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Isolation and anti-oomycete activity of nyasol from *Anemarrhena asphodeloides* rhizomes

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Abstract

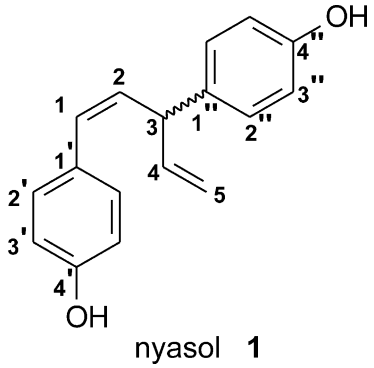
The methanol extract of *Anemarrhena asphodeloides* rhizomes exhibited strong antifungal activity against the plant pathogenic fungi *Magnaphothe grisea, Rhizoctonia solani,* and the plant pathogenic oomycete *Phytophthora capsici*. The antifungal substance isolated from the rhizomes of *A. asphodeloides* was identified to be nyasol, (*Z*)-1,3-bis(4-hydroxyphenyl)-1,4-pentadiene by NMR and mass spectral analysis. Nyasol effectively inhibited the mycelial growth of *Colletotrichum orbiculare, P. capsici, Pythium ultimum, R. solani,* and *Cladosporium cucumerinum* in a range of 1–50 μg/ml, but did not affect the growth of bacteria and yeast. In a greenhouse test, treatment with the antifungal compound nyasol was significantly effective in suppressing the Phytophthora blight on pepper plants.

Keywords: *Anemarrhena asphodeloides*; Liliaceae; Norlignans; Nyasol; Anti-oomycete activity; Phytophthora blight

1. Introduction

Anemarrhena asphodeloides Bunge belongs to the family Liliaceae growing in China, Korea and Japan. The *A. asphodeloides* rhizomes, having antipyretic, anti-inflammatory, sedative, diuretic and anti-diabetic properties are used in Chinese medicine, have been demonstrated not only to have anti-diabetic activity (Takahashi et al., 1985; Nakashima et al., 1993; Ichiki et al., 1998), platelet aggregation inhibitory activity (Niwa et al., 1988; Dong and Han, 1991), diuretic activity, molluscicidal activity (Takeda et al., 1989), antifungal activity (Iida et al., 1999) and anti-yeast activity (Iida et al., 2000), but also to have inhibiting effects on cyclic AMP phosphodiesterase (Nikaido et al., 1981). Chemical constituents from the rhizomes of *A. asphodeloides* that have been studied included steroidal saponins (Kawasaki and Yamauchi, 1963; Dong and Han, 1991; Nakashima et al., 1993; Saito et al., 1994; Ma et al., 1997; Meng et al., 1999), xanthone C-glycosides (Aritomi and Kawasaki, 1970; Ichiki et al., 1998), polysaccharides (Takahashi et al., 1985), and norlignans (Nikaido et al., 1981; Iida et al., 1999; Jeong et al., 1999).

In the present study, we focused on the search for antifungal compounds from medicinal plants for the control of plant diseases. Methanol extracts from various medicinal plants were evaluated for their potential antifungal activities against plant pathogenic fungi and oomycete, with the methanol extract from *A. asphodeloides* rhizomes being found effective in inhibiting mycelial growth of some plant pathogens. The antifungal compound nyasol **1** was then isolated and identified from the rhizomes of *A. asphodeloides*. This paper reports the isolation of nyasol **1**, and its in vitro and in vivo activities against plant pathogenic fungi and oomycetes.



nyasol **1**

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2. Results and discussion

2.1. Extraction and purification of the anti-oomycete compound *1* from *A. asphodeloides* rhizomes

The concentrated MeOH extract of powdered *A. asphodeloides* rhizomes (1 kg) were dissolved in H_2O and partitioned with EtOAc. The organic layer was concentrated in vacuo and subjected to silica gel cc (63–200 μm, Merck) with a stepwise gradient of $CHCl_3$ and MeOH. Each fraction was concentrated and bioassayed against *Phytophthora capsici* by the paper disk-agar diffusion method. The 90 and 80% $CHCl_3$ fractions were highly active against mycelial growth of *P. capsici*, and the combined eluates were re-chromatographed on the silica gel column chromatograhpy. The fractions eluted with a stepwise gradient of *n*-hexane and Me_2CO were concentrated and bioassayed against *P. capsici*. The 90, 80 and 70% *n*-hexane fractions were active against *P. capsici*. These fractions were pooled and loaded on the column packed with Sephadex LH-20 resin (Pharmacia Biotech, Uppsala, Sweden). Each fraction (5 ml) eluted with $CHCl_3$ and MeOH (1:1, v/v) were bioassayed against *P. capsici*. Anti-oomycete activity was detected in the fractions nos. 67–109. The concentrated active fractions were combined and applied to a Sephadex LH-20 column eluted with methanol. The fractions nos. 34–154 were active against *P. capsici*. The anti-oomycete substances from the Sephadex LH-20 column chromatography were separated by preparative TLC on silica gel plates ($60F_{254}$, Merck). The methanol extracts of the band (*Rf* 0.37) with anti-oomycete activity were purified by preparative reversed phase HPLC. The pure anti-oomycete compound **1** against *P. capsici* was obtained from a single peak with retention time of 23.83 min at 250 nm, and was identified as (−)-*cis*-nyasol **1**, by comparison of EIMS and ^1H and ^{13}C NMR data with those reported in the literature (Iida et al., 1999). Nyasol has been purified from *A. asphodeloides* rhizomes in the evaluation process of capacity of Bio-cell Tracer, which consists of a computer-aided growth analyzing system to search for the existence of active compounds in most medicinal plants (Oh et al., 1996; Iida et al., 1999).

Three isomers of nyasol have been reported earlier. The *trans*-nyasol, *trans*-hinokiresinol, was isolated from *Chamaecyparis obtusa* (Hirose et al., 1965; Beracierta and Whiting, 1976), (−)-*cis*-nyasol (**1**) was obtained by hydrolysis of the glycoside nyasosides from *Hypoxis nyasica* (Marini-Bettolo et al., 1985; Messana et al., 1989) and from *Amenarrhena asphodeloides* (Iida et al., 1999; Jeong et al., 1999), and (+)-*cis*-nyasol from *Asparagus cochinchinensis* (Tsui and Brown, 1996) and *Asparagus africanus* (Oketch-Rabah et al., 1997). The specific rotation of (−)-nyasol **1** isolated from *Anemarrhena asphodeloides* (Iida et al., 1999) was −134°, while optical rotation of (−)-nyasol obtained from *Hypoxis nyasica* (Marini-Bettolo et al., 1985) and (+)-nyasol isolated from *Asparagus cochinchinensis* (Tsui and Brown, 1996) was −147° and +112°, respectively.

2.2. In vitro and in vivo anti-oomycete activity of nyasol *1*

The minimum inhibitory concentrations (MIC) of the anti-oomycete substance, nyasol **1**, against various microorganisms were examined to determine its antimicrobial spectrum (Table 1). Nyasol **1** inhibited the mycelial growths of *C. orbiculare, P. capsici* and *F. oxysporum* f.sp. *lycopersici* at a concentration of 50 μg/ml, but not those of *A. mali, C. destructans, M. grisea, B. cinerea, S. sclerotiorum* and *D. bryoniae*. Nyasol **1** showed neither anti-yeast nor antibacterial activity. The anti-oomycete activity of nyasol for the control of Phytophthora blight in pepper plants at the first branch stage was examined under greenhouse conditions (Fig. 1). At concentrations over 100 μg/ml, nyasol **1**, was significantly effective in suppressing Phytophthora blight in pepper plants, but was less effective than the commercial fungicide metalaxyl (Fig. 1).

Nyasol **1** has shown various biological activities such as antiprotozoal (Oketch-Rabah et al., 1997), antibacterial (Akendengue et al., 1999), antileishmania (Akendengue et al., 1999), hyaluronidase inhibition (Jeong et al., 1999), and the inhibition of LTB4 binding to human neutrophils (Lee and Ryu, 1999). The (−)-*cis*-nyasol **1** isolated from the extracts of A. *asphodeloides*

Table 1
Minimum inhibitory concentrations (MICs) against various microorganisms of (−)-nyasol from rhizomes of *Anemarrhena asphodeloides*

Test organism	MIC (μg/ml)[a]
Alternaria mali	>100[b]
Botrytis cinerea	>100
Cladosporium cucumerinum	1
Colletotrichum orbiculare	50
Cylindrocarpon destructans	>100
Didymella bryoniae	>100
Fusarium oxysporum f.sp. *lycopersici*	50
Magnaporthe grisea	>100
Phytophthora capsici	50
Pythium ultimum	5
Rhizoctonia solani	10
Sclerotinia sclerotiorum	>100
Candida albicans	>100
Saccharomyces cerevisiae	>100
Bacillus subtilis	>100
Erwinia carotovora subsp. *carotovora*	>100
Ralstonia solanacearum	>100
Xanthomonas campestris pv. *vesicatoria*	>100

[a] The lowest concentration that completely inhibit the growth of microorganisms was examined after incubation for 4–7 days.

[b] >100 represents that the growth of microorganisms was not inhibited at the concentration of 100 μg/ml.

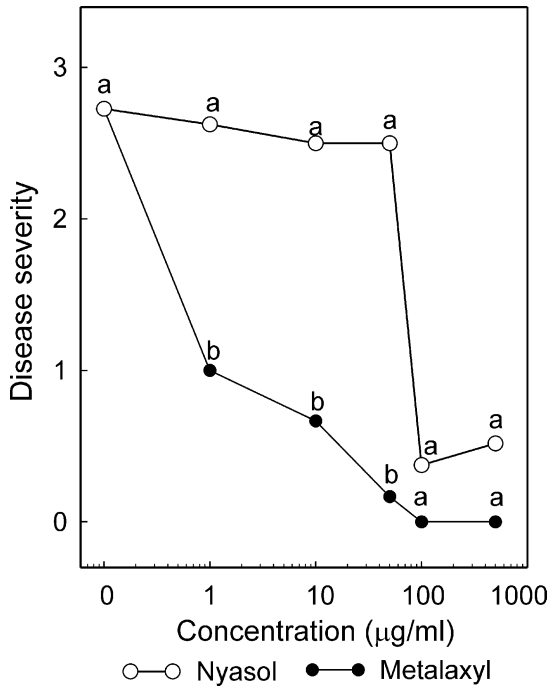

Fig. 1. Effects of nyasol and the commercial fungicide metalaxyl at different concentrations on the disease development in pepper plants inoculated with *Phytophthora capsici* at the first-branch stage. Disease severity is based on a 0–5 scale, where 0 = no visible symptom and 5 = plant dead. Means at each concentration followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

rhizomes was found to be active against the fungi *Curvularia lunata, Eurotium* sp., *Mucor* sp., and *Trychophyton* sp. (Iida et al., 1999). It has recently been demonstrated that nyasol **1** could be used as an adjuvant in anti-mycotic therapy clinic (Iida et al., 2000). However, (−)-nyasol **1** isolated from *A. asphodeloides* rhizomes in the present study did not inhibit the growth of yeast and bacteria at the concentration of 100 μg/ml. In the tests for antifungal spectrum, nyasol **1** was active against some plant pathogenic fungi and oomycetes such as *C. orbiculare, R. solani*, *C. cucumerinum*, *Py. ultimum,* and *P. capsici*. The growth of *P. capsici* was completely inhibited at the concentration of 50 μg/ml. The antifungal activity of (−)-nyasol **1** is supported by the findings of Takasugi (1993) that a norlignan-related C_6–C_2–C_3–C_6 compound, (Z)-hinokiresinol (nyasol) is a phytoalexin that forms in *Asparagus officinalis* inoculated with plant pathogens. More recently, Suzuki et al. (2001) has demonstrated that a fungal treatment elicited the production of a norlignan, (Z)-hinokiresinol, in an *Asparagus officinalis* cell culture. In addition, in vitro formation of the compound (Z)-hinokiresinol from phenylpropanoid monomers was further found to be catalyzed by an enzyme preparation from the fungal-elicited *Asparagus* cell culture (Suzuki et al., 2002).

Nyasol **1** showed no phytotoxicity against pepper plants even at of 500 μg/ml. The rhizomes of *Aemarrhena asphodeloides* have been prescribed as a crude drug in traditional Chinese medicine, which suggests low biotoxicity to humans, when used at proper concentrations.

We conclude that (−)-nyasol **1** from rhizomes of *A. asphodeloides* has not only a potent in vitro antifungal and anti-oomycete activity against some plant pathogenic fungi, but also in vivo control efficacy against Phytophthora blight on pepper plants. However, further studies should be done to determine whether or not nyasol could be developed as an agricultural fungicide for the control of plant diseases or as a lead compound for chemical synthesis.

3. Experimental

3.1. General

CC was performed in silica gel (63–200 μm, Merck) and Sephadex LH-20. TLC was carried out on silica gel $60F_{254}$ (2 mm in thickness, Merck) and the spots on TLC were detected under UV 254 nm. Prep. reversed-phase HPLC (Gilson, Middleton, WI) was conducted using a symmetry Prep™ C_{18} column (7 μm, 7.8×300 mm, Waters, Milford, MA) and a linear gradient of 10% CH_3CN in H_2O to 100% CH_3CN for 30 min at a flow rate of 2 ml/min. The UV absorbance of the eluates was monitored at 250 nm by a Gilson spectrophotometer (118 UV/VIS detector). UV spectrum was recorded on Beckman DU® 650 spectrometer (Beckman instruments, Inc., Fullerton, CA). ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX 500 NMR spectrometer (Bruker, Rheinstetten, Germany) using CD_3OD with TMS as internal standard. Two-dimensional NMR spectra, such as ^1H–^1H COSY, ROSEY, HMQC and HMBC also were measured using the Bruker AMX 500 NMR spectrometer. EI Mass spectrum was measured on JEOL JMS-700 MStation mass spectrometer (JEOL, Tokyo, Japan).

3.2. Plant material

The rhizomes of Anemarrhena asphodeloides were commercially purchased from Jingun Co. in Seoul, Korea. A voucher specimen has been deposited at Laboratory of Molecular Plant Pathology, Korea University, Seoul, Korea.

3.3. Purification of the antifungal compound from *A. asphodeloides* rhizomes

Powder of rhizomes (1 kg) was extracted with 80% aqueous MeOH at room temperature for 5 days. The MeOH extracts were concentrated in vacuo using a rotary evaporator and partitioned with equal volume of EtOAc. The crude extracts in organic solvent layer were

concentrated in vacuo, dissolved in methanol, and applied to silica gel (63–200 μm, Merck) flash column. The silica gel column was eluted with a stepwise gradient of $CHCl_3$-MeOH (90:10, 80:20, 70:30, 50:50, 30:70, and 10:90, v/v). Each fraction was concentrated and bioassayed against *P. capsici* by paper disk-agar diffusion method. The active fractions were pooled and re-chromatographed on a silica gel column. The solvent system was *n*-hexane–Me_2CO with the stepwise gradients (100:0, 90:10, 80:20, 50:50, 30:70, 10:90, v/v). Each fraction was concentrated *in vacuo* and bioassayed against *P. capsici*. The active fractions were pooled and loaded on the column (50×900 mm, Pharmacia) which was packed with Sephadex LH-20 resin. The mobile phase used in Sephadex LH-20 cc was the $CHCl_3$–MeOH (1:1, v/v). The Sephadex LH-20 column was eluted at a flow rate of 0.35 ml/min. Each fraction (5 ml) was collected by a fraction collector (RediFrac, Pharmacia, Uppsala, Sweden) and then measured for anti-oomycete activity against *P. capsici* by paper disk-agar diffusion method. The concentrated active fractions were re-chromatographed on the Sephadex LH-20 column (26×950 mm, Pharmacia) and eluted with MeOH at a flow rate of 0.15 ml/min. The fractions active against *P. capsici* were separated using prep. TLC plates, which were developed in $CHCl_3$–MeOH (9:1, v/v). The active band was scraped under 254 nm, extracted with MeOH, concentrated under reduced pressure and analyzed by prep. TLC.

3.4. Biological activity of nyasol **1**

3.4.1. In vitro antimicrobial activity

The minimum inhibitory concentration (MIC) was evaluated using several plant pathogenic fungi, oomycetes, yeast and bacteria. One ml of PDA or NB was pipetted into wells of a 24-well microtiter dish (Cell Well™, Corning Glass Works, Corning, NY) and nyasol **1** in the range 0–50 μg/ml was added into the microwells. The inocula used were zoospore suspension (10^5 zoospores/ml) of *P. capsici*, mycelial suspensions of *Rhizoctonia solani* and *Phythium ultimum*, spore suspensions (10^5 spores/ml) of the following fungi: *Altermaria mali, Botrytis cinerea, Clasdosporium cucumerinum, Colletotrichum orbiculare, Cylindrocarpon destructans*, *Didymella bryoniae, Fusarium oxysporum* f.sp. *lycopersici, Magnaporthe grisea, Sclerotinia sclerotiorum,* yeast (10^4 cfu/ml): *Candida albicans, Saccharomyces cerevisiae*, and bacteria (10^4 cfu/ml): *Bacillus subtilis, Erwinia carotovora* subsp. *carotovora, Ralstonia solanacearum, Xanthomonas campestris* pv. *vesicatoria*. The 10 μl of inocula suspension was added into each microwell. The microtiter containing *B. cinerea, C. cucumerinum*, and *S. sclerotiorum* was incubated at 20 °C, and the rest at 28 °C for 4–7 days.

3.4.2. Evaluation of in vivo control efficacy

The control efficacy of nyasol **1** was evaluated against Phytophthora blight of pepper (*Capsicum annuum* cv. Hanbyul). Pepper seeds were sown in steam-sterile soil mix (peat moss:soil = 2:1) in a plastic tray (55×10×5 cm) and raised in the growth room at 28±2 °C under 5000 lux illumination for 16 h a day. Six seedling plants at four-leaf stage were transplanted into a plastic pot (5×15×10 cm) containing the above described soil mix. When pepper plants were the first branch stage, stems of the plants were treated with either nyasol **1** or the commercial fungicide metalaxyl (Ridomil, Novartis Crop Protection, Inc., Greensboro, NC). Nyasol **1** and metalaxyl dissolved in methanol were diluted with 0.05% Tween 20 (Showa, Tokyo, Japan) to give the concentrations of 1, 10, 50, 100 and 500 μg/ml. The chemical substances were sprayed on the plants. The plants treated with each solution were wounded by making 1-cm slits approximately 1 cm above the soil surface. Zoospores of *P. capsici* were produced, as previously described (Kim et al., 1989). The sterile cotton soaked in zoospore suspensions (10^5 zoospores/ml) was enclosed around the wounded position. The inoculated sites were covered with plastic tapes to maintain moisture. Disease severity was measured daily after inoculation based on a scale of 0–5: 0 = no visible disease symptoms, 1 = leaves slightly wilted with brownish lesions beginning to appear on stems, 2 = 30–50% of entire plant diseased, 3 = 50–70% of entire plant diseased, 4 = 70–90% of entire plant diseased, and 5 = plant dead. Statistical analysis was performed by the least significant difference (LSD) test at $P = 0.05$ with SAS software (SAS Institute, Cary, NC).

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