{-1}$) by comparison with *G. masseei* extract. Chowdhury et al. (2018) reported that the highest total phenol $27.65 \pm 1.20 \text{ mg QE.g}^{-1}$ and total flavonoid $2.06 \pm 0.10 \text{ mg GAE.g}^{-1}$ contents were observed in *Curvularia geniculata* isolated from *C. roseus*. In addition, *Nigrospora sphaerica* isolated from *C. roseus* also showed the highest contents in total phenol and total flavonoid contents, which were $0.030 \pm 0.01 \text{ mg GAE.g}^{-1}$ and $0.038 \pm 0.001 \text{ mg QE.g}^{-1}$ respectively (Ayob et al., 2019).

3.2. Effects of endophytic fungi on callus growth

Induction of callus was observed in four weeks after initiation of *in vitro* cultures. Four week-old calli were separated from primary explants and proliferated on the same media used for the induction of callus. In view to enhance the growth of callus culture, calli were subcultured on Murashige and Skoog medium supplemented with the same growth regulators as used for induction medium and containing cell extract and culture filtrate from two endophytic fungi, *S. amaranthi* and *G. masseei*, at various concentrations (0, 2.50, 5 and 10%). Calli were incubated for 7 and 14 days. The control calli showed the multiplication index of 0.32 and 0.41 g after 7 and 14 days of culture respectively. The calli growth treated with cell extract and culture filtrate from the two endophytic fungi was calculated by measurement of the fresh weight (FW) of the callus and the corresponding results are shown in figure 3. The main effects of endophytic fungi species, periods of time, endophytic fungi extract and their

concentration level, and also quadrilateral interaction of fungal species \times time \times endophytic extract (cell extract or culture filtrate) \times endophytic extract concentration were significant on fresh weight of calli. The significant interaction effect of endophytic fungi (*S. amaranthi* and *G. masseei*), concentration level (0, 2.5, 5, and 10%) (CE and CF) in 7th and 14th days based on mean comparison revealed that adding *S. amaranthi* and *G. masseei* CE and CF increased callus growth as compared with the control. The highest fresh weight of callus (1.42 g) was observed with 10% of cell extract of *S. amaranthi* after 14 days of culture and the lowest fresh weight of callus (0.323 g) was recorded in control cultures. About *G. masseei* endophytic fungus, 10% of the culture filtrate used for the calli treatment led to a higher fresh weight (1.02 g) comparatively to the control calli (0.41 g) after 14 days of culture. It was reported that cell extract of *Chaetomium globosum* decreased cell growth of *Corylus avellana* cell suspension (Salehi et al., 2019). In contrary, cell suspension growth of *C. roseus* increased until the 10th day using *Fusarium oxysporum* fungal elicitor (Tang et al., 2011). Tonk et al. (2016) demonstrated that after treatment with a low-dose of *flavus* fungal elicitor, the callus growth rate of *C. roseus* could effectively increase.

3.3 Effects of endophytic fungi on alkaloid accumulation

Four week old callus cultures established on MS solid medium were treated during 7 and 14 days with 2.5, 5 and 10% concentrations of cell extract or culture filtrate of the two endophytic fungi, *S. amaranthi* and *G. masseei*, in order to enhance the alkaloid synthesis. After harvesting the calli growing on solid MS medium, the alkaloids were extracted using methanol, purified with SPE cartridges and the compounds were identified using LCMS TQ. Ajmalicine and vinblastine were identified by comparison with authentic compounds and quantified (Figure 4). This system was able to detect the compounds in amount below 0.02 $\mu\text{g/mL}$.

The LCMS chromatogram of the various extracts presented excellent separation of ajmalicine and vinblastine peaks and permitting the quantification of these alkaloids (Figure 4). Ajmalicine and vinblastine were detected in control callus with an average of 0.075 $\mu\text{g/g FW}$ and 0.04 $\mu\text{g/g FW}$ respectively after 7 days of culture (Figure 5, Figure 6). Surprisingly vinblastine alkaloids was not detected in all cultures at the end of the second week of the experiment. These results can be clarified by the biotransformation of these alkaloids in other terpenoid indole alkaloids like vincristine (Hamada and Furuya, 1999). Also due to cell

division and longitudinal growth, *in vitro* cultures growth continues for a short time and are stopped because of accumulation of toxic substances and termination of nutrients within the medium (Bhojwani and Razdan, 1986). Thus, a predictable reason for the low performance of secondary metabolites production in cell and plant tissue culture is the short phase of *in vitro* tissue differentiation that somehow inhibits the activity of the present and active enzymes in differentiated or mature plants (Dias et al., 2016). Another reason that justifies the low performance of these compounds is the lack of knowledge on the biosynthesis of secondary metabolites and how they are regulated (Verpoorte et al., 2002).

Ajmalicine contents ($\mu\text{g/g}$ fresh weight of plant material) in *C. roseus* callus culture treated with CE and CF of *S. amaranthi* and *G. masseei* are presented in Figure 5. The results showed that the amount of ajmalicine in *C. roseus* callus was significantly affected by all the treatments.

Whatever the concentration of the both endophytic extracts tested, the content in ajmalicine was higher than control callus cultures except the calli treated with 2.5% and 5% of *G. masseei* CE after 7 days of culture. The main effects of treatments and their quadrilateral interactions effect (fungal species, CE or CF extract of endophyte, their concentration and time duration) on ajmalicine were significant. The highest content of ajmalicine ($0.735 \mu\text{g.g}^{-1}$ FW) was observed with 5% CE extract of *S. amaranthi* during 7 days of culture. The concentration of this alkaloid decreased after two weeks of culture however the ajmalicine content was still higher ($0.456 \mu\text{g.g}^{-1}$ FW) comparatively to the other conditions of treatment.

About vinblastine accumulation in calli, stimulating or inhibitory effect were observed according to the fungal species, time of culture, Cell Extract (CE) or Culture Filtrate (CF) and its concentration level. Highest content of vinblastine ($0.059 \mu\text{g.g}^{-1}$ FW) was obtained with 5% of CF from *S. amaranthi* after 7 days of culture. Also CE (2.5, 5 and 10%) of this endophytic fungus increased vinblastine accumulation after one week of treatment. In contrary, extracts from *G. masseei* (CE and CF) led to the lowest content of vinblastine ($0.022 \mu\text{g.g}^{-1}$ FW) whatever the concentration used (Figure 6) and vinblastine was not detected with 10% of *G. masseei* CF in first week. In 14th day, vinblastine accumulation in callus was stopped in all treatments even control that maybe it was metabolized to another alkaloids.

In plant *in vitro* culture, fungal elicitors are known to be the prominent enhancement technique for specialized metabolites (Takeuchi et al., 2013). The use of fungal elicitors to enhance specialized metabolites has a long history (Christen et al., 1991).

In fact, fungal elicitors are known as one of the most interesting techniques for improving specialized metabolites (Zhang et al., 2011). The present research demonstrates the impact of fungal elicitors on callus growth, production of ajmalicine and vinblastine in *C. roseus* cv. Icy Pink callus culture. The results obviously present that the elicitors isolated from *C. roseus* cv. Icy Pink have a considerable impact on enhancement of production of these two alkaloids. The most effective elicitor for biosynthesis of vinblastine and ajmalicine was *S. amaranthi* isolated from *C. roseus* cv. Icy Pink leaf.

Fungal elicitors can be defined as signaling molecules that trigger specialized metabolites formation in *in vitro* cultures by inducing proteins related to plant defense, hypersensitive response or pathogenesis (Baldi et al., 2009). However no elicitor was identified to have a general impact on different cultures, and no system was found to react to all elicitors which this issue is related to receptors. There are receptors in plasma membrane of plants cell which are responsible for the identification of fungal elicitors and transferring activation system for defense system of plant cell. Specific receptor structure allowed the specific elicitors to be recognized. So, all fungal elicitors cannot induce plants system. In order to produce a desired compound, various elicitors need to be screened (Singh et al., 2018). In this research, the highest content of ajmalicine was related to the CE of *S. amaranthi* with 5% level in 7th day and the highest amount of vinblastine was produced from callus culture exposed with 5% (v/v) CF of *S. amaranthi* in 7th day. Consequently, it could be inferred that the fungal elicitor's effect on the yield of vinblastine and ajmalicine depends on the sampling time and elicitor concentrations. Indeed, in order to obtain the highest indole terpenoid alkaloid production by fungal elicitor, it is necessary to optimize the concentration and different sampling time to culture medium. The earlier researches (Namdeo et al., 2002; Tonk et al., 2016) confirmed that the concentration, type and elicitors sampling time affect the inducing of specialized metabolites production. It was reported that the amount of vinblastine was increased by using *Trichoderma tomentosum* and *Priformospora indica* fungal extracts (Ramezani et al., 2018). Salehi et al. (2019) reported also the favorable impact of elicitors obtained from *Paraconiothyrium brasiliense* and *Chaetomium globosum* isolated from *Corylus avellana* and *Taxus baccata* on production of paclitaxel in cell suspension culture of *Corylus avellana*. In another study cleared that the IBA and the endophytic fungus

Zasmidium syzygii elicitor could increase the content of podophyllotoxin of *in vitro* roots of *Dyosma versipellis* after 3 weeks (Tan et al., 2019).

4. Conclusion

Fungal endophytes synthesize different compounds that induce biosynthesis of specialized metabolites. This research focused on phytochemical analysis of two fungal endophytes isolated from *C. roseus* cv. Icy Pink (*S. amaranthi* and *G. masseei*) and their effects on specialized metabolites of *C. roseus* cv. Icy Pink callus as an elicitor. Considering the results, endophytic fungal extracts, added to the *in vitro* cultures, increased the amount of vinblastine, ajmalicine and also callus growth. The maximum elicitation of these parameters dependent on fungal endophyte species, concentration, sampling time and type of elicitors. So that the highest amount of vinblastine and ajmalicine and callus growth related to *Stemphylium amaranthi* but with different concentration, sampling time and type of elicitors. Also *G. masseei* had positive impact on ajmalicine production and callus growth but not as much as *S. amaranthi*. Further work into the different sampling times and concentrations is needed to obtain an enough amount of specialized metabolites. These researches also are required to improve the low cost protocol for vinblastine and ajmalicine bulk production.

Acknowledgements

The authors thankfully acknowledge "Impact Biomolecules" project of the "Lorraine Université d'Excellence"(Investissements d'avenir – ANR) and Institut Jean Barriol for financial support, the Ministry of Science, Research and Technology of the Islamic republic of Iran for providing a scholarship and the EspaceBio laboratory for their technical support (LCMS TQ).

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Table 1

Primers used for molecular identification

Locus	Primer	Primer sequence 5' to 3'	Orientation	Reference
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	Forward	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	Reverse	

Table 2

Multiple reaction monitoring (MRM) transitions for the determination of ajmalicine and vinblastine

Compounds	Time window (min)	Parent masse	Fragment mass	Qualifier ratio	Collision energie
Ajmalicine	15.2	353	144	-	25
			117	6.1	46
			115	3.9	58
			355	34.0	34
Vinblastine	16.0	811	224	-	39
			337	13.1	43

Table 3Identification of metabolites in the endophyte *Stemphylium amaranthi* by GC-MS analysis.

Compounds	Retention Time	Area (%)	Formula
Dodecanol	10.45	8.9	C ₁₂ H ₂₆ O
1-Hexadecene	16.06	6.9	C ₁₆ H ₃₂
2,7(1H,3H)-Naphthalenedione	16.87	1.4	C ₁₀ H ₁₄ O ₂
2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-	17.55	7.5	C ₁₀ H ₁₈ O ₃
cis-2-Ethyl-2-hexen-1-ol	18.14	11.3	C ₈ H ₁₆ O
1-Octadecene	18.32	3.5	C ₁₈ H ₃₆
Olivetol, dimethyl ether	19.01	5.9	C ₁₃ H ₂₀ O ₂
n-Hexadecanoic acid	20.05	14.8	C ₁₆ H ₃₂ O ₂
Hexadecane, 1-(ethenyloxy)-	20.36	2.0	C ₁₈ H ₃₆ O
1,E-11,Z-13-Octadecatriene	21.66	10.5	C ₁₈ H ₃₂
Oleic Acid	21.71	11.7	C ₁₈ H ₃₄ O ₂
Octadecanoic acid	21.93	4.0	C ₁₈ H ₃₆ O ₂
Gamma-sitosterol	30.40	8.3	C ₂₉ H ₅₀ O

Table 4Identification of metabolites in the endophyte *Gliomastix maseei* by GC-MS analysis.

Compounds	Retention Time	Area %	Formula
2-Tridecene, (Z)-	10.45	0.8	C13H26
3-Heptenoic acid	12.88	1.2	C7H12O2
2-Undecenal	12.96	0.4	C11H20O
Butyric acid, 4-pentadecyl ester	13.65	0.8	C19H38O2
3-Tetradecyn-1-ol	14.19	0.2	C14H26O
2-Nonenoic acid	14.37	0.6	C9H16O2
Cyclobutane, 1,2-dicyclopropyl-	15.10	0.6	C10H16
6-Methyl-1,4-naphthoquinone	15.50	1.2	C11H8O2
7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl-	15.63	0.4	C8H12O2
Cyclobutaneethanol, 1-methyl-2-(1-methylethenyl)-, cis-	15.91	3.8	C10H18O
1-Dodecene	16.06	1.9	C12H24
1,3-Cyclopentadiene, 5-(1,3-dimethylbutylidene)-	16.23	1.0	C11H16
3,5-Hexadien-2-ol	16.40	0.7	C6H10O
5-Decen-1-ol, acetate, (E)-	17.11	2.6	C12H22O2
10-Dodecyn-1-ol	17.20	1.8	C12H22O
4-Methoxy-7-methylindan-1-one	17.36	0.4	C11H12O2
9-Tetradecen-1-ol, acetate, (E)-	17.51	4.4	C16H30O2
Octadecanoic acid	17.96	0.2	C18H36O2
Benzaldehyde, 4-[(acetyloxy)methyl]-	18.26	0.8	C10H10O3
4-Methylphthalaldehyde	18.53	0.6	C9H8O2
3-Nonen-5-yne, 4-ethyl-, (E)-	18.73	1.7	C11H18
2-Allyl-1,4-dimethoxy-3-methyl-benzene	18.87	9.8	C12H16O2
Phenol, 2-(2-penten-4-yl)-4-methyl-	19.03	4.3	C12H16O
9,17-Octadecadienal, (Z)-	19.33	1.1	C18H32O
3-Tridecen-1-yne, (Z)-	19.41	0.2	C13H22
4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	19.54	1.8	C13H18O
Pentafluoropropionic acid, tetradecyl ester	20.36	0.4	C17H29F5O2
13-Tetradecen-1-yn-1-ol	20.71	0.4	C14H24O
1,1-Dodecanediol, diacetate	21.24	0.1	C16H30O4
9,12-Octadecadienoic acid, methyl ester, (E,E)-	21.69	19.4	C19H34O2
Oleic Acid	21.74	15.0	C18H34O2
Eicosanoic acid	21.93	0.8	C20H40O2
Oxalic acid	22.07	0.4	C19H34O4
Cyclopentanone, 2-(2-nitro-2-heptenyl)-	22.22	0.9	C12H19NO3
Fucosterol	30.50	0.5	C29H48O

Table 5

Antioxidant activity of endophytic fungal extracts

Samples	DPPH	ABTS
	IC ₅₀ (µg/mL)	IC ₅₀ (µg/mL)
<i>Gliomastix masseei</i>	944.87±9.5	78.83±0.9
<i>Stemphylium amaranthi</i>	454.02±3.6	24.15±0.4
Vitamin C	70.08±7	65.14±1.5
Trolox	110.60±2.5	18.21±3.3

DPPH: 1,1-Diphenyl-2-picrylhydrazyl,

ABTS: 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid.

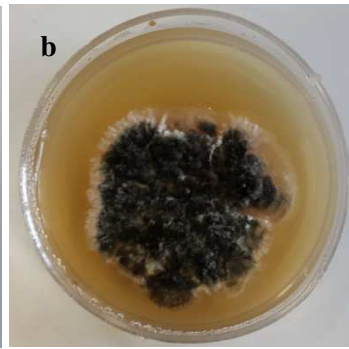
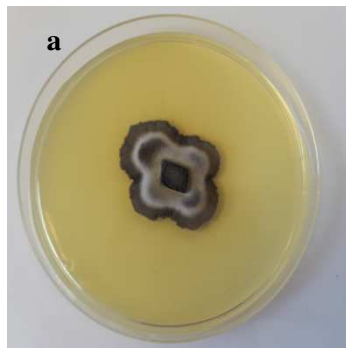
Values are representative of three independent determinations.

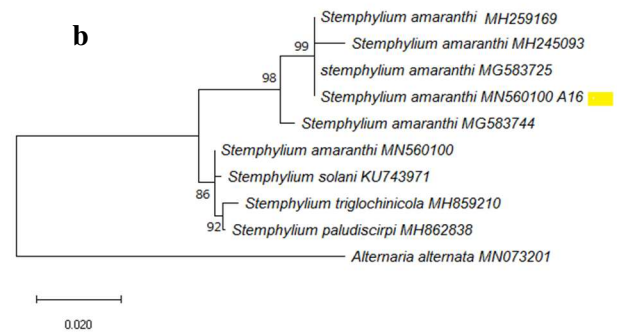
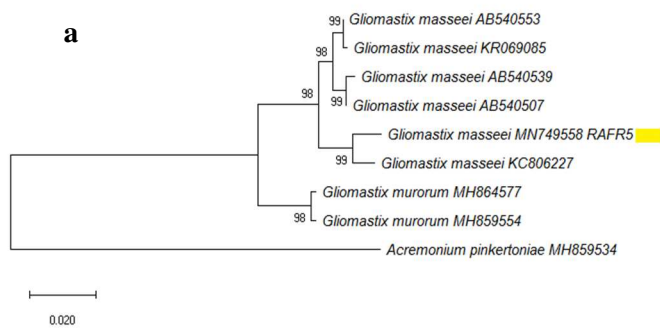
Table 6

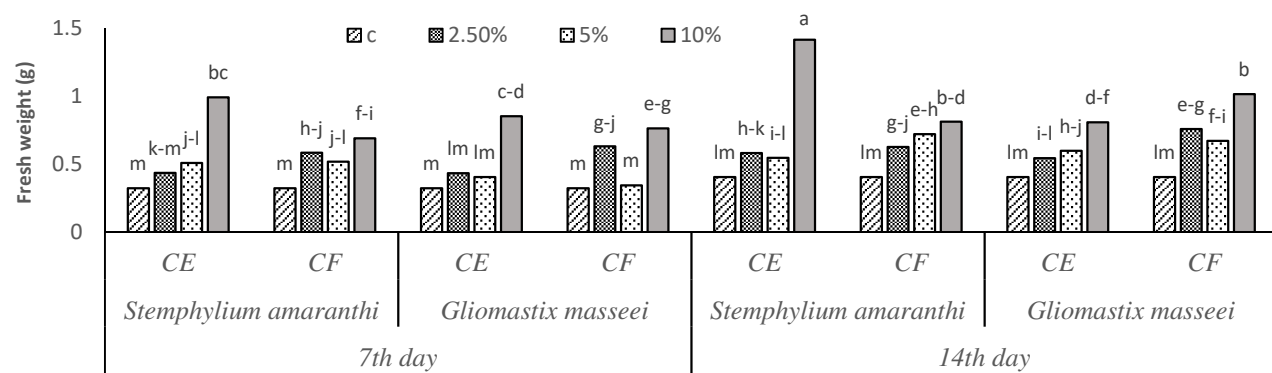
Phenol and flavonoid contents in extracts of endophytic fungi

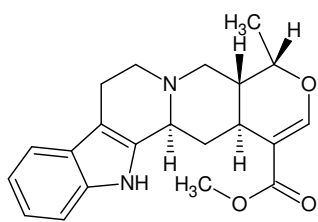
Fungal endophytes	Total flavonoids (mg quercetin equivalents.g ⁻¹)	Total phenols (mg gallic acid equivalents.g ⁻¹)
<i>Gliomastix masseei</i>	0.10±0.0001 ^b	16.63±0.340 ^b
<i>Stemphylium amaranthi</i>	0.12±0.001 ^a	18.29±0.035 ^a

Different letters indicate the statistically significant differences (LSD's multiple range test, *p* ≤ 0.05) among the two species of fungi.

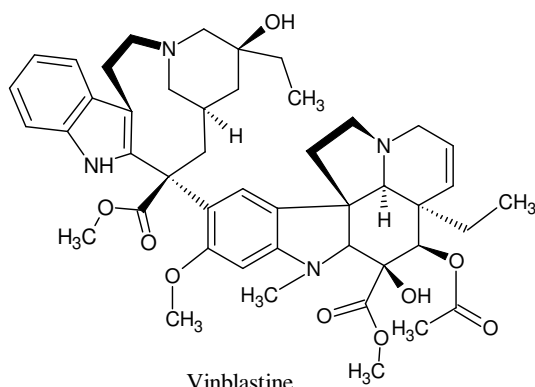




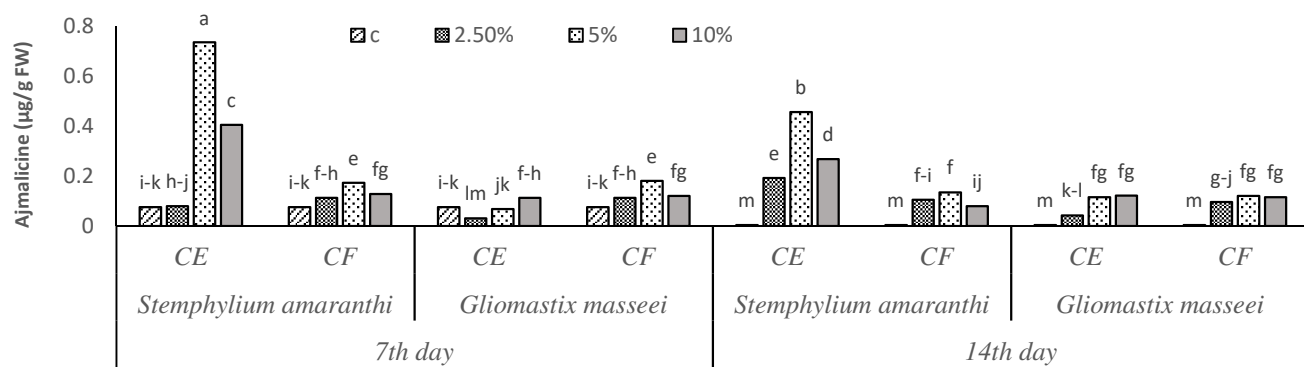




Ajmalicine



Vinblastine



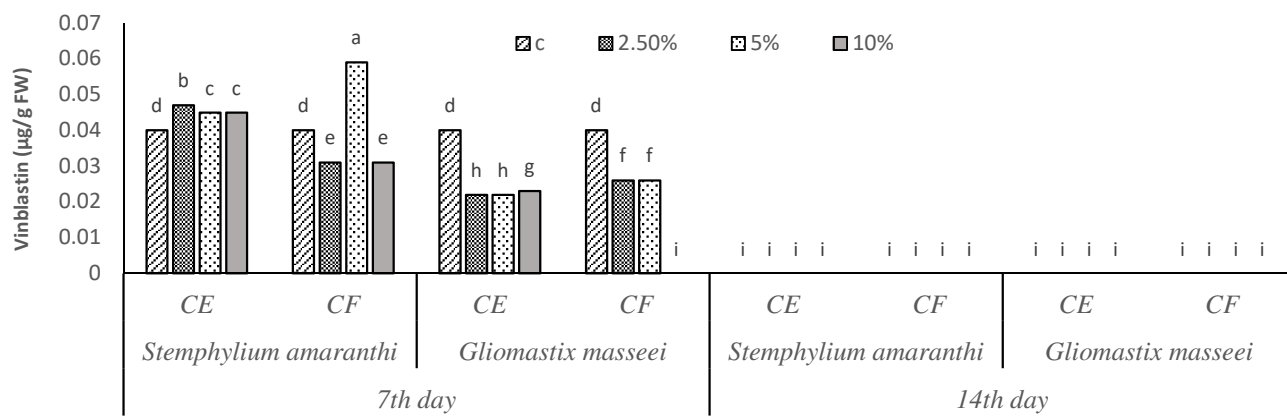


Fig. 1. Endophytic fungi isolated from *Catharanthus roseus* L. G.Don cv. Icy pink grown in PDA medium:

a) *Stemphylium amaranthi* b) *Gliomastix masseei*.

Fig. 2. Reconstruction of the phylogenetic trees of endophytic fungi isolated from root (a) and leaf (b) of *Catharanthus roseus* L. G. Don cv. Icy pink from Neighbor-Joining analysis.

Fig. 3. Effects of the addition of cell extract (CE) and culture filtrate (CF) of *S. amaranthi* and *G. masseei* on 7th and 14th days of sampling on fresh weight of *Catharanthus roseus* L. G.Don cv. Icy pink callus. Different letters indicate the statistically significant differences (LSD's multiple range test, $p \leq 0.05$) among the two species of fungi.

Fig. 4. Structures of ajmalicine and vinblastine

Fig. 5. Effects of cell extract (CE) and culture filtrate (CF) of *S. amaranthi* and *G. masseei* after 7 and 14 days of culture on ajmalicin accumulation in *Catharanthus roseus* L. G.Don cv. Icy pink callus culture. Different letters indicate the statistically significant differences (LSD's multiple range test, $p \leq 0.05$) among the two species of fungi.

Fig. 6. Effects of cell extract (CE) and culture filtrate (CF) of *S. amaranthi* and *G. masseei* after 7 and 14 days of culture on vinblastine accumulation in *Catharanthus roseus* L. G.Don cv. Icy pink callus culture. Different letters indicate the statistically significant differences (LSD's multiple range test, $p \leq 0.05$) among the two species of fungi.