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Photochemical dimerization of wasalexins in UV-irradiated *Thellungiella halophila* and *in vitro* generates unique cruciferous phytoalexins

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ABSTRACT

The production of phytoalexins in *Thellungiella halophila* exposed to UV-radiation and NaCl was investigated over a period of 8 days. UV-radiation induced substantially larger quantities of wasalexins A and B than NaCl irrigation or $CuCl_2$ spray. Isolation of two metabolites and their chemical structure determination using X-ray diffraction analysis provided the phytoalexins biswasalexins A1 and A2, that resulted from head-to-tail photodimerization of wasalexin A. The production of biswasalexins A1 and A2 in stressed *T. halophila*, as well as their chemical synthesis and antifungal activity are reported. Biswasalexins A1 and A2 (60 nmol/g and 15 nmol/g fresh wt, respectively, 2 days after UV elicitation) are cruciferous phytoalexins whose formation *in planta* appears to result from a photochemical reaction, which might protect the plant from fungal attack and UV-radiation.

1. Introduction

Thellungiella halophila (salt cress) is an annual crucifer and an important model plant due to its small genome and resistance to salinity and drought (Inan et al., 2004). To date, two ecotypes of *T. halophila* have been described: the Shandong ecotype, native to high-salinity coastal areas of the Shandong province in northeast China (Inan et al., 2004) and the Yukon ecotype, isolated from saline meadows in the Yukon Territories, Canada (Wong et al., 2005, 2006). Both the Shandong and Yukon ecotypes originate from extreme climatic conditions and have been reported to be tolerant to salinity, drought, and cold stresses. *T. halophila*, a species close to *Arabidopsis thaliana*, produces the phytoalexins wasalexins A (**1**) and B (**2**) in plants stressed with $CuCl_2$ but not in healthy tissues (Pedras and Adio, 2008). Wasalexins were first isolated from $CuCl_2$ stressed wasabi plants (*Wasabia japonica*, Pedras et al., 1999, 2007), and more recently found to be produced in stressed leaves of water cress (*Thlaspi arvense*, Pedras et al., 2003, 2007). In addition to wasalexins, *T. halophila* produces the phytoalexins 1-methoxybrassenin B (**3**) and rapalexin A (**4**), the latter in trace amounts (Pedras and Adio, 2008).

In continuation of previous work (Pedras and Adio, 2008), we investigated the production of phytoalexins in *T. halophila* exposed to UV-radiation, NaCl irrigation and $CuCl_2$ spray. Interestingly, UV-radiation induced substantially larger quantities of wasalexins A (**1**) and B (**2**) together with less polar metabolites than NaCl or $CuCl_2$ spray. Isolation of these less polar metabolites and chemical structure determination using X-ray diffraction analysis provided unique wasalexin photoaddition products, the biswasalexins A1 (**10**) and A2 (**11**). Here we report the production and accumulation of biswasalexins A1 and A2 in stressed *T. halophila*, as well as their chemical synthesis and antifungal activities (Fig. 1).

2. Results and discussion

2.1. Elicitation and isolation of metabolites

T. halophila Shandong ecotype plants were elicited with UV light, NaCl or $CuCl_2$, as described in the Section 4. Plants were harvested 2, 4, 6 and 8 days after elicitation, the aerial parts were frozen in liquid nitrogen, ground, and extracted with MeOH. Control plants were extracted similarly. The methanolic extracts were concentrated to dryness and the residues were rinsed with CH_2Cl_2 to yield the non-polar fractions containing wasalexins and other less polar metabolites. The non-polar residues of UV-irradiated, NaCl irrigated, $CuCl_2$ sprayed and control plants were analyzed by

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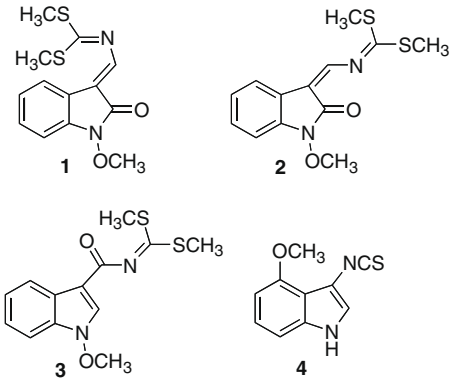


Fig. 1. Phytoalexins produced in leaves of *Thellungiella halophila* sprayed with $CuCl_2$ (Pedras and Adio, 2008).

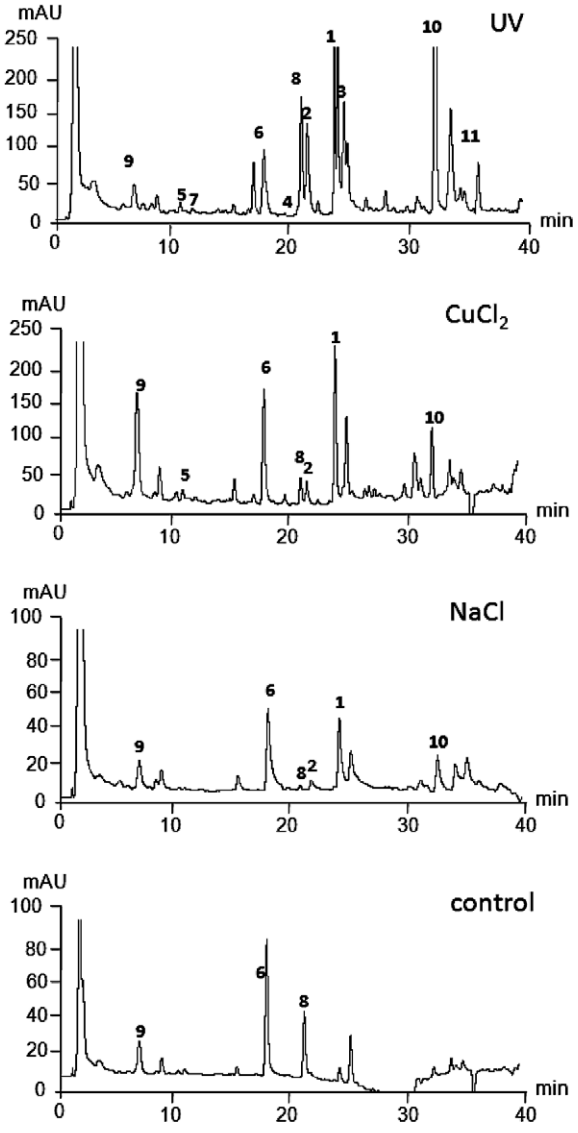
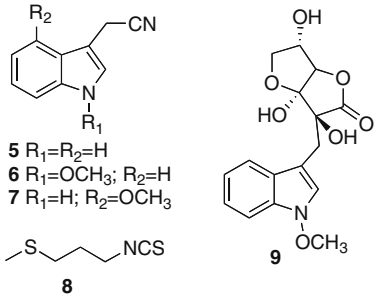


Fig. 2. HPLC-DAD chromatograms of non-polar extracts of *Thellungiella halophila* Shandong 2 days after elicitation using UV light, NaCl and $CuCl_2$ and non-polar extract of non-elicited plants. Peaks: 1, wasalexin A (**1**); 2, wasalexin B (**2**); 3, brassenin B (**3**); 4, rapalexin (**4**); 5, indolyl-3-acetonitrile (**5**); 6, caulilexin C (**6**); 7, arvelexin (**7**); 8, methylthiopropylisothiocyanate (**8**); 9, neoascorbigen (**9**); 10, biswasalexin A1 (**10**); 11, biswasalexin A2 (**11**).

HPLC-DAD (Fig. 2) as well as LC–ESI-MS. Compounds detected in each extract were identified either by direct comparison with authentic samples available in our metabolite libraries (HPLC-DAD and HPLC–ESI-MS), or by isolation and structure determination using spectroscopic data (Pedras et al., 2006, 2007, 2008). The structural assignments of the new metabolites biswasalexins A1 (**10**) and A2 (**11**) were confirmed by single crystal X-ray diffraction analysis.

The HPLC chromatograms of all samples displayed readily identifiable peaks corresponding to caulilexin C (**6**, 1-methoxyindolyl-3-acetonitrile) and 3-methylthiopropylisothiocyanate (**8**). Indolyl-3-acetonitrile (**5**) and arvelexin (**7**, 4-methoxyindolyl-3-acetonitrile) were identified only after fractionation of the non-polar extracts due to their low concentration. The HPLC chromatograms of plants elicited with UV light (Fig. 2) displayed several peaks not present in control plants, namely wasalexin A (**1**), wasalexin B (**2**), 1-methoxybrassenin B (**3**), together with peaks with retention times between 30 and 36 min, which were likely due to unknown metabolites. In addition, minor components were identified after fractionation of the plant extract, namely neoascorbigen (**9**) and rapalexin A (**4**).



The elicited compounds responsible for peaks with t_R = 32.0 min (**10**) and t_R = 35.4 min (**11**) were isolated from larger scale experiments and purified using chromatography. The ^1H NMR spectroscopic data of each metabolite were similar to those of wasalexin A (**1**) except for the signal due to the methylene proton on the 2-oxoindole side chain that was displayed at substantially higher field (δ_H 8.12 in **1** vs. 5.25 ppm in **10** and 5.14 ppm in **11**). Since HRMS-ESI data suggested the molecular formula of $C_{26}H_{29}N_4O_4S_4$ for the compounds with t_R = 32.0 min (**10**) and t_R = 35.4 min (**11**), a structure resulting from wasalexins A and/or B dimerization was considered.

2.2. Synthesis of biswasalexins A2 (*11*) and A1 (*10*)

A literature search indicated that photodimerization of 2-oxoindoles in the solid state had been reported for (*E*)-2-furylidenoxindole (**12**), while *E-Z* isomerization was the only photochemical process observed in solution (Milanesio et al., 2000). Because wasalexin A (**1**) was sufficiently stable to purify (Pedras and Suchy, 2006), its photodimerization was examined under different light sources. As expected, in solution under UV-radiation (315–400 nm) *E-Z* isomerization of wasalexin A (**1**) was the major transformation detected, yielding about a 3:1 ratio of *E*/*Z* isomers; longer UV-radiation periods yielded traces of undetermined compounds. However, when wasalexin A (**1**) was irradiated in a film on a Pyrex Petri dish, two major products with t_R = 32.0 min and t_R = 35.4 min were obtained that appeared identical to those isolated from UV-irradiated leaves. Crystals of wasalexin A (**1**), and both photoaddition products suitable for X-ray diffraction analysis were obtained by slow evaporation of a solution of each compound. X-ray diffraction analyses clearly showed that both photoaddition products, compounds **10** and **11**, resulted from the