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Cis- and *trans*-protopinium, a novel nematicide, for the eco-friendly management of root-knot nematodes

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ABSTRACT

Plant-derived nematicides are being increasingly used while select synthetic nematicides are phased out for environmental concerns. This is the first report on the in vitro nematicidal activity of *cis*- and *trans*-protopinium isolated from roots of *Fumaria parviflora* and its in vivo effect on the southern root-knot nematode *Meloidogyne incognita*. *Cis*- and *trans*-protopinium was isolated from the methanolic fraction FM2.1, and its structure elucidated using ^{13}C and ^{1}H nuclear magnetic resonance (NMR). The NMR spectra were characterized using deuterated dimethyl sulfoxide (DMSO) at temperatures of 25 and 80 °C. In an in vitro study, over 120 h of incubation, the area under cumulative percent hatch inhibition and mortality of *cis*- and *trans*-protopinium reached 100% at a concentration of 200 μg mL^{-1}. In the greenhouse and field settings, *cis*- and *trans*-protopinium was evaluated against *M. incognita* on tomato at a concentration of 100, 200, and 300 μg mL^{-1} for two consecutive seasons, that is, spring and autumn, in 2010. At a concentration of 300 μg mL^{-1} in the greenhouse and field trials during spring and autumn, *cis*- and *trans*-protopinium significantly reduced the nematode galling index, the number of females per gram of root, and the reproduction factor, as well as increased plant height, fresh and dry shoot weights, and root length. Therefore, *cis*- and *trans*-protopinium can be used as an effective and safe nematicide against *M. incognita* on tomato in an organic and sustainable agricultural production system. Phytochemicals have various agricultural applications, especially to control economically important nematode pests.

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is an important crop in tropical and subtropical countries, and root-knot nematodes of the genus *Meloidogyne* are pests that reduce tomato yields significantly (Luc et al., 2005). The southern root-knot nematode *M. incognita* causes extensive damage to and significantly reduces the yield of tomato in Pakistan, especially in the Khyber Pakhtunkhwa province (Naz et al., 2013a). The indiscriminate use of synthetic nematicides has caused environmental and health problems, leading to a ban on most of these nematicides (Backman, 1997).

Nematologists continue to develop control methods, particularly focusing on endoparasitic nematodes such as root-knot and cyst nematodes (Roberts, 1995). Plant-derived secondary metabolites and their derivatives have been proposed as novel chemicals for eliminating nematode pests; alkaloids, flavonoids, glycosides, tannins, saponins, steroids, phenols, and many essential oils have been investigated in this respect (Chitwood, 2002). More than 30,000 secondary plant metabolites have been reported (Harborne, 1998); further, a variety of phytochemicals from several families (such as Meliaceae, Asteraceae, Myrtaceae, Lauraceae, Lamiaceae, and others) have been used for insecticidal, acaricidal, antibacterial, antifungal, and anti-nematodal applications (Chitwood, 2002). Many plants with known anti-nematodal properties may be used as green or dry amendments or plant-derived natural products (biopesticides) to minimize the populations of root-knot nematodes in

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agriculture (Chitwood, 2002). Most of these plants can be safely used in integrated disease management (IDM) and integrated pest management (IPM), as phytochemicals are relatively safer and more eco-friendly, pose lesser risks to human and animal health, have a selective mode of action, and will prevent the buildup of resistance in pests (Isman, 2006).

We have already reported the nematicidal activity of extracts from roots and stem of *Fumaria parviflora* Lam., identifying seven classes of bioactive compounds. We found that the *n*-hexane and methanolic (MeOH) root extracts showed the strongest nematicidal activity against egg-hatching and juvenile (J2) viability in both the in vitro and in planta experiments at different concentrations (Naz et al., 2013a). The secondary metabolites of *F. parviflora* are reported to affect bacteria, fungi, viruses, and gastrointestinal nematodes (Orhan et al., 2007; Al-Shaibani et al., 2009). To the best of our knowledge, alkaloids of *F. parviflora* have not been investigated for their nematicidal activity in the laboratory, greenhouse, and more importantly the field setting on tomato. We report on the following points in the present paper: a) the isolation of *cis*- and *trans*-protopinium from the MeOH root extracts and the elucidation of its structure using spectra obtained at 25 and 80 °C, b) the in vitro nematicidal effect of the alkaloid *cis*- and *trans*-protopinium of *F. parviflora* against *M. incognita*, and c) the in planta nematicidal effect against *M. incognita* on tomato under artificially inoculated greenhouse and naturally infested field conditions.

2. Materials and methods

2.1. Chemicals

The solvents used were chloroform, ethyl acetate, and methanol after distillation for column chromatography. The dried methanolic root fractions/compound was dissolved in dimethyl sulfoxide (DMSO, 99.9%, Merck, Germany).

2.2. General techniques for extraction, isolation, and identification of the compound

All fractions were filtered through Whatman No. 1 (10-μm) filter paper (Sigma-Aldrich) and dried at 60 °C in a rotary evaporator (Heidolph, Schwabach, Germany). For column chromatography (CC), the stationary phase was silica gel (Si 60, 200 mesh, Sigma Aldrich) and the mobile-phase solvents were MeOH (methanol), EtOAC (ethyl acetate), and $CHCl_3$ (chloroform). For thin-layer chromatography (TLC), TLC plates (Merck Silica gel 60 F254) and glass plates (20 × 20 cm, 10-μm pore size, Merck, Darmstadt, Germany) were used. The alkaloids in MeOH fractions were detected by spraying TLC plates with Dragendorff's reagents (solution of bismuth nitrate + potassium iodide). The retardation factor values were obtained. The melting points were determined in a glass capillary (M-560, BÜCHI, Flawil, Switzerland), which were left uncorrected. Mass spectra (by electron ionization mass spectrometry (*EIMS*)) were obtained at the James Hutton Institute (JHI), Scotland, on JEOL MSRoute, using a direct insertion probe. Spectroscopic data (^1H and ^{13}C NMR spectra) were recorded in deuterated DMSO at 25 and 80 °C on a Bruker AVANCE 400 instrument, and the *J* values were measured in Hertz. Carbon atoms were assigned by a combination of DEPTq135 and heteronuclear single-quantum coherence (HSQC) experiments. A combination of 1-D and 2-D NMR experiments was conducted, and the spectra were processed by Bruker TOPSPIN software. $^1H–^1H$ and $^1H–^{13}C$ NMR correlations were established by standard procedures (Naz et al., 2013b).

2.3. Plant collection, column chromatography, and activity-guided fractionation

Mature *F. parviflora* plants were collected in February and March of 2009 from wheat fields at the Agriculture Research Farms, Peshawar. The plants were identified and deposited in the herbarium (voucher specimen; No. ISH-1732) of the Weed Science Department, the University of Agriculture (Naz et al., 2013a). Roots (1 kg) of full-grown *F. parviflora* plants were washed in tap water and freeze-dried at −80 °C for 24 h. The roots were lyophilized and crushed in a grinder to around 1 mm, and then extracted with methanol for 4 h in a Soxhlet apparatus. The methanol extract from 22.0 g of roots was dried in vacuo and extracted with 10% aqueous hydrochloric acid. This was further extracted with EtOAC to give two layers, that is, EtOAC (FM1, 3.0 g) and aqueous layers. The aqueous layer was basified with ammonia hydroxide (NH_4OH) to a pH of 8.0, followed by extraction with EtOAC to give FM2 (4.0 g, alkaloids) and the aqueous layer FM3 (120.0 mg). The FM1 and FM2 layers were combined and dissolved in $CHCl_3$, then subject to column chromatography over silica gel, and eluted with solvents of increasing polarity to afford a major fraction FM2.1. The elutes from MeOH–CH_3Cl (1:17) of FM2.1 produced an alkaloid after crystallization from methanol as a colorless amorphous powder. Its structure was determined as *cis*- and *trans*-protopinium based on spectroscopic data and comparisons with the literature (Tousek et al., 2005). A nematicidal bioassay was conducted using microwell plates, as described by Naz et al. (2013a).

2.4. Maintenance of M. incognita and preparation of stock solutions

Females of *M. incognita* were harvested from roots of susceptible tomato (*S. lycopersicum*) cv. Moneymaker 2–4 months after inoculation. The eggs used in the bioassay were extracted using a 1% NaOCl solution (Naz et al., 2013a), and the concentration of the egg suspension was adjusted to 1000 ± 50 eggs mL^{-1}. For the bioassay, J2s from freshly extracted eggs were separated after 48 h and concentrated to a final optimized concentration of 200 J2s mL^{-1}, according to the reported procedures (Naz et al. 2013a). The J2 larvae and eggs of *M. incognita* were subjected to a microwell bioassay following the standard procedures (Naz et al., 2013a). The stock solution (5 mg mL^{-1}) was prepared by reconstituting *cis*- and *trans*-protopinium in DMSO (1% v/v), and further dilutions were made in distilled water. A total of four final concentrations (i.e., 50, 100, 150, and 200 μg mL^{-1}) of *cis*- and *trans*-protopinium were prepared (Naz et al., 2013b). The nematode eggs were disinfected with 1% NaOCl for 5 min and rinsed on an aperture sieve (36 μm) with distilled water (Meyer et al., 2004). In an assay, the eggs (1000 ± 50) and J2s (200) of *M. incognita* were used in a final volume of 1 mL at final concentration. Simple distilled water (SDW) dissolved in 1% DMSO was used as the control. The experiments were performed twice under identical conditions in a completely randomized design and replicated five times. Finally, the microwell plates were incubated under humidified conditions at 25 °C (±2 °C) for 3 days in the dark. Unhatched eggs and J2 larvae in each well were recorded after a range of incubation hours (i.e., 24, 48, 72, 96, and 120 h) (Naz et al., 2013b). Both the eggs and larvae were transferred to SDW for 24 h to observe their reclamation. Motionless juveniles with straight posture were declared as dead (Naz et al., 2013a). The percentage of unhatched eggs and J2 mortality in each well was calculated (Naz et al., 2013b). Data were statistically analyzed by analysis of variance (ANOVA) using Statistix (NH Analytical Software, Roseville, MN, USA). The area under cumulative number of nematode percentage hatch inhibition (AUCPHI) and mortality (AUCPM) were estimated by trapezoidal integration (Campbell and Madden, 1990) for in vitro experiments on the hatching and mortality of J2s exposed to

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