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► To cite this version:

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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1 Accumulation of ajmalicine and vinblastine in cell cultures is enhanced by endophytic 2 fungi of *Catharanthus roseus* cv. Icy Pink

4 Abstract

5 *Catharanthus roseus* belonging to the Apocynaceae family synthesizes indole alkaloids like
6 vinblastine and ajmalicine which are used in cancer treatment and for decrease high blood
7 pressure, respectively. However, these compounds with a high value added are accumulated
8 at low level in the plants. For the first time, endophytic fungi isolated from leaves
9 (*Stemphylium amaranthi*) and roots (*Gliomastix masseei*) of *C. roseus* cv. Icy Pink were used
10 as fungal elicitors to improve the biomass of *C. roseus* callus culture as well as the
11 accumulation of indole alkaloids in the calli. Polyphenols and the antioxidant activity of
12 endophytes extracts were examined. The ethyl acetate extract of *S. amaranthi* showed the
13 maximum scavenging activities in 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-
14 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays. The maximum amount of total
15 flavonoid and total phenol content were found in *S. amaranthi* (18.29±0.03 mg gallic acid
16 equivalents.g⁻¹ and 0.117±0.00001 mg quercetin equivalents.g⁻¹, respectively). Also 5% cell
17 extract and 5% culture filtrate extract of this endophytic fungus during 7 days of calli culture
18 led to the highest contents of ajmalicine (0.735 µg.g⁻¹ fresh weight) and vinblastine (0.059
19 µg.g⁻¹ FW) respectively. Thus, endophytic fungi isolated from *C. roseus* could be used as
20 fungal elicitor to improve the indole alkaloid production.

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

24 1. Introduction

25 The periwinkle (*Catharanthus roseus* (L.) G. Don) is a perennial tropical plant which belongs
26 to Apocynaceae family. Periwinkle is capable of synthesizing valuable alkaloids, including
27 vincristine, vinblastine, vindoline, ajmalicine, catharanthine and serpentine, which some of
28 them like vinblastine and vincristine are commonly used in treating cancer. Also ajmalicine is
29 an antihypertensive compound which can decrease high blood pressure (Ramezani et al.,
30 2018). The yield of some indole terpenoid alkaloid compounds such as vinblastine and
31 vincristine are relatively low in the aerial part of this plant (approx. 0.0005%) (Tonk et al.,
32 2016). Due to the high price, importance and trace amounts of these alkaloids in *C. roseus*,
33 attempts to enhance the production of these compounds have led to comprehensive researches
34 on this plant as a model for plant specialized metabolism (Anjum and Chandra, 2019). The *in*

35 *in vitro* culture technique is one of the promising methods to give the opportunity to regulate
36 metabolism, cell growth and to direct the culture to produce the specialized metabolites
37 (Zhang et al., 2011). In the study of Mekky et al. (2018), they announced that the
38 biosynthesis of vincristine and vinblastine showed a 3.39-fold increase in callus culture
39 compared with the wild *C. roseus* plant. It has been recognized that a large number of biotic
40 compounds are effective to enhance specialized metabolites biosynthesis when they were
41 added in plant tissue cultures. Arbuscular mycorrhizal fungal induction increased levels of
42 hypericin and pseudohypericin in *Hypericum perforatum* root culture (Zubek et al., 2012).
43 Various fungi like *Colletotrichum lindemuthianum*, *Trichoderma harzianum*, and *Fusarium*
44 *oxysporum* enhanced asiaticoside accumulation and biomass in shoot culture of *Centella*
45 *asiatica* (Prasad et al., 2013). About *C. roseus*, *Aspergillus flavus*, as a fungal elicitor,
46 improved biomass of callus culture and alkaloid yield (Tonk et al., 2016). Also *in vitro* shoot
47 cultures of *C. roseus* indicated the highest biosynthesis of vindoline and catharanthine after
48 16 h of *Pythium aphanidermatum* treatment (Shukla et al., 2010). Endophytic fungi, that
49 typically coexist with their hosts asymptotically, represent a wide source of
50 uncharacterized and unexplored microorganisms capable of producing novel and different
51 metabolites (Parthasarathy et al., 2020). Some of fungal endophytes synthesized compounds
52 such as phenols, alkaloids and flavonoids that have biological functions (Hughes, 2016). So
53 far, less than 16% of the identified fungal species have been studied for their biosynthesis
54 capacity and fewer than 5% of the total fungal species have been characterized, they present
55 an important source of natural compounds (Bedi et al., 2018). In cell suspension culture of *C.*
56 *roseus*, endophytic fungi could increase yield of total alkaloids (Tang et al., 2011).
57 It seems that fungal endophytes produce some compounds that can play elicitor role and this
58 fungal elicitor may increase the alkaloid content in the *C. roseus*. So this research was done
59 in order to investigate both the phytochemical characteristics of two endophytic fungi isolated
60 from *C. roseus* cv. Icy Pink plant and the possibility of improving the biomass of *C. roseus*
61 callus culture as well as the accumulation of indole terpenoid alkaloids in the calli through
62 the application of these two new endophytes used as fungal elicitors. Also it should be
63 mentioned that this study has been performed on icy pink cultivar for the first time.

64

65 **2. Material and methods**

66 *2.1 Isolation of endophytic fungi*

67 Healthy tissues including roots and stems were collected from *Catharanthus roseus* (L.) G.
68 DON cv. Icy Pink grown in May 2018 in Gorgan, Iran and were stored at 4°C (Waksman et
69 al., 1916). The stems and roots of the plant were cut into 5 cm pieces and rinsed with running
70 water for 10 minutes to remove soil and other particles. The samples were surface sterilized
71 with 70% alcohol for one minute and 1.5% sodium hypochlorite for 10 minutes and washed
72 with sterilized water 3 times. Samples were dried under the laminar on sterile blotters and
73 then the sterilized small pieces were put on the Potato Dextrose Agar (PDA, Merck,
74 Darmstadt, Germany) petri dish (100×15 mm) combined with tetracycline (50 mg. L⁻¹) to
75 prevent the bacterial growth and incubated for up to 6 to 8 weeks at 25°C with 12 h light and
76 dark cycles to enable the endophytic fungi growth. By the hyphal tip culture, the pure fungal
77 cultures of endophytic isolates were acquired. To identification of colonies that emerged from
78 different tissue parts, colonies were transferred to antibiotic-free PDA (Hatamzadeh et al.,
79 2020).

80

81 *2.2 Molecular Studies*

82 Fungal endophytes were grown for 7 days in 200 mL of PDB (Potato Dextrose Broth) at 28
83 °C. The fungal mycelia were freeze-dried. The DNA was extracted as described by Saar et al.
84 (2001). Strains were sequenced with ITS primer (Table 1). The PCR amplifications were
85 carried out on a GeneAmp PCR System 9600 (Perkin Elmer, USA) with a total volume of
86 12.5 µL solution containing 10–20 ng of template DNA, 0.7 µL DMSO (99.9%), 1 × PCR
87 buffer, 2 mM MgCl₂, 0.5 µM of each primer, 1.0 U Taq DNA polymerase (NEB) and 25 µM
88 of each dNTP. The amplification procedure was started with pre-heating at 95°C for 1 min,
89 accompanied by 40 denaturation cycles at 95°C for 30 s, extension at 72°C for 10 s and a
90 final extension at 72°C for 5 minutes. Electrophoresis was used to examine the products of
91 the PCR reaction using 1% (w/v) agarose gel. BLAST analysis and sequences were
92 documented in the GenBank Database at NCBI.

93

94 *2.3 Endophytic fungi extraction*

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

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

102

103 *2.4 Determination of antioxidant activities*

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

109

110 *2.5 Determination of total flavonoids*

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

114

115 *2.6 Determination of total polyphenols*

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

119

120 *2.7 Catharanthus roseus cv. Icy Pink callus cultures*

121 Seeds of *C. roseus* (L.) G. DON cv. Icy pink were provided by Syngenta seeds Co,
122 Enkhuizen, Netherlands. Sterilized seeds were placed in glassy jar containing 50 ml of solid
123 MS medium without any plant growth regulator (Murashige and Skoog, 1962). The
124 germinated seedlings were maintained in *in vitro* conditions as long as seedlings grow and
125 produce leaves. Young leaves parts were used as explants to produce callus. For callus
126 induction, the MS medium was supplemented with 1 mg.L⁻¹ of 2,4-dichlorophenoxyacetic
127 acid (2,4-D) and 0.5 mg.L⁻¹ 6-benzylaminopurine (BA). The medium was solidified with
128 0.8% agar. Each petri dish contained 15 mL of medium. The medium pH was set to 5.7
129 before autoclaving. All cultures were incubated at 25 ± 2°C under 16/8 (light/dark)
130 photoperiod.

131

132 2.8 Endophytic fungi elicitor preparation and elicitation conditions

133 The fungal endophyte elicitors were prepared with some modifications in accordance to the
134 process defined by Baldi et al. (2009). Two fungal endophytes cells (agar plugs containing
135 mycelia) were put into a 250 mL Erlenmeyer flask containing 50 mL of PDB media. The
136 flasks were incubated on an incubator shaker at $25 \pm 2^\circ\text{C}$ and rotating at 200 rpm to allow the
137 fungal endophytes cells to grow until the end of the log phase (6 days). Then the culture broth
138 was collected by passing the fungal culture through a Whatman N°1 filter paper and fungal
139 mycelia were kept. Afterwards the culture broth was centrifuged at 5000 rpm for 15 min to
140 eliminate fungal cells. The centrifuged culture broth was filtered by passing through $0.22 \mu\text{m}$
141 cellulose acetate syringe filters and labeled as Culture Filtrate (CF). The fungal mycelia were
142 separated from the broth by filtration and was rinsed 3 times with sterile distilled water and
143 dried at 40°C and crushed. The fungal powders were immersed in sterile distilled water (3
144 $\text{mg}\cdot\text{ml}^{-1}$) and were autoclaved. Then the fungal suspensions were centrifuged and the
145 supernatant was collected and designated as Cell Extract (CE).

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

149
150

151 2.9 GC-MS identification

152 The ethyl acetate extracts of endophytic fungi were analyzed using GC-MS QP2010-
153 Shimadzu equipment operating in the EI mode at 70 eV. An agilent DB5MS column ($30 \text{ m} \times$
154 $0.25 \text{ mm} \times 0.25 \mu\text{m}$) was used with a 26 min temperature program of $60\text{-}320^\circ\text{C}$ at $5^\circ\text{C}/\text{min}$.
155 The temperature for the injector was 250°C , the carrier gas (helium) flow rate was $1 \text{ mL}/\text{min}$,
156 and the split ratio was 1:5. The identification of the compounds was done by comparing the
157 measured data with literature data as indicated in the text.

158

159 2.10 LC-MS analysis

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

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

166 and solvent B consisted of pure methanol. The linear gradient solvent system used was: 0
167 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-
168 20 min, 99% of B, 20-22 min, 99-10% of B, 22-25 min, 10% of B. The LC system was
169 coupled to a Bruker Elite TQ MS mass spectrometer (LCMS TQ) with an atmospheric
170 pressure electrospray interface. Bruker MS Workstation version 8.2.1 was used for LC-MS
171 system control and data analysis. Alkaloid identification was done by multiple reaction
172 monitoring (MRM) transition and retention time. The parameters used for the mass
173 spectrometer under the ESI⁺ mode were as follows: spray voltage 4500 V, cone temperature
174 300°C, cone gas flow 20 psi, heated probe temperature 250°C, probe gas flow 35 psi,
175 nebulizer gas (nitrogen) flow 40 psi. Parameters for the cone energy and collision energy are
176 listed in Table 2. All parameters were automatically optimized by Workstation.

177

178 *2.11 Data Analysis*

179 Treatments with 3 replications were arranged in a completely randomized factorial design.
180 The data are reported as means ± standard deviation (SD). Variance analysis was carried out
181 by using the SAS software (9.1 version) and means were measured by using LSD's test ($p \leq$
182 0.05).

183

184 **3. Results and discussion**

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

189

190 *3.1. Endophytic fungi*

191 *3.1.1. Identification of fungal endophytes*

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

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

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

207

208 3.1.2. Identification of natural compounds

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

218

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

222

223 3.1.3. Antioxidant activity

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

231 induced by plant-derived materials at different wavelengths. It was reported that the crude
232 supernatant extract of the endophytic fungus *Nigrospora sphaerica*, isolated from *C. roseus*,
233 showed antioxidant activity (Ayob et al., 2019). Also Khiralla et al. (2015) reported that the
234 endophytic extracts of *C. roseus* had extremely wide range of antioxidant activity (IC₅₀
235 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
236 DPPH assay. Other researchers confirmed that fungal endophyte extracts isolated from *C.*
237 *roseus* such as *Chaetomium nigricolor* (Dhayanithy et al., 2019), *Mucor* sp. (Shukla et al.,
238 2018) and *Curvularia geniculata* (Chowdhury et al., 2018) had a great potential as
239 antioxidant agent.

240

241 3.1.4. Determination of total phenol and total flavonoid contents

242 Total phenol and flavonoid contents of ethyl acetate extracts of *S. amaranthi* and *G. masseei*
243 endophytic fungi were evaluated (Table 6). *S. amaranthi* extract contained both more total
244 phenols ($18.29 \pm 0.03 \text{ mg gallic acid equivalents.g}^{-1}$) and total flavonoids ($0.12 \pm 0.00001 \text{ mg}$
245 $\text{quercetin equivalents.g}^{-1}$) by comparison with *G. masseei* extract. Chowdhury et al. (2018)
246 reported that the highest total phenol $27.65 \pm 1.20 \text{ mg QE.g}^{-1}$ and total flavonoid $2.06 \pm 0.10 \text{ mg}$
247 GAE.g^{-1} contents were observed in *Curvularia geniculata* isolated from *C. roseus*. In addition,
248 *Nigrospora sphaerica* isolated from *C. roseus* also showed the highest contents in total
249 phenol and total flavonoid contents, which were $0.030 \pm 0.01 \text{ mg GAE.g}^{-1}$ and 0.038 ± 0.001
250 mg QE.g^{-1} respectively (Ayob et al., 2019).

251

252 3.2. Effects of endophytic fungi on callus growth

253

254 Induction of callus was observed in four weeks after initiation of *in vitro* cultures. Four week-
255 old calli were separated from primary explants and proliferated on the same media used for
256 the induction of callus. In view to enhance the growth of callus culture, calli were subcultured
257 on Murashige and Skoog medium supplemented with the same growth regulators as used for
258 induction medium and containing cell extract and culture filtrate from two endophytic fungi,
259 *S. amaranthi* and *G. masseei*, at various concentrations (0, 2.50, 5 and 10%). Calli were
260 incubated for 7 and 14 days. The control calli showed the multiplication index of 0.32 and
261 0.41 g after 7 and 14 days of culture respectively. The calli growth treated with cell extract
262 and culture filtrate from the two endophytic fungi was calculated by measurement of the fresh
263 weight (FW) of the callus and the corresponding results are shown in figure 3. The main
264 effects of endophytic fungi species, periods of time, endophytic fungi extract and their

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

280

281 3.3 Effects of endophytic fungi on alkaloid accumulation

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

290

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

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

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

310

311

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

321 About vinblastine accumulation in calli, stimulating or inhibitory effect were observed
322 according to the fungal species, time of culture, Cell Extract (CE) or Culture Filtrate (CF) and
323 its concentration level. Highest content of vinblastine ($0.059 \mu\text{g.g}^{-1}$ FW) was obtained with
324 5% of CF from *S. amaranthi* after 7 days of culture. Also CE (2.5, 5 and 10%) of this
325 endophytic fungus increased vinblastine accumulation after one week of treatment. In
326 contrary, extracts from *G. masseei* (CE and CF) led to the lowest content of vinblastine
327 ($0.022 \mu\text{g.g}^{-1}$ FW) whatever the concentration used (Figure 6) and vinblastine was not
328 detected with 10% of *G. masseei* CF in first week. In 14th day, vinblastine accumulation in
329 callus was stopped in all treatments even control that maybe it was metabolized to another
330 alkaloids.

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

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

341 Fungal elicitors can be defined as signaling molecules that trigger specialized metabolites
342 formation in *in vitro* cultures by inducing proteins related to plant defense, hypersensitive
343 response or pathogenesis (Baldi et al., 2009). However no elicitor was identified to have a
344 general impact on different cultures, and no system was found to react to all elicitors which
345 this issue is related to receptors. There are receptors in plasma membrane of plants cell which
346 are responsible for the identification of fungal elicitors and transferring activation system for
347 defense system of plant cell. Specific receptor structure allowed the specific elicitors to be
348 recognized. So, all fungal elicitors cannot induce plants system. In order to produce a desired
349 compound, various elicitors need to be screened (Singh et al., 2018). In this research, the
350 highest content of ajmalicine was related to the CE of *S. amaranthi* with 5% level in 7th day
351 and the highest amount of vinblastine was produced from callus culture exposed with 5%
352 (v/v) CF of *S. amaranthi* in 7th day. Consequently, it could be inferred that the fungal
353 elicitor's effect on the yield of vinblastine and ajmalicine depends on the sampling time and
354 elicitor concentrations. Indeed, in order to obtain the highest indole terpenoid alkaloid
355 production by fungal elicitor, it is necessary to optimize the concentration and different
356 sampling time to culture medium. The earlier researches (Namdeo et al., 2002; Tonk et al.,
357 2016) confirmed that the concentration, type and elicitors sampling time affect the inducing
358 of specialized metabolites production. It was reported that the amount of vinblastine was
359 increased by using *Trichoderma tomentosum* and *Priformospora indica* fungal extracts
360 (Ramezani et al., 2018). Salehi et al. (2019) reported also the favorable impact of elicitors
361 obtained from *Paraconiothyrium brasiliense* and *Chaetomium globosum* isolated from
362 *Corylus avellana* and *Taxus baccata* on production of paclitaxel in cell suspension culture of
363 *Corylus avellana*. In another study cleared that the IBA and the endophytic fungus

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

366 367 **4. Conclusion**

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

381 382 **Acknowledgements**

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

388 389 **References**

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496 fungi. Mycorrhiza, 22(2), 149-156.

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-Tetradecene-11-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 IC ₅₀ (µg/mL)	ABTS IC ₅₀ (µg/mL)
<i>Gliomastix maseei</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.

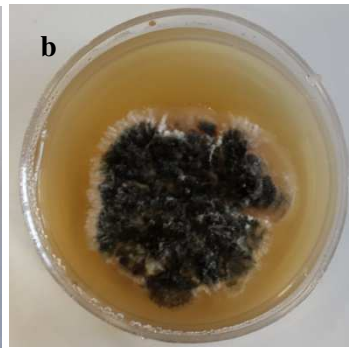
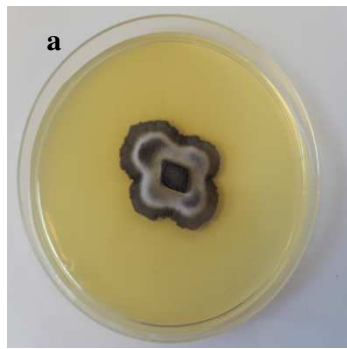
Values are representative of three independent determinations.

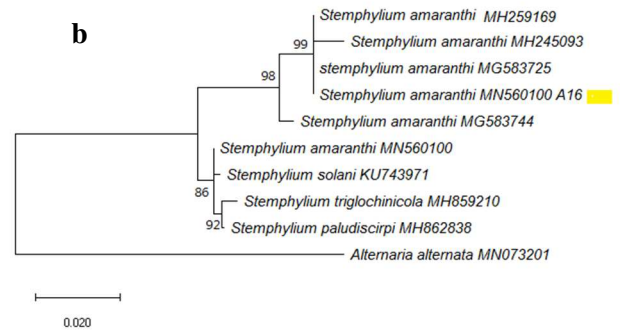
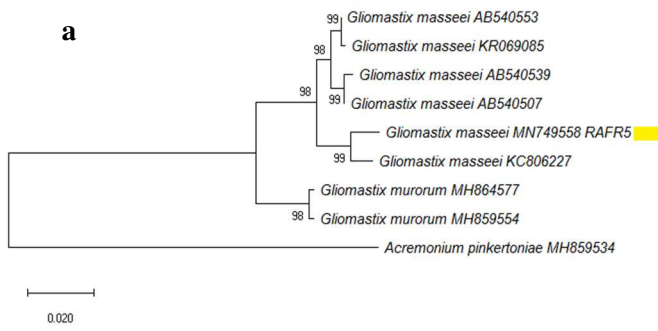
Table 6

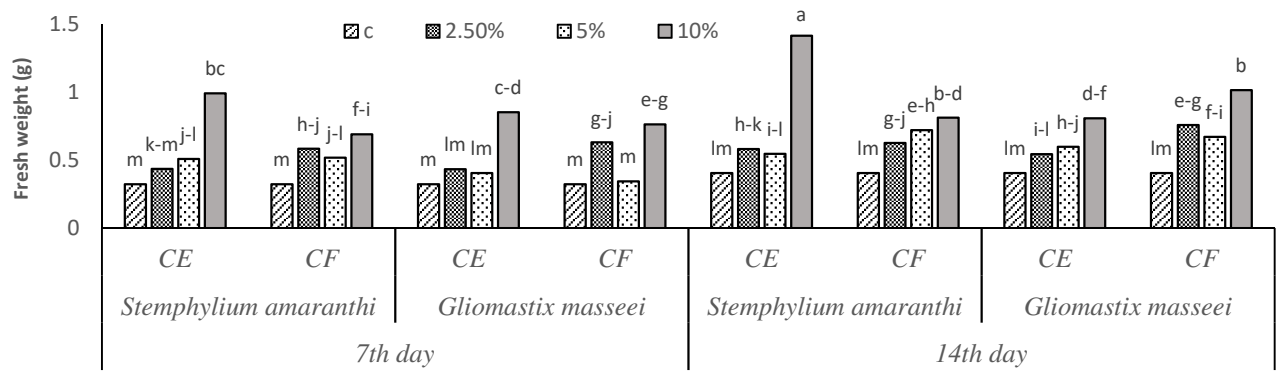
Phenol and flavonoid contents in extracts of endophytic fungi

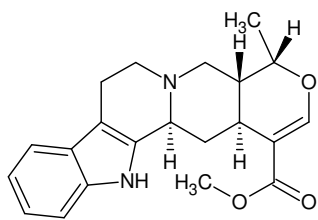
Fungal endophytes	Total flavonoids (mg quercetin equivalents.g ⁻¹)	Total phenols (mg gallic acid equivalents.g ⁻¹)
<i>Gliomastix maseei</i>	0.10±0.0001 ^b	16.63±0.340 ^b
<i>Stemphylium amaranthi</i>	0.12±0.001 ^a	18.29±0.035 ^a

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

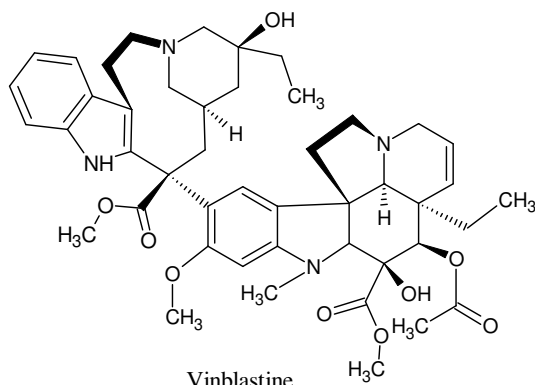




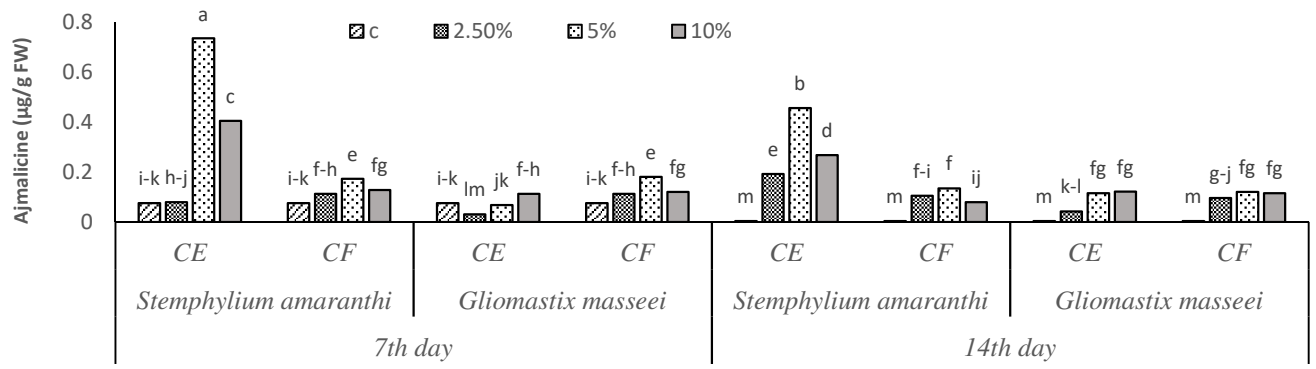




Ajmalicine



Vinblastine



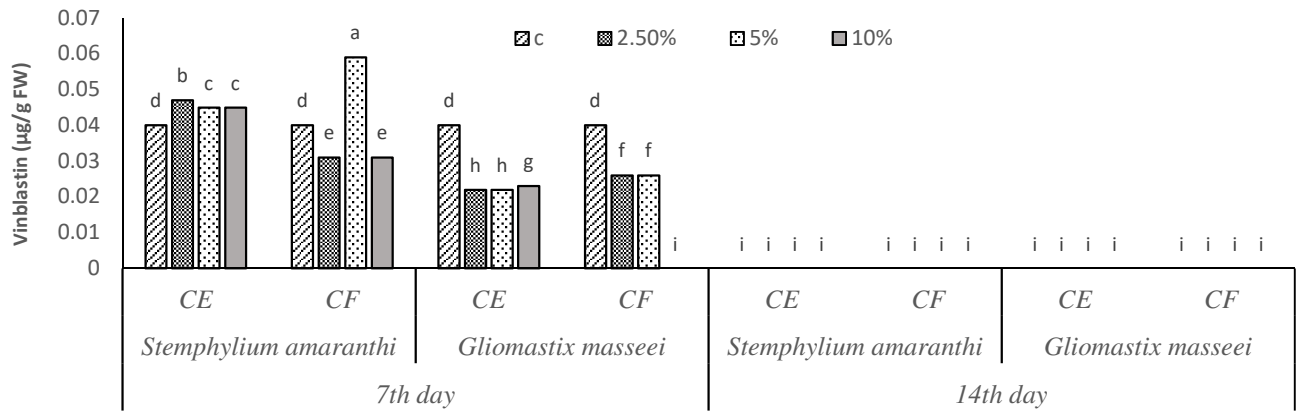


Fig. 1. Endophytic fungi isolated from *Catharanthus roseus* L. G. Don cv. **Icy pink** grown in PDA medium:

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.