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Exudation of fluorescent β-carbolines from *Oxalis tuberosa* L. roots

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Abstract

Root fluorescence is a phenomenon in which roots of seedlings fluoresce when irradiated with ultraviolet (UV) light. Soybean (*Glycine max*) and rye grass (*Elymus glaucus*) are the only plant species that have been reported to exhibit this occurrence in germinating seedling roots. The trait has been useful as a marker in genetic, tissue culture and diversity studies, and has facilitated selection of plants for breeding purposes. However, the biological significance of this occurrence in plants and other organisms is unknown. Here we report that the Andean tuber crop species *Oxalis tuberosa*, known as oca in the highlands of South America, secretes a fluorescent compound as part of its root exudates. The main fluorescent compounds were characterized as harmine (7-methoxy-1-methyl-β-carboline) and harmaline (3, 4-dihydroharmine). We also detected endogenous root fluorescence in other plant species, including *Arabidopsis thaliana* and *Phytolacca americana*, a possible indication that this phenomenon is widespread within the plant kingdom.

Keywords: Harmine; Harmaline; *Oxalis tuberosa*; Oca; Oxalidaceae; β-carbolines

1. Introduction

After potato, oca (*Oxalis tuberosa* L.) is the most common tuber crop in the Andean region (King, 1987). Oca, a member of the Oxalidaceae family, is an annual herbaceous plant which adapts to divergent environments and is cultivated at elevations between 2800 and 4000 m (National Research Council, 1989; King, 1987). As is true for most Andean tuber crops, there are very few reports on the basic biology, agronomy or biochemistry of oca. To better understand the biology of oca, we established an in vitro system to study the expression and manipulation of constitutive secondary metabolites. Interestingly, we observed that in vitro-grown oca plants turned the sterile media fluorescent upon UV irradiation.

The phenomenon of the roots of plants/seedlings fluorescing under ultraviolet (UV) light has been termed root fluorescence (Delannay and Palmer, 1982). The only documented plant species exhibiting this phenomenon are soybean and rye grass in germinating seedling roots (Delannay and Palmer, 1982), and the biological significance of this occurrence in plants is unknown. We report here the isolation and characterization of the fluorescent β-carbolines harmine **1** and harmaline **2** (3,4-dihydroharmine) in in vitro grown *O. tuberosa* root exudates. Additionally, we observed that green-house grown plants of *O. tuberosa* also exuded harmine **1** and harmaline **2** into soil. We hypothesize that harmine **1** and harmaline **2** might play a defensive role in the rhizosphere, and that this activity is regulated by light activation and UV irradiation under natural circumstances.

2. Results and discussion

In vitro cultures of oca plants grown in sterile liquid media produce purplish-blue fluorescence in the culture flask medium when irradiated with UV light (Fig. 1A). The roots of oca, however, did not fluoresce, nor did stems, leaves or flowers. Furthermore, when these organs were placed in liquid culture they did not induce fluorescence of the medium as the roots did. The fluorescent

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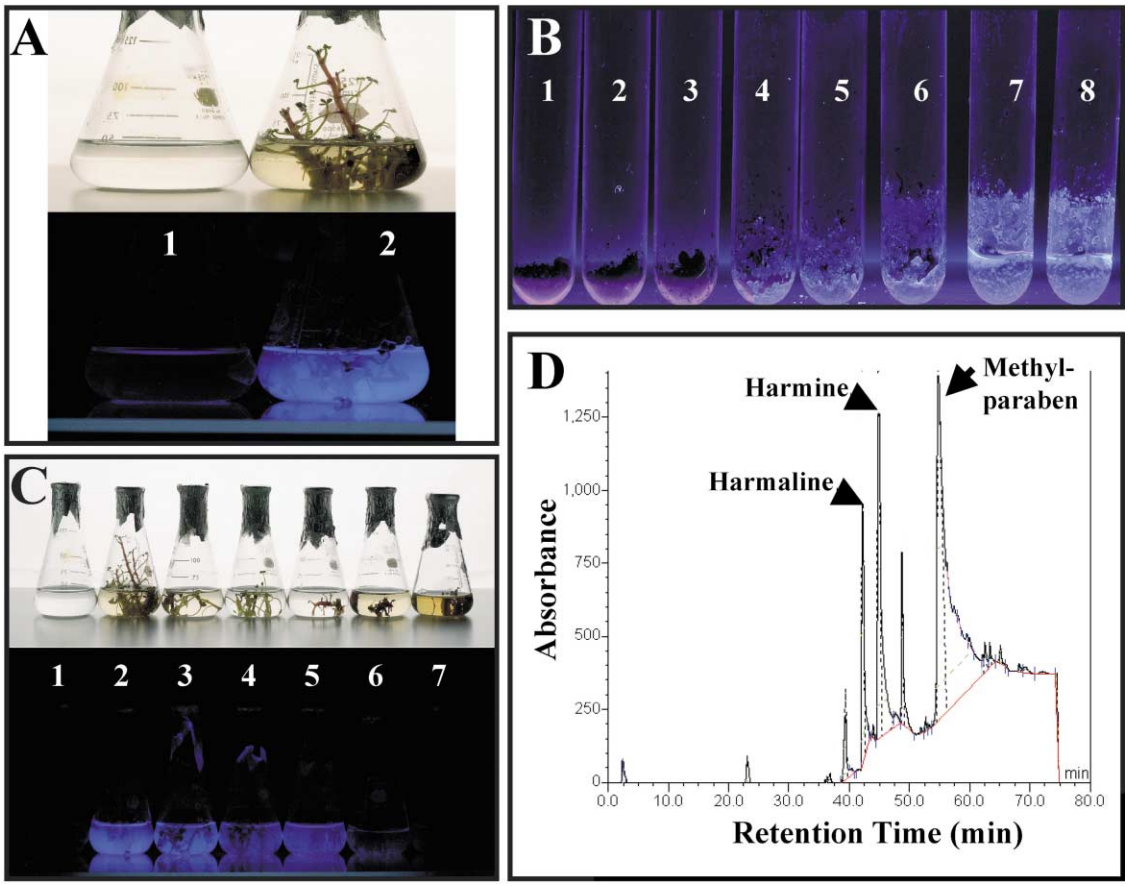

Fig. 1. Fluorescent roots. A. Sterile liquid medium (1), and in vitro culture of oca grown in sterile liquid medium (2) under white and UV light exposure. B. Soil samples showing fluorescence obtained from greenhouse-grown oca (*Oxalis tuberosa*) plants. Samples were taken 5 cm from the stem-girth of the plant, and the numbers (1–8) denote the depth by every 1 cm towards the top-layer soil. In vitro-grown oca plants and soil samples collected from oca's rhizosphere were visualized for blue-purplish fluorescence under UV light exposure with a short wavelength of UV∼254 nm. Each experiment was repeated twice with five replicates. C. Oca plants at different stages of development under white and UV light exposure. 1: sterile medium, 2–5: youngest (1-month old plant); 5: (3-month-old plant); 6–7: (4-month-old plant). 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. D. Separation and characterization of harmine **1**, harmaline **2** and methyl paraben **3** from root exudates of *O. tuberosa*. HPLC profiles of *O. tuberosa* exudates showing the presence of harmine **1**, harmaline **2** and methyl paraben **3**. A multistep gradient was applied for all separations with an initial injection volume of 15 μL and a flow rate of 1 ml min^{-1}. The multistep gradient was as follows: 0–5 min 5.0% B, 5–10 min 20.0% B, 15–20 min 20.0% B, 20–40 min 80.0% B, 40–60 min 100% B, 60–70 min 100% B, 70–80 min 5.0% B.

exudates seem to be specifically secreted from roots. In vitro root secretions of secondary metabolites and proteins can in general be compared with root secretions under natural soil settings (Flores et al., 1999; Bais et al., 2001, 2002; Vivanco et al., 2002), which suggested that oca may secrete fluorescent compounds into the soil as well as into a culture flask. Accordingly, fluorescence was detected in the soil surrounding greenhouse-grown oca roots (Fig. 1B). Ordinary light can cause fluorescence; however, there are reports that UV light may penetrate soil and induce a fluorescent reaction (Lakowicz, 1999; Schober and Lohmannsroben, 2000). Fluorescence in the tissue culture medium decreased as plants senesced (Fig. 1C), and exhibited a direct correlation with the accumulation of fluorescent compounds (data not shown). This direct correlation suggests that fluorescent compound secretion by roots may play a significant role in the plant's life cycle, and that it is not simply a by-product linked to senescence. Similarly, the fluorescent plumage of parrots is an adapted sexual signal rather than a by-product of plumage pigmentation (Arnold et al., 2002).

Using HPLC conditions developed in the present study (see Experimental), harmine **1**, harmaline **2** and methyl paraben **3** (methyl 4-hydroxybenzoate) in the root exudates of *O. tuberosa* were successfully separated and identified (Fig. 1D). As the chromatogram shows, root exudates contained three major peaks (Fig. 1D). Harmine **1**, harmaline **2** and methyl paraben **3** isolated from HPLC eluant were further characterized using ^{1}H NMR spectroscopy (data not shown). The main fluorescent compound was identified as harmine **1** (7-methoxy-1-methyl-β-carboline) (Fig. 2A) using a combination of HPLC and ^{13}C and ^{1}H nuclear magnetic resonance

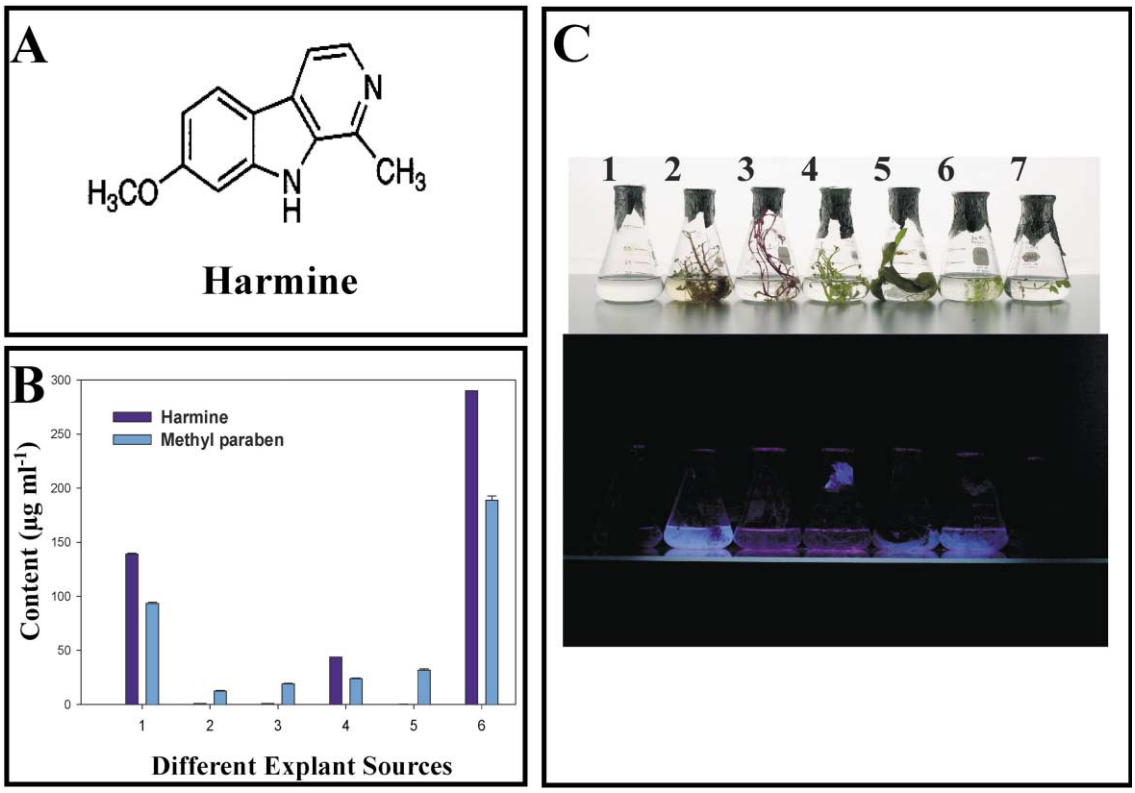

Fig. 2. A. Chemical structure of harmine **1** as determined by ^{1}H and ^{13}C NMR spectroscopy analyses. B. Intracellular and exuded content of harmine **1** and methyl paraben **3** in roots and different plant parts of oca. 1: Roots' exudation; 2: intracellular content in roots; 3: intracellular content in leaves; 4: intracellular content in shoots; 5: intracellular content in tubers; 6: soil from greenhouse-grown oca plants. Each experiment was repeated twice with five replicates (values are mean ± S.D., $n = 5$). C. Sterile liquid medium (1), and in vitro cultures of oca (2), *Ullucus tuberosus* (3), *Tropaeolum tuberosum* (4), *Phytolacca americana* (5), *Arabidopsis thaliana* (6) and *Nicotiana 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.

spectrometry analyses (data not shown). Harmine **1** exhibits a strong purplish-blue fluorescence (Al-Shamma et al., 1981), which correlates with the fluorescence observed in the medium (Fig. 1A,C; Fig. 2C). Harmine **1** is a widespread photoactive β-carboline, a well-known central nervous system stimulant, and has been reported as a major component in the seeds of *Peganum harmala* (Al-Shamma et al., 1981). It also occurs in *Banisteriopsis caapi,* a component of the shamanistic Amazonian hallucinogenic mixture "ayahuasca", as well as in a number of other plant species (Allen and Holmstedt, 1980). Oca tubers are a staple food crop in the highlands of South America and, as far as we could find, no hallucinogenic effect has been reported from this crop (King and Gershoff, 1987). We detected a high content of harmine **1** in the media and soil where oca was grown (Fig. 2B), suggesting that this compound was being exuded from roots, both in culture and under natural conditions in the soil. In contrast, various organs of oca plants such as leaves, roots and stems harbored only traces of endogenous harmine **1** (Fig. 2B). At present we can only suggest that harmine is not found in oca because it plays a crucial role in the rhizosphere.

In fall armyworms (Lepidoptera: Noctuidae), harmine **1** increases the activity of juvenile hormone-III and ecdysone 20-monooxygenase, which causes insect mortality (Yu, 2000). Harmine **1** shows strong phototoxicity against a polyphagous feeder, *Trichoplusia ni,* suggesting that its insecticidal activity may be linked to photoactivation (Larson et al., 1988). The Andean highlands are subjected to a high incidence of UV radiation, and we found that the strongest fluorescence intensity occurred with oca varieties that showed resistance to the larvae of *Mycrotrypes* spp., the Andean tuber weevil (Flores, 1999; National Research Council, 1989). All together these data suggest that UV light penetrating soil layers could photoactivate harmine **1** secreted by oca roots to create an insecticidal defense response. Roots of oca exuded other compounds including methyl paraben **3**, a known insect feedant (Andow and Stodola, 2001), and the less fluorescent alkaloid harmaline **2**. β-carbolines, including harmine **1** and harmaline **2**, are reported as active photosensitizers with a possible conversion to different isoforms (McKenna and Towers, 1981). Additionally, harmine **1** and harmaline **2** also exhibit UV-mediated cytotoxic

activity against various plant pathogens (McKenna and Towers, 1981; Hudson et al., 1986).

Flowers of certain species emit wavelength patterns in the UV spectrum that are perceived by butterflies (Deinert et al., 1994). This evolutionary trait has helped plants take advantage of arthropod behavior to improve pollination rates. It is also possible that plant roots use similar strategies by which photoactive fluorescent compounds exuded by roots, such as harmine **1**, may attract and/or deter insects. Larvae of most root-feeding insects possess stemmata as their visual organs; these have visual powers comparable to that of compound eyes in adult insects which can perceive UV light (Deinert et al., 1994). Some scorpions use harmine **1** localized on their cuticle as an evening mating marker that fluoresces in the dark (Stachel et al., 1999). Thus, harmine **1** may be used by some plants and arthropods as a fluorescent signal under dark conditions; however, the biological implications of fluorescence are not clear.

Other Andean root and tuber crop species such as *Ullucus tuberosus* and *Tropaeolum tuberosum*, as well as *Nicotiana tabacum*, *Arabidopsis thaliana* and *Phytolacca americana,* were established in liquid culture for fluorescence comparison purposes (Fig. 2B). We did not observe fluorescence of the media in any of these cultures but oca. We did however, detect strong endogenous blue fluorescence in the roots of *A. thaliana* and *P. americana*, a possible indication that this phenomenon is widespread within the plant kingdom. However, our preliminary results show that blue endogenous fluorescence in Arabidopsis roots is not due to the presence of harmine **1**/harmaline **2**. Thus, there may be plethora of unexplored fluorescent compounds in the plant kingdom. To our knowledge, this is the first reported characterization of fluorescent compounds exuded by plant roots. Perhaps the underground world of plant roots may not be as dark as previously thought.

3. Experimental

3.1. Plant material

Seeds of oca (*Oxalis tuberosa* L.) were obtained from the International Potato Center (Lima, Peru) and from the laboratory of Dr. Hector E. Flores at Pennsylvania State University. Seeds were washed five times with sterile water and were surface sterilized with 10% (v v^{-1}) commercial bleach for 15 min followed by 3–4 washes in sterile distilled water. Surface sterilized seeds were placed on static Murashige and Skoog (1962) (MS) basal solid media without any growth regulators for germination and incubated at 25±2 °C with a light intensity of 4.41 J m^{-2} s^{-1}.

3.2. Chemicals

Harmine **1**, harmaline **2** and methyl paraben **3** standards were obtained from Sigma, USA; all other chemicals were of analytical and HPLC grade. Solvents were from Fisher, USA.

3.3. In vitro liquid cultures

Ten-day old *O. tuberosa* seedlings were transferred to MS basal liquid media without any growth regulators, and the cultures were incubated under 16 h light and 8 h dark conditions. Plant cultures were kept at 25±2 °C. Plants and media exudates were collected at weekly time intervals and were solvent extracted for HPLC and UV analyses.

3.4. Sample extraction and HPLC analysis of root exudates

Oca plants grown in liquid media were subjected to solvent extraction prior to HPLC analysis by taking 2 ml of media exudates followed by extraction with hexane (5 ml) (Fisher, USA). Similarly, different plant tissues such as in vitro-grown roots, stems and leaves (200 mg each) were extracted using MeOH (2 ml) (Fisher, USA). Soil samples (500 mg) collected from oca's rhizosphere were extracted using hexane (5 ml) (Fisher, USA) at 4 °C for 24 h. The extracts from exudates, tissues and soil samples were centrifuged at 10,000 rpm for 10 min; supernatants were concentrated in vacuo and were re-suspended in methanol (500 μl). To filter any cellular debris, the extract was passed through a nylon syringe filter of pore size 0.45 μm (Scientific Resources Inc.). The extracts were vortexed and stored for 24 h at room temp. The supernatant was collected and transferred with a Pasteur pipette to a separate test tube, and hexane (1 ml) (Fisher, USA) was added. The supernatant was concentrated by freeze-drying (Vir Tis, USA), and the weighed powder was dissolved in MeOH (200 μl) (Fisher, USA). Extracts from the media were subjected to HPLC, by gradient elution on a reversed phase 5 μm, C_{18} column (25 cm×4.6 mm) (Supelco, USA), using an HPLC system (Summit Dionex, USA) consisting of P580 pumps (Dionex, USA) connected to an ASI-100 Automated Sample Injector (Dionex, USA). The UV absorbance at 210 nm was measured by a PDA-100 Photodiode array variable UV/VIS detector (Dionex, USA), although the injected samples were subjected to a broad range wavelength scan between 190–800 nm. Mobile phase consisted of double distilled water (Solution A) and MeOH (Solution B) (Fisher, USA). A multi-step gradient was applied for all separations with an initial injection volume of 15 μl and a flow rate of 1 ml min^{-1}. The multistep gradient was as follows: 0–5 min 5.0% B, 5–10 min 20.0% B, 15–20 min

20.0% B, 20–40 min 80.0% B, 40–60 min 100% B, 60–70 min 100% B, 70–80 min 5.0% B. HPLC eluants were collected and checked for fluorescence under UV light. Fluorescent fractions were re-injected into the HPLC to check for purity and were subjected to NMR spectroscopic analysis.

3.5. H and C^{13} NMR analysis

^{1}H and ^{13}C NMR spectra of exuded harmine **1** and harmaline **2** were identical to those of authentic standards **1** and **2** (Varian INOVA 400 MHz Fourier Transform spectrometer). Harmaline **2** samples were converted to harmaline–hydrochloride for comparison. Spectral data also corresponded to published spectra in the Aldrich library of ^{1}H FT NMR Spectra (Pouchert and Behnke, 1993).

3.6. UV analysis and imaging

In vitro-grown oca plants and soil samples collected from oca's rhizosphere were visualized for blue-purplish fluorescence under UV light exposure using a Mineralight UV lamp (Model UVG-54, Short wave UV~254 nm) (Ultra-Violet, USA). Images were taken using a Sony Digital Still Camera (DCS-S85) (Sony, Japan) with a supplementary Carl Zeiss ACC terminal. Subsequently, digital images were processed at the Department of Photographic Services (Colorado State University).

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