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Original article

Root specific elicitation and exudation of fluorescent β -carbolines in transformed root cultures of *Oxalis tuberosa*

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8 Abstract

9 Stable transformation was achieved in oca (*Oxalis tuberosa* L.) using an *Agrobacterium rhizogenes*-mediated system. Transformation
10 frequencies varied with the use of different types of strains of *A. rhizogenes* and the age of explants. The transfer of *rol A* gene into the oca
11 genome was confirmed by PCR analysis. In vitro transformed root cultures of oca grown in sterile liquid media induced purplish-blue
12 fluorescence of the culture flask medium when irradiated with UV light. We have previously observed a similar phenomenon, the exudation of
13 fluorescent compounds by the roots of in vitro and field-grown oca plants. Hairy root cultures of *O. tuberosa* transformed with *A. rhizogenes*
14 (ATCC-15834) exuded constitutive levels of harmine (7-methoxy-1-methyl- β -carboline) and harmaline (3,4-dihydroharmine), the main
15 fluorescent compounds detected from oca's root exudates. Transformed roots showed better growth and exudation of harmine and harmaline
16 compared to the untransformed normal roots. Upon elicitation with fungal cell wall elicitors from *Phytophthora cinnamoni*, the production
17 and exudation of harmine/harmaline was enhanced in both transformed and non-transformed roots. Harmine and harmaline showed a wide
18 range of antimicrobial activity against soil-borne microorganisms. Biologically, these findings suggest that in nature β -carbolines are
19 constitutive antimicrobial compounds released into the rhizosphere upon microbial challenge. Transformed root cultures of oca make a simple,
20 reliable and well-defined model system to investigate the molecular and metabolic exudation of fluorescent β -carboline biosynthesis, and to
21 evaluate the biological significance of the phenomenon of root exudation of fluorescent metabolites.

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23 **Keywords:** *Agrobacterium rhizogenes*; Elicitation; Harmine; Harmaline; Oca; *Oxalis tuberosa*

25 1. Introduction

26 The Andean root and tuber crops constitute a unique
27 reservoir of germplasm biodiversity. They have been bred to
28 grow at altitudes of 2400-4000 m; therefore, they have great
29 potential for introduction into other highland areas where
30 crops from the Old World are not well adapted [14,15]. After
31 potato, oca (*Oxalis tuberosa* L.) is the most well-known
32 tuber crop from the Andean region [14]. It is an annual,
33 herbaceous plant in the Oxalidaceae family. Despite an as-
34 sortment of valuable features, including high nutritional
35 value, the Andean root and tuber crops have been largely
36 overlooked and poorly studied at the biological level [21]. As
37 is true for most Andean root and tuber crops, there are very

few reports on the basic biology, agronomy and biochemistry 38
of oca. Our laboratory has been involved in the investigation 39
of oca for some time, focusing on its unique root biology. 40
Upon elicitation, plants produce a wide diversity of biologi- 41
cally active secondary metabolites termed phytoalexins 42
[11,29]. Some of these compounds are synthesized and 43
stored during normal growth and development while others 44
are absent in healthy plants, accumulating only in response to 45
pathogen attack, stress conditions or artificial elicitation. 46
Elicitors are defined as molecules that stimulate defense or 47
stress-induced responses in plants [11,24]. The exogenous 48
application of elicitors to in vitro cultures is useful for study- 49
ing plant responses to potential microbe attack as well as for 50
enhanced biotechnological production of value-added sec- 51
ondary metabolites in fermentation systems. Fungal elicitors, 52
mostly derived from the cell walls of fungal pathogens, are 53
known to induce de novo synthesis of antimicrobial phytoal- 54
exins. Under specific conditions fungal elicitation operates 55
by induction of methyl jasmonate, leading to phytoalexin 56

Abbreviations: CWE, cell wall elicitors; FW, fresh weight; JA, jasmonic acid; OD, optical density; SA, salicylic acid.

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57 production [28,29]. In this manuscript we explored the in-
58 duction of root secretion of fluorescent compounds upon
59 elicitation.

60 Root fluorescence is a phenomenon in which roots of
61 plants/seedlings fluoresce when irradiated with ultraviolet
62 (UV) light. Only two plant species have been documented to
63 exhibit this phenomenon: soybean and rye grass, and both
64 only in germinating seedling roots [10]. The biological sig-
65 nificance of this occurrence in plants is unknown. We have
66 previously reported that the Andean tuber crop species *O.*
67 *tuberosa* secretes fluorescent compounds as part of its root
68 exudates [5,7]. The main fluorescent compounds from oca's
69 root exudates were identified as harmine (7-methoxy-1-
70 methyl- β -carboline) and harmaline (3,4-dihydroharmine)
71 [7]. Harmine and harmaline are widespread photoactive
72 β -carbolines, alkaloids, and well-known central nervous sys-
73 tem stimulants, reported as a major component of the seeds
74 of *Peganum harmala* (Peha) [1]. They also occur in *Baniste-*
75 *riopsis caapi*, an ingredient in the shamanistic Amazonian
76 hallucinogenic mixture "ayahuasca", as well as in a number
77 of other plant species [2]. The study of roots, the "hidden
78 half" of the plant, and the identification of compounds they
79 excrete pose special challenges. To better understand the
80 functional significance of β -carbolines secretion by roots, we
81 developed a hairy root system of *O. tuberosa* for the expres-
82 sion and manipulation of constitutive and inducible second-
83 ary metabolites. Hairy roots show stable expression of bio-
84 synthetic pathways, and thus have been used as an
85 experimental system to study the biology and biochemistry
86 of underground organs [4,10,22,27].

87 In this communication, we report the isolation and func-
88 tional characterization of fluorescent β -carbolines along with
89 methyl paraben in *O. tuberosa* hairy roots. We discovered
90 that the exudation of these fluorescent metabolites along with
91 methyl paraben was triggered upon fungal cell wall elicitat-
92 ion. Furthermore, harmine and harmaline were found to be
93 highly inhibitory against an array of rhizosphere microorgan-
94 isms. Our results highlight a novel defense mechanism by
95 which hairy roots of *O. tuberosa* secrete antimicrobial fluo-
96 rescent metabolites.

97 2. Methods

98 2.1. Plant material

99 Seeds of oca (*O. tuberosa* L.) were obtained from the
100 International Potato Center (Lima, Peru) and from the labo-
101 ratory of Dr. Hector E. Flores (Pennsylvania State Univer-
102 sity). Seeds were washed five times with sterile water and
103 were surface sterilized with 10% (v/v) commercial bleach for
104 15 min followed by three to four washes in sterile distilled
105 water. Surface sterilized seeds were placed on static Murash-
106 ige and Skoog (MS) [20] basal media for germination and
107 incubated at $25 \pm 2^\circ\text{C}$ with a light intensity of $4.41 \text{ J m}^{-2} \text{ s}^{-1}$.

2.2. Chemicals

Harmine, harmaline and methyl paraben were obtained
from Sigma Co (St. Louis, USA); all other chemicals were of
analytical and HPLC grade. Solvents were from Fisher Co.
(Glendale, CA, USA).

2.3. Root cultures

Leaves from in vitro germinated *O. tuberosa* were used
for transformation. Shoot cultures were placed separately in
Magenta GA-7 vessels containing (MS) basal medium soli-
dified with 0.3% Phytigel (Sigma Co.). Cultures were kept
in a light chamber maintained at 24°C with a fluence rate of
 $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$. *A. rhizogenes* strains ATCC-15834 and
LBA-9402 were obtained from Dr. Eugene W. Nester, (Uni-
versity of Washington, Seattle). Bacterial colonies were cul-
tured for 3 d on solid YEM medium. Transformed roots
(hairy roots) were initiated by inoculating the wounded hy-
pocotyls of 2-10-day-old seedlings of *O. tuberosa* with *A.*
rhizogenes strains. Hairy roots were found to appear within
10 d of infection (Fig. 1). Roots of $3 \pm 0.2 \text{ cm}$ size were
excised and immediately transferred to 40 ml MS basal
media containing carbenicillin (500 mg l^{-1}) in 150 ml conical
flasks. Transformed roots were periodically subcultured at 3
d intervals in antibiotic-containing medium. The roots were
subsequently transferred to MS basal liquid media and incu-
bated in the dark on a rotary shaker at 90 rpm and maintained
at $25 \pm 2^\circ\text{C}$, to obtain axenic hairy root cultures (Fig. 1).
Hairy root cultures derived from ATCC-15834 were prefer-
red over those derived from LBA-9402 due to a faster
growth rate and better transformation efficiency (data not
shown), and were used for the elicitation studies. Root
growth was monitored on a weekly basis. In all experiments
an initial biomass of $100 \pm 0.09 \text{ mg}$ was used for 40 ml of
culture medium. Roots were collected and fresh weight was
measured in triplicate.

2.4. Polymerase chain reaction (PCR) analyses

Total genomic DNA was isolated using the DNeasy mini-
prep kit (Qiagen Co., Germany) from the original hairy root
clone (derived from the strain ATCC-15834), and from the
untransformed shoots, leaves, and roots of oca. Plasmid
DNA from *A. rhizogenes* strain ATCC-15834 was used as a
positive control. Isolated DNA was analyzed by polymerase
chain reaction (PCR) for *rol A* gene. The oligonucleotide
primers used to amplify *rol A* gene on the T-DNA of *A.*
rhizogenes (ATCC 15834) were designed according to
Rhodes et al. [25] and obtained from the Macromolecular
Facility (Colorado State University). The primers designed
to amplify *rol A* were 5'-CAGAATGGAA-
TTAGCCGGACTA-3' and the reverse primer 5'-
CGTATTAATCCCGTAGGTTTGTTT-3'. PCR for *rol A* was
performed by amplification under the following conditions:
initial denaturation at 94°C for 4 min, annealing at 58°C for
1 min and extension at 72°C for 1 min for 35 cycles, with a

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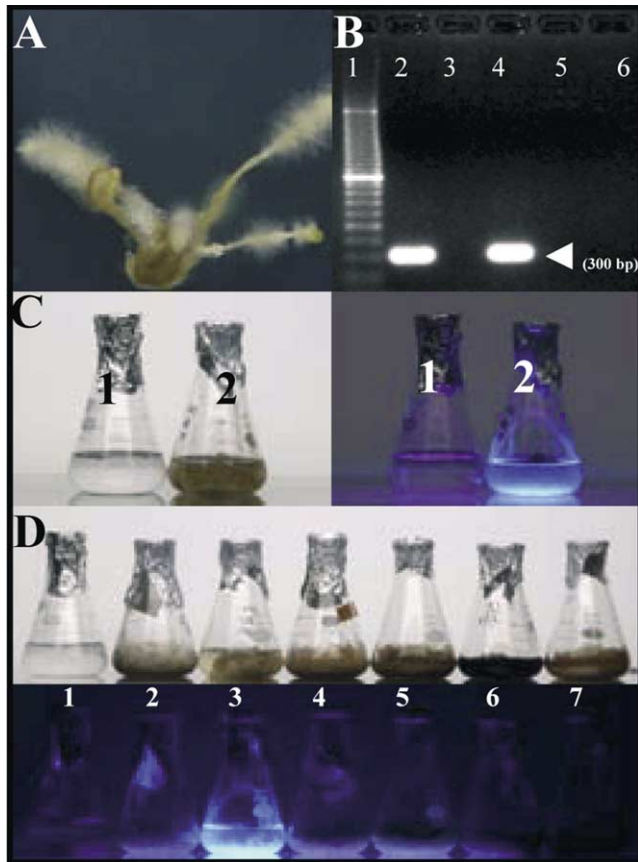


Fig. 1. Initiation and maintenance of transformed root cultures of *O. tuberosa* by *A. rhizogenes* mediated transformation. **A**, Initiation of hairy root cultures from leaf explant derived from *A. rhizogenes* (ATCC-15834); **B**, the presence of *rol A* (ca. 0.3 kb) in transformed roots of *O. tuberosa* analyzed by PCR. Lanes 1, molecular size markers (100 bp markers); 2, plasmid DNA from ATCC-15834 (*A. rhizogenes*) as a positive control; 3, untransformed roots of *O. tuberosa*; 4, transformed roots of *O. tuberosa* derived from ATCC-15834 infection; 5, untransformed shoots of *O. tuberosa*; 6, untransformed leaves of *O. tuberosa*; **C**, sterile liquid medium (1), and in vitro culture of transformed roots of oca grown in sterile liquid medium (2) under white and UV light exposure; **D**, sterile liquid medium (1); in vitro transformed root cultures of *U. tuberosus* (2); transformed root cultures of *O. tuberosa* (3); transformed root cultures of *H. muticus* (4); transformed root cultures of *P. americana* (5); transformed root cultures of *L. erythrorhizon* (6); and transformed root cultures of *N. tabacum* (7), under white and UV light exposure. Photographic parameters were adjusted to capture the purple and blue fluorescence. However, photographs cannot fully reproduce the color intensity or distinguish simultaneously across the range of blue and purple tones as perceived by the eye.

160 final extension at 72 °C for 5 min. The amplicons were
161 analyzed by electrophoresis on 2% agar gel. *Rol A* gene was
162 analyzed by means of enzymatic digestions with Eco RI and
163 Hind III (Fig. 1).

164 2.5. Preparation and addition of elicitor

165 An initial inoculum of 100 ± 0.09 mg of hairy roots was
166 used for all the growth and elicitation experiments. The hairy
167 roots of *O. tuberosa* were analyzed periodically for growth
168 and metabolite content during the culture period of 28 d.
169 Hairy root cultures of *O. tuberosa* were elicited with fungal

cell wall preparations, JA and SA. Fungal cell wall extracts 170
(CWE) from fungi such as *P. cinnamoni* were used. The 171
fungal CWEs were prepared and used according to McKin- 172
ley et al. [18]. Fungal elicitors were administered at various 173
concentrations (1–3 ml), into 125 ml flasks containing 40 ml 174
of MS basal media. Solutions of SA and JA were prepared in 175
absolute ethanol and were added individually to roots grown 176
in 40 ml MS flasks to yield final concentrations of 50–200 177
and 100–500 μ M, respectively. A time-course study of the 178
influence of CWE on root growth and metabolite production 179
was conducted by harvesting the roots at 7 d intervals for 4 180
weeks; a non-elicited control was harvested during the same 181
period for biomass and metabolite content as a positive 182
control. Each experiment was repeated twice with five repli- 183
cates each. 184

2.6. Bacterial growth inhibition assay 185

Plant pathogenic bacterial isolates covering a broad phy- 186
logenetic range were tested for inhibition of growth by both 187
filter disc and broth method using 10–40 μ g ml⁻¹ of standard 188
harmine, harmaline and methyl paraben. Bacterial cultures 189
were grown overnight at 24 °C in liquid Luria-Bertani (LB) 190
media [17] to an OD of 0.2 at 600 nm. Different concentra- 191
tions (10–40 μ g ml⁻¹) of harmine, harmaline and methyl 192
paraben were administered to the bacteria-containing LB 193
media. OD at 600 nm was checked after 24 h of incubation to 194
measure bacterial inhibition. Each experiment was repeated 195
twice for each isolate tested. *X. campestris* pv. *vesicatoria*, *P.* 196
fluorescens, *E. carotovora*, *Erwinia herbicola*, *Bacillus* 197
subtilis, *Citrobacter ferundi* and yeast (*Saccharomyces cer-* 198
visiae), were obtained from Dr. Hector E. Flores (Pennsyl- 199
vania State University). 200

2.7. Fungal growth inhibition assay 201

Hyphal growth inhibition was tested by linear growth 202
assay. Eleven fungal isolates were tested and showed a wide 203
range of growth responses on medium containing 10–40 μ g 204
ml⁻¹ of harmine, harmaline, and methyl paraben. Fungal 205
isolates were maintained on potato dextrose agar medium in 206
the dark at 24 °C. Harmine, harmaline and methyl paraben 207
were applied at different concentrations (10, 20, 30, 40 μ g 208
ml⁻¹) to the filter discs. The discs were allowed to air dry and 209
were placed in a circle on the 35 mm petri dish. A 4 mm plug 210
of fungal hyphae was placed at the center of the petri dish and 211
inhibition was observed on a daily basis. Each fungal isolate 212
was tested at all concentrations in three separate replicates. 213
Fungal isolates of *Phytophthora drechsleri*, *Aspergillus niger*, 214
Rhizoctonia solani, *Fusarium oxysporium*, *Alternaria* 215
solani, and *Alternaria brassicae* were obtained from the 216
culture collections of Dr. Hector E. Flores (Pennsylvania 217
State University). 218

2.8. HPLC analysis of root exudates 219

Hairy roots were harvested on a periodic basis, and 200
mg of fresh wet tissues were extracted in 2 ml of methanol for 221

222 24 h (Fisher Co.). Similarly, different plant tissues such as in
 223 vitro-grown roots, stems and leaves (200 mg each) were
 224 extracted using 2 ml of absolute methanol (Fisher Co.). Oca
 225 plants were grown in sterilized soil under greenhouse condi-
 226 tions. Soil samples (500 mg) collected from the plants' rhizo-
 227 sphere were extracted using 5 ml hexane (Fisher Co.) at 4 °C
 228 for 24 h. The extracts from exudates, tissues and soil samples
 229 were centrifuged at 10 000 rpm for 10 min; supernatants
 230 were concentrated under vacuum and were re-suspended in
 231 500 µl of methanol. The media exudate samples (1 ml) from
 232 all treatments were extracted using 5 ml of hexane (Fisher
 233 Co.). To filter any cellular debris, the extract was passed
 234 through a nylon syringe filter of pore size 0.45 µm (Scientific
 235 Resources Inc.). The extracts were vortexed and stored for 24
 236 h at room temperature. The supernatant was collected and
 237 transferred with a Pasteur pipette to a separate test tube, and 1
 238 ml of hexane (Fisher Co.) was added. The supernatant was
 239 concentrated by freeze-drying (Vir Tis, Genesis), and the
 240 weighed powder was dissolved in 200 µl of absolute metha-
 241 nol (Fisher Co.). Extracts from the media were injected into a
 242 high performance liquid chromatography (HPLC) system.
 243 Compounds in the root exudates were chromatographed by
 244 gradient elution on a reverse phase 5 µm, C₁₈ column (25 cm
 245 × 4.6 mm) (Supelco Co.). The chromatographic system
 246 (Summit Dionex Co. Ltd.) consisted of P580 pumps (Dionex
 247 Co., USA) connected to an ASI-100 Automated Sample
 248 Injector (Dionex Co.). The visible absorbance at 210 nm was
 249 measured by a PDA-100 Photodiode array variable UV/VIS
 250 detector (Dionex Co.), although the injected samples were
 251 subjected to a broad range wavelength scan between 190 and
 252 800 nm. Mobile phase Solution A consisted of double dis-
 253 tilled water and Solution B (methanol) (Fisher Co.). A multi-
 254 step gradient was applied for all separations with an initial
 255 injection volume of 15 µl and a flow rate of 1 ml min⁻¹. The
 256 multi-step gradient was as follows: 0–5 min 5% B, 5–10 min
 257 20% B, 15–20 min 20% B, 20–40 min 80% B, 40–60 min
 258 100% B, 60–70 min 100% B, 70–80 min 5% B [7].

259 2.9. UV analysis and imaging

260 In vitro-grown transformed roots, untransformed roots
 261 and soil samples collected from oca's rhizosphere were visu-
 262 alized for blue-purplish fluorescence under UV light expo-
 263 sure using a Mineralight UV lamp (Model UVG-54, Short
 264 wave UV ~254 nm) (Ultra-Violet, Co. USA). Images were
 265 taken using a Nikon still camera (Nikon Co. Japan) and
 266 Kodak 100 R slide film.

267 2.10. ¹H and C¹³ NMR analysis

268 ¹H and C¹³ NMR spectra of exuded harmine and harma-
 269 line were identical to those of commercial standard harmine
 270 and harmaline (Varian INOVA 400 MHz Fourier transform
 271 spectrometer). Harmaline samples were converted to harma-
 272 line hydrochloride for comparison. Spectral data also corre-
 273 sponded to published spectra in the Aldrich library of ¹H FT
 274 NMR Spectra [23].

3. Results

3.1. Influence of different *A. rhizogenes* strains and age of explant on transformation efficiency

278 We established transformed roots of *O. tuberosa* with
 279 *Agrobacterium rhizogenes* (ATCC-15834) using leaf ex-
 280 plants (Fig. 1). The presence of *rol A* gene [25] in hairy root
 281 cultures of *O. tuberosa* upon PCR analysis confirmed its
 282 transformed nature (Fig. 1). Plasmid DNA from *A. rhizo-*
 283 *genes* (ATCC-15834) was used as a positive control (Fig. 1).
 284 Of the two strains of *A. rhizogenes* used in transformation
 285 studies, ATCC-15834 and LBA-9402, it was observed that
 286 ATCC-15834 at a concentration of 10⁸ cells ml⁻¹ and a
 287 co-cultivation of 3 d gave the maximum frequency explant
 288 infection compared to the other strain (data not shown).
 289 Hypocotyls of 6-day-old seedlings infected with 10⁸ cells
 290 ml⁻¹ of ATCC-15834 gave the maximum frequency of ex-
 291 plant infection as compared to the 10-day-old explant (data
 292 not shown). Of the two strains of *A. rhizogenes*, it was seen
 293 that the hairy roots derived from ATCC-15834 were faster
 294 growing and hence were selected for further elicitation stud-
 295 ies. The transformed oca roots when placed in liquid medium
 296 induced fluorescence of the medium (Fig. 1). In contrast,
 297 hairy root cultures of *Ullucus tuberosus*, *Hyoscyamus muti-*
 298 *cus*, *Phytolacca americana*, *Lithospermum erythrorhizon*
 299 and *Nicotiana tabacum* did not fluoresce (Fig. 1).

3.2. Distribution of harmine, harmaline and methyl paraben in oca plants, roots and exudates

302 To analyze the distribution of β-carbolines and methyl
 303 paraben, in vitro grown plants of oca were raised. Thirty-day-
 304 old plants of oca were used for estimation of constitutive
 305 levels of harmine, harmaline, and methyl paraben. The levels
 306 of these metabolites were measured in various plant parts
 307 such as leaves, shoots, untransformed roots and exudates
 308 (Fig. 2). Leaves, shoots and normal untransformed roots of
 309 *O. tuberosa* were extracted in methanol to measure endog-
 310 enous metabolite titers. It was observed that hairy roots
 311 harbored the maximum endogenous titers of methyl paraben
 312 compared to leaves and shoots (Fig. 2). In contrast, endog-
 313 enous titers of harmine and harmaline were higher in shoots
 314 when compared to leaves, untransformed and transformed
 315 roots (Fig. 2). Maximum titers of harmine, harmaline and
 316 methyl paraben were found in the root exudates (Fig. 2). It
 317 was observed that transformed roots exuded maximum con-
 318 centrations of harmine, harmaline and methyl paraben com-
 319 pared to the exudates from whole plants and the intracellular
 320 metabolite content from different parts of the plant (Fig. 2).
 321 Interestingly, the sterilized soil in which the oca plants were
 322 grown under greenhouse conditions accumulated maximum
 323 concentrations of harmine, harmaline and methyl paraben in
 324 comparison to various other tested soil types [7].

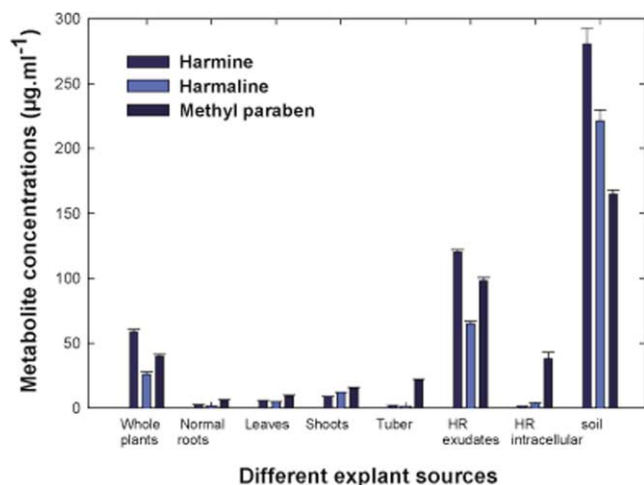


Fig. 2. Variation of harmine, harmaline and methyl paraben content in various parts of the plant and exudates. Treatments were as follows. 1, Whole plant exudation; 2, intracellular content in untransformed oca roots; 3, intracellular content in untransformed oca leaves; 4, intracellular content in untransformed oca shoots; 5, intracellular content in untransformed oca tubers; 6, exudation from transformed root cultures of oca; 7, intracellular content in transformed oca roots; 8, exudation in soil from greenhouse grown oca plants (values are mean \pm S.D., $n = 5$).

3.3. Root growth

Hairy root clones were selected on the basis of faster growth rate and were maintained in 125 ml flasks as indicated in Methods. *O. tuberosa* hairy root cultures showed stable growth producing a substantial biomass yield (Fig. 3). As shown in Fig. 4, *O. tuberosa* hairy roots showed a typical exponential root growth curve during the culture period. A representative growth curve showed that roots achieved log phase after the 14th day (Fig. 3). Maximum tissue accumulation under these culture conditions was $28.6 \pm 1.2 \text{ g l}^{-1}$ (fresh weight (FW) basis), representing about a 8.93-fold increase in biomass over growth in untreated untransformed roots (Fig. 3). Extracellular exuded harmine, harmaline and methyl paraben found in the culture medium were analyzed over the culture period by comparing them with the standards in terms of chromatographic retention and peak area. Hairy root cultures exuded higher titers of extracellular harmine, harmaline and methyl paraben over time compared to the normal untransformed roots (Fig. 3).

3.4. Separation and identification of harmine, harmaline and methyl paraben by HPLC-¹H NMR

Using HPLC conditions developed in the previous study (see Section 2; [7]), harmine, harmaline and methyl paraben in exudates of hairy root cultures of *O. tuberosa* were successfully separated and identified (Fig. 4). As the typical chromatogram (Fig. 4) shows, hairy root exudates contained three major peaks (Fig. 4) under non-elicited treatments. It was observed that with 2% v/v *Phytophthora cinnamoni* treatment, *O. tuberosa* roots exuded seven additional and unknown compounds along with higher titers of harmine,

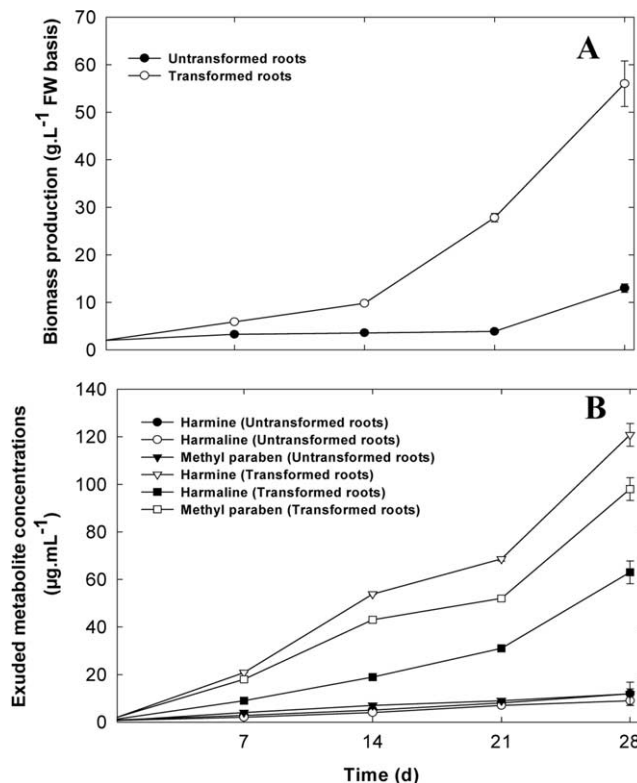


Fig. 3. A typical growth and production curve in hairy root cultures of oca over time. A, Biomass production in hairy root cultures compared to the normal untransformed roots of *O. tuberosa*; B, harmine, harmaline and methyl paraben content in exudates of hairy root cultures compared to the normal untransformed roots of *O. tuberosa* (values are mean \pm S.D., $n = 5$).

harmine and methyl paraben, as evidenced by the quantitative and qualitative variation in production of harmine, harmaline and methyl paraben upon elicitation (Fig. 4). Harmine, harmaline and methyl paraben isolated from the HPLC eluant were further characterized using ¹H NMR (data not shown).

3.5. Growth and elicitation of hairy root cultures

To analyze the biological role in exudation of β -carbolines and methyl paraben, hairy root cultures of *O. tuberosa* were challenged with stress-related compounds such as salicylic acid (SA), jasmonic acid (JA) and fungal cell wall elicitors (CWE) as described in Methods. CWE from *P. cinnamoni* at 2% v/v produced the maximum root growth ($130.8 \pm 5.1 \text{ g l}^{-1}$), which was 2.23-fold higher than the control ($58.6 \pm 2.1 \text{ g l}^{-1}$) (Fig. 5). Root cultures treated with other elicitors such as SA and JA showed a negative growth correlation and a decrease in biomass compared to the untreated control (Fig. 5).

Hairy roots treated with 2% v/v CWE from *P. cinnamoni* showed an increased growth in root biomass over the control (Fig. 5). Harmine, harmaline and methyl paraben showed a growth-associated production and exudation in root cultures elicited with CWE from *P. cinnamoni* at 2% v/v (Fig. 5). This feature was observed in all the treatments wherein the secondary metabolite production remained strictly correlated

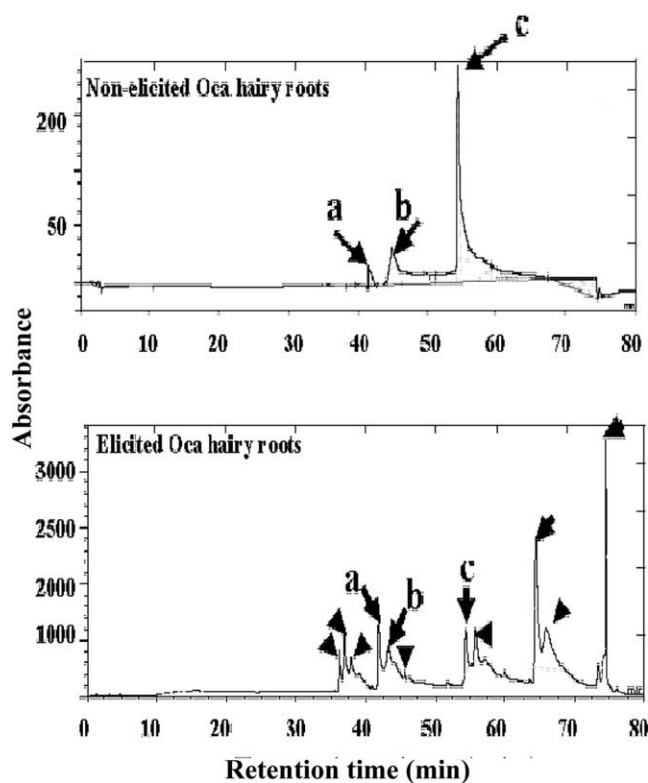


Fig. 4. Separation and characterization of harmine, harmaline and methyl paraben from hairy root cultures of *O. tuberosa*. HPLC profiles of hairy root extracts from *O. tuberosa* showing the presence of harmine, harmaline and methyl paraben. Non-elicited (I) and elicited with 2% v/v cell wall extracts from *P. cinnamomi* (II) treatments. Peaks a, harmaline; b, refers to harmine; c, methyl paraben. A multi-step gradient was used for all separations with an initial injection volume of 15 μl and a flow rate of 1 ml min^{-1} . The multi-step gradient was as follows: 0–5 min 5% B, 5–10 min 20% B, 15–20 min 20% B, 20–40 min 80% B, 40–60 min 100% B, 60–70 min 100% B, 70–80 min 5.0% B. Other arrows depict the unknown compounds eluted under elicited conditions.

380 with growth (Fig. 5). Our results clearly show that
381 β -carbolines and methyl paraben contained in roots can be
382 over-exuded upon elicitation (Fig. 5). Elicitation by the use of
383 SA and JA had a negative effect on the growth of hairy root
384 cultures causing blackening of the tissue in conjunction with
385 reduced titers of endogenous and exuded metabolites at the
386 end of the time course (data not shown, Fig. 5). This result
387 implies that SA and JA may not be involved in elicitation of
388 these metabolites.

389 3.6. Effects of harmine, harmaline and methyl paraben on 390 microbial growth

391 It is known that β -carbolines are photoactive antimicrobi-
392 als [1], and these compounds were over-exuded upon elicitat-
393 ion with fungal CWE. We tested whether they were inhibi-
394 tory to soil-borne microorganisms. Both bacterial and fungal
395 isolates were assayed with harmine and harmaline purified
396 from root cultures as well as commercially purchased stan-
397 dards.

398 Of the seven bacterial strains tested, most showed a degree
399 of inhibition in response to harmine and harmaline treatment

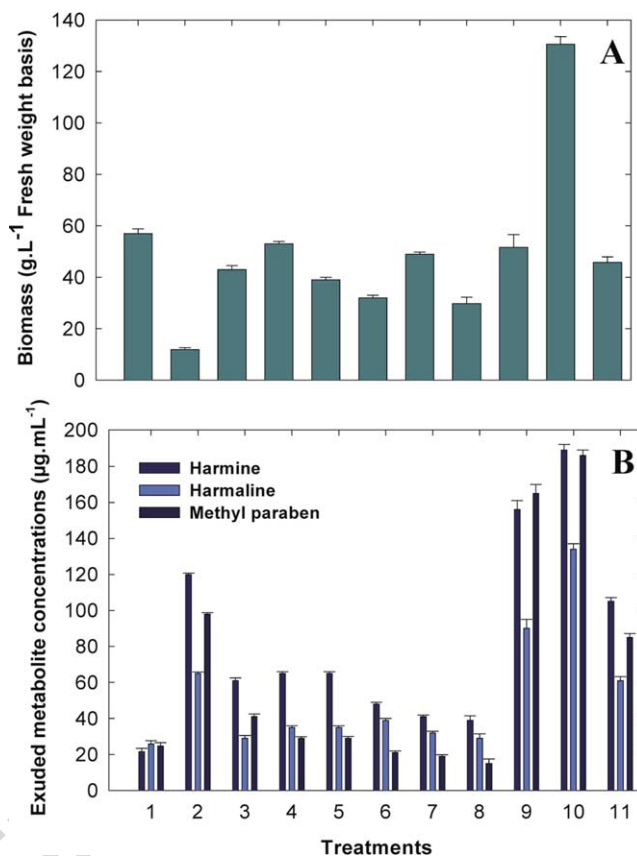


Fig. 5. Comparison of biomass accumulation and harmine, harmaline and methyl paraben content in hairy root cultures of *O. tuberosa* treated with various elicitors. Elicitors were added on day 0 at varying concentrations (see Section 2). Roots were analyzed at the beginning of the stationary phase (28 d). **A**, Biomass accumulation in g L^{-1} ; **B**, harmine, harmaline and methyl paraben content in $\mu\text{g mL}^{-1}$ exuded metabolite concentrations; Treatments: 1, untransformed roots; 2, untreated transformed roots; 3–5, (50, 100, 200 μM) SA; 6–8, (100, 250, 500 μM) JA; 9–11, (1, 2, 3% v/v) CWE *P. cinnamomi* (values are mean \pm S.D., $n = 5$).

(Fig. 6). *Xanthomonas campestris*, *Pseudomonas fluorescens* and *Erwinia carotovora* showed a distinct inhibition of growth under harmine and harmaline (30–40 $\mu\text{g mL}^{-1}$) treatment, which was shown by a decrease in optical density (OD) at higher alkaloid concentrations (Fig. 6). In contrast, *A. rhizogenes* (15834) and yeast were not affected even at higher concentrations of these alkaloids (30–40 $\mu\text{g mL}^{-1}$) (Fig. 6). Successful transformation and production of hairy roots in oca (*O. tuberosa*) plants confirm that harmine and harmaline do not inhibit growth of *A. rhizogenes* (Fig. 6). Methyl paraben failed to show any antibacterial activity, although it showed mild antifungal activity at 40 $\mu\text{g mL}^{-1}$ against specific fungi (Table 1; data not shown).

We tested the antifungal activity of harmine and harmaline against an array of plant pathogenic fungi (see Section 2). Harmine and harmaline inhibited growth of six different fungi in varying degrees (Table 1). Comparisons of growth patterns among all of the fungi tested were made by measurement of the inhibition zone of hyphal growth for each concentration of alkaloids as compared to the untreated control (Table 1). All the concentrations (10–40 $\mu\text{g mL}^{-1}$) were active

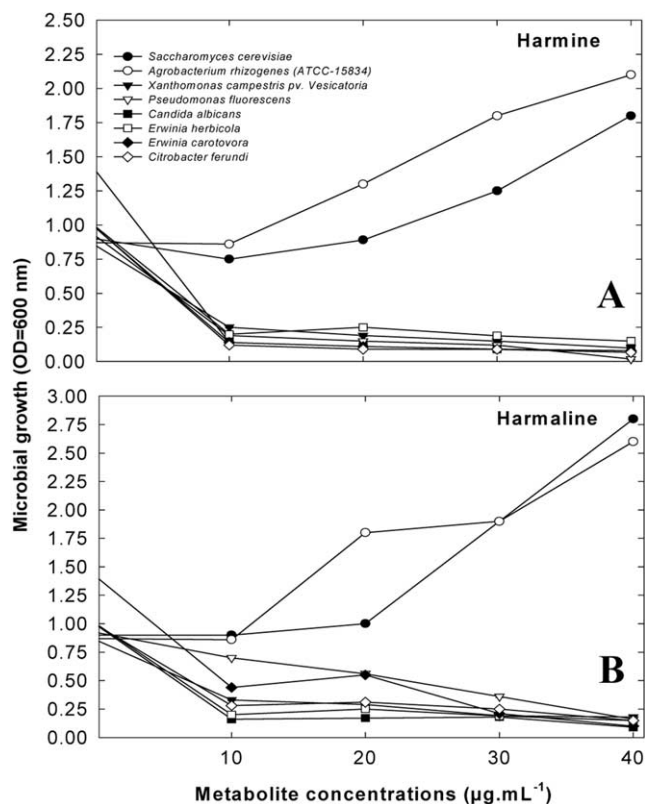


Fig. 6. Antibacterial activity of authentic harmine and harmaline on different bacterial strains. **A**, Harmine; **B**, harmaline; tube antibacterial assay 1, control (without harmine/harmaline); 2, 10 $\mu\text{g ml}^{-1}$ harmine/harmaline; 3, 20 $\mu\text{g ml}^{-1}$ harmine/harmaline; 4, 30 $\mu\text{g ml}^{-1}$ harmine/harmaline; 5, 40 $\mu\text{g ml}^{-1}$ harmine/harmaline. Bacterial cultures were grown overnight at 24 °C in liquid LB media in tubes to an OD of 0.2 at 600 nm. Different concentrations (10–40 $\mu\text{g ml}^{-1}$) of harmine/harmaline were administered to the bacteria-containing LB media. OD at 600 nm was checked after 24 h of incubation to measure bacterial inhibition in tubes (values are mean \pm S.D., $n = 5$).

421 against the tested fungi, suggesting a biological significance
422 for root exudation.

423 4. Discussion

424 *A. rhizogenes* has been used to obtain transformed and
425 transgenic roots in many plant systems [4,12]. We estab-
426 lished transformed roots of *O. tuberosa* with *A. rhizogenes*
427 (ATCC-15834) that displayed similar morphological charac-
428 teristics to *O. tuberosa* primary roots (Fig. 1) with the excep-
429 tion of a rapid growth rate. Hairy roots of *O. tuberosa*
430 (ATCC-15834) showed the presence of the *rol A* gene
431 (Fig. 1), which confirms the transformed nature of the hairy
432 root cultures. The hairy root cultures of *O. tuberosa* showed
433 a stable and fast growth rate, and produced harmine, harmal-
434 ine and methyl paraben constitutively. We found only traces
435 of intracellular harmine, harmaline and methyl paraben in
436 leaves, transformed roots, untransformed roots and tubers
437 (Fig. 2), whereas all three metabolites were exuded from the
438 roots in maximum quantity (Fig. 2). That maximum exuda-
439 tion occurs normally in roots, compared to leaves and shoots

(Fig. 2), implies a vital biological/biochemical role of β -carbolines in the rhizosphere.

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441
442 We have previously reported that oca plants grown in vitro
443 exhibit purplish-blue fluorescence of the medium when sub-
444 jected to UV light [4,5,7]. It was observed that fluorescent
445 compounds are specifically exuded from roots, suggesting
446 that oca may secrete similar compounds into its rhizosphere.
447 Accordingly, we observed similar fluorescence in the soil
448 surrounding greenhouse-grown oca roots [4]. Previously re-
449 searchers have shown that UV light may penetrate soil, thus
450 triggering a fluorescent reaction for yet unknown purposes
451 [26].

452 In vitro production and exudation of these metabolites
453 within the root varied with conditions of stress including
454 challenge by elicitors. We found that root cultures of *O.*
455 *tuberosa* offer a useful system for studying the production
456 and potential function(s) of a suite of antimicrobial com-
457 pounds such as harmine and harmaline in plant roots. We
458 observed a strong regulation of harmine/harmaline produc-
459 tion in roots as evidenced by the quantitative and qualitative
460 variation in production of the compounds upon elicitation
461 with appearance of eight additional and unknown com-
462 pounds (Fig. 4). Maximum growth and exudation of
463 harmine/harmaline occurred after the 21st day of the culture
464 period (Fig. 4). The specific elicitation of harmine/harmaline
465 with fungal CWEs was confirmed by the over-exudation
466 under elicitor treatments compared to the non-elicited condi-
467 tions (Figs. 3–5). β -carbolines like harmine and harmaline
468 are found constitutively in species of the Zygophyllaceae
469 [13,16]. Due to the known constitutive and antimicrobial
470 properties of indole alkaloids, these compounds may play the
471 role of phytoanticipins in plants [2,11]. Although preformed
472 defense compounds are present in healthy plants at levels that
473 are thought to be antimicrobial, their levels may increase in
474 response to pathogenic challenge [19]. Fungal elicitation has
475 been proven to enhance accumulation and exudation of vari-
476 ous classes of secondary metabolites in root cultures of
477 different plant systems [4–7]. In hairy root cultures of oca,
478 fungal cell wall elicitation enhanced the constitutive and
479 exuded level of alkaloids (Figs. 3–5).

480 Our studies showed that harmine and harmaline are the
481 major compounds produced in *O. tuberosa* root exudates
482 upon microbial challenge compared to the unchallenged root
483 controls (Figs. 3–5). This observation validates the antimi-
484 crobial activity of harmine/harmaline under in vitro and in
485 vivo conditions. This phenomenon is similar to that docu-
486 mented for shikonin production in hairy root cultures of *L.*
487 *erythrorhizon*, wherein shikonin is secreted under stress con-
488 ditions, including microbial attack [9]. The ratio of com-
489 pounds produced in different induced states is influenced by
490 the type of pathogen and plant tissue attacked [8]. It has been
491 reported that accumulation of glucosinolates in oil seed rape
492 (*Canola* sp.) varies with type of challenging organism and
493 the type of tissue attacked [8]. In our studies, we found that
494 an elicitor from crude cell wall extracts of *P. cinnamoni*
495 altered the ratio and quantity of compounds produced in

Table 1

Antifungal activities of the characterized secondary metabolites ^a in root exudates of hairy root cultures of *O. tuberosa* L. Data are presented as follows: (–), no inhibition of fungal growth; (+), slight inhibition; (++) , weak inhibition; (+++) , moderate inhibition; (++++), strong inhibition, and are the average of two separate experiments with two replicates in each treatment. In the table, each (+) represents 5 mm from the filter disc. (–) Depicts no fungal inhibition. All antifungal experiments were performed with standard, commercially available compounds. Control discs contained the highest volume of methanol used for each treatment

Treatment ($\mu\text{g ml}^{-1}$)	<i>F. oxysporium</i>	<i>P. drechsleri</i>	<i>R. solani</i>	<i>A. niger</i>	<i>A. solani</i>	<i>A. brassicae</i>
Control	--	--	--	--	--	--
Harmine						
10	--	--	--	--	--	--
20	--	--	--	--	--	--
30	++	++	--	--	+++	++++
40	++++	+++	+	+++	++++	++++
Harmaline						
10	--	--	--	--	--	--
20	--	--	--	--	--	--
30	++	+	--	--	--	+++
40	++	++	++	--	++	+++
Methyl paraben						
10	--	--	--	--	--	--
20	--	--	--	--	--	--
30	--	--	+	--	--	--
40	+	+	+	--	--	+

496 hairy root cultures of oca compared to the non-elicited control (Figs. 3–5). The repeated production of a set of very similar compounds upon fungal elicitation raises the question of whether or not duplication is part of a survival strategy to preclude development of resistance by microorganisms in the rhizosphere.

502 The mechanism of action of harmine and harmaline against microorganisms is not yet known. The composition of microbial populations in the rhizosphere of *O. tuberosa* is also unknown, and further work is required to determine the nature of the rhizosphere communities in various *O. tuberosa* populations. It is reasonable to hypothesize that *O. tuberosa* roots and soil borne pathogens are likely to be in competition in their natural environment, the soil, and hence that *O. tuberosa* roots have developed strategies such as alkaloid exudation to combat potential infections. Harmine causes mortality in armyworms (*Pseudaletia unipuncta*) [21,30], supporting the hypothesis of harmine/harmaline as defense compounds. Interestingly, harmine shows strong phototoxicity against a polyphagous feeder (*Trichoplusia ni*) and *Escherichia coli*, suggesting that its biological activity may be linked to photoactivation [16]. All together these data suggest that UV light penetrating soil layers may photoactivate harmine secreted by oca roots to create an insecticidal and microbicidal defense response.

521 Transformed roots and plants of oca exuded other compounds including methyl 4-hydroxybenzoate (methyl paraben), a known insect feedant stimulant [3]. That methyl paraben failed to show any antimicrobial activity (Table 1), in contrast its known insect feedant properties, suggests that oca plant roots may have developed an attractant-killing/bait

mechanism by the combined exudation of harmine, harmaline and methyl paraben.

The results of this study are consistent with the possibility that β -carbolines and their derivatives function as both preformed and inducible microbial inhibitors that are regulated in a root-specific manner to maximize the production and exudation of harmine/harmaline against invading plant pathogens with minimal expense to the plant. The system described here may allow us to understand the complex relationships between multiple compounds influencing multiple organisms in the rhizosphere. Our preliminary results have shown that several other plant species, including *Arabidopsis thaliana*, exhibit root fluorescence, a possible indication that this phenomenon is widespread within the plant kingdom (Bais and Vivanco, unpublished data). Our results suggest that root fluorescence might be a widespread phenomenon among plants and that its biological implications are worthy of detailed investigation.

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