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Effect of light-irradiation on allelopathic potential of germinating maize

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Abstract

The effect of visible light-irradiation on the allelopathic potential of germinating maize (*Zea mays*) was investigated. Six substances with inhibitory activity were found in the acetone extract of the seedlings, and one substance was higher in light-grown seedlings than in dark-grown ones. By spectral analysis, the substance was identified as 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). At concentrations greater than 0.03 mM, DIBOA inhibited the growth of roots and hypocotyls of lettuce seedlings. The concentrations of DIBOA in the light-grown maize seedlings and their root exudates were 43 and 0.38 μmol kg^{-1} fr. wt., respectively, and concentrations in the dark-grown seedlings and their root exudates were 19 and 0.17 μmol kg^{-1} fr. wt., respectively. The level of DIBOA in the dark-grown seedlings increased rapidly upon visible light irradiation. These results suggest that visible light may enhance allelopathic activity of germinating maize due to an increase in the level of DIBOA.

Keywords: *Zea mays*; Gramineae; Maize; Allelopathy; Light-irradiation; Phytotoxicity; Hydroxamic acid; 2,4-dihydroxy-1,4-benzoxazin-3-one; Root secretion

1. Introduction

A number of secondary metabolites in plants can act as allelochemicals to other plants. Under certain conditions, these compounds are released into the environment by exudation from living plant tissues and decomposition of plant material and may affect the growth of neighboring or successional plants (Rice, 1984; Putnam, 1988; Einhellig, 1996).

Some crop and weed species have been reported to change their allelopathic potential depending on abiotic and biotic environmental conditions such as mineral availability (Williamson, Obee & Weidenhamer, 1992; Bulm, Gerig, Worsham & King, 1993), temperature (Qasem & Abu-Irmaileh, 1985; Fischer, Williamson, Weidenhamer & Richardson, 1994), water deficiency (Einhellig, 1989; Richardson & Bacon, 1993), UV and ionizing radiation (Alsaadawi, Al-Uqaili, Al-Hadithy & Alrubeaa, 1985; Balakumar, Vincent & Paliwal, 1993), pathogens (Lynch, 1987; Perice & Colby, 1987) and chemicals (Bulm et al., 1993; Gross & Parthier, 1994). Generally, stressed donor plants increased allelopathic activity due to increased production of allelochemicals (Niemeyer, 1988; Putnam, 1988). Thus, it was concluded that stress-induced elevation of allelopathic activity was a strategy for defense of plants to environmental stress (Bell, 1981; Rice, 1984; Einhellig, 1996).

Bhowmik and Doll (1983) reported that photosynthetic visible light also affected the allelopathic potential of weed residues. Light perception for germinating plant seeds is one of the most important events of early plant development. However, little is known about the effect of visible light on the allelopathic potential of germinating plants (Rice, 1984; Putnam & Tang, 1986; Einhellig, 1996).

The purpose of the present experiments was to investigate whether visible light has an effect on the allelopathic potential of germinating maize, and to

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determine the variation in level of the allelochemical influenced by light irradiation.

2. Results and discussion

2.1. Effect of light irradiation on inhibitory substance in maize seedlings

To investigate the effect of light irradiation on allelopathic constituents of germinating maize, neutral and acidic fractions obtained from the Me_2CO extract of dark-and light-grown maize seedlings were subjected to silica gel chromatography, and inhibitory activity of their eluted fractions was determined in a lettuce root bioassay (Figs. 1 and 2). Three peaks of inhibitory activity were detected in the eluted fractions: 40–60% and 80–90% EtOAc in C_6H_6, and MeOH in both neutral fractions; active principles were named N-1, N-2 and N-3 in order of the elution (Fig. 1). In both acidic fractions, three peaks of activity were also detected in eluted fractions: 20–30% and 60–80% EtOAc in C_6H_6 and MeOH; active principles were named A-1, A-2 and A-3 in order of the elution (Fig. 2).

The inhibitory activity of N-3 from the light-grown seedlings was higher than that from the dark-grown ones (Fig. 1), while the inhibitory activities of N-1, N-2, A-1, A-2 and A-3 in the light-grown seedlings were almost equal to those in the dark-grown ones (Figs. 1 and 2). These results indicate that substances with inhibitory activity were present in the Me_2CO extract of the germinating maize and that the activity of N-3 was increased by light irradiation.

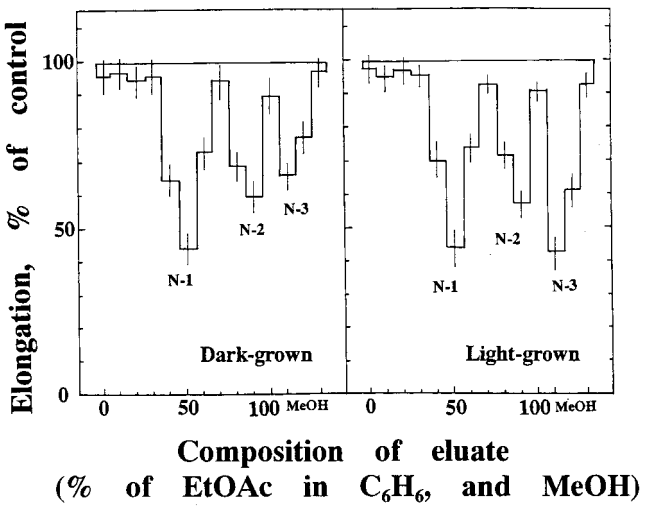
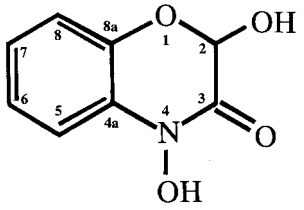


Fig. 1. Bioassay of neutral fractions isolated from dark-grown and light-grown maize seedlings, separated by column chromatography. All tested samples corresponded to 3 g fr. wt. equivalent of the seedlings. Biological activity was determined in a lettuce root bioassay as described in Section 3. Mean ± s.e. from three replicate experiments with 15 plants each are shown. Root elongation of control plants was 19.3 ± 1.4 mm.

2.2. Identification of N-3

N-3 was purified from the neutral fraction obtained from light-grown maize seedlings. Mass spectroscopy indicated a molecular formula of $C_8H_7NO_4$ (m/z 181.0343; calculated 181.0373) and EI-MS (element composition, relative intensity); m/z 181 ($[M]^+$, 4), ($[M–OH]^+$, 5), 135 ($[M–CO–H_2O]^+$, 100). The ^{1}H-NMR spectrum (CD_3OD) showed signals for δ 5.68 (1H, *s*, C-2), 7.01 (1H, *dd*, J = 7.7, 1.8 Hz, C-5), 7.05 (1H, *ddd*, J = 7.7, 7.3, 1.5 Hz, C-6), 7.09 (1H, *ddd*, J = 7.5, 7.3, 1.8 Hz, C-7) and 7.36 (1H, *dd*, J = 7.5, 1.5 Hz, C-8). The ^{13}C-NMR spectrum (CD_3OD) showed signals for δ 93.7 (*d*, C-2), 114.4 (*d*, C-8), 118.5 (*d*, C-5), 123.8 (*d*, C-7), 125.6 (*d*, C-6), 129.7 (*s*, C-4a), 142.6 (*s*, C-8a) and 160.1 (*s*, C-3). From the interpretation of these data and comparison with literature data (Klun, Tipton, Robinson, Ostrem & Beroza, 1970; Wolf, Spencer & Plattner, 1985; Barnes & Putnam, 1987; Kato-Noguchi, Sakata, Takenokuchi, Kosemura & Yamamura, 1999), compound **1** was identified as hydroxamic acid (2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA)).



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Hydroxamic acids are secondary plant metabolites found in the Gramineae, and are associated with resistance of the plants to insects (Klun & Robinson, 1969; Argandoña, Luza, Niemeyer & Corcuera, 1980; Hibbard, Peairs, Pilcher, Schroeder, Jewett & Bjostad, 1995; Dowd & Vega, 1996) and pathogens (Pérez, 1990; Xie, Arnason, Philogéne, Olechowski & Hamilton, 1992; Richardson & Bacon, 1995). In addition, hydroxamic acid is involved in allelopathic effects of the plants (Willard & Penner, 1976; Wolf et al., 1985; Barnes & Putnam, 1987; Niemeyer, 1988). Although the main hydroxamic acid in maize plants was reported to be 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (Kumar, Gagliardo & Chilton, 1993; Iwamura, Nakagawa & Hirai, 1996), DIBOA, its demethoxy analog, was isolated here from light-grown maize seedlings.

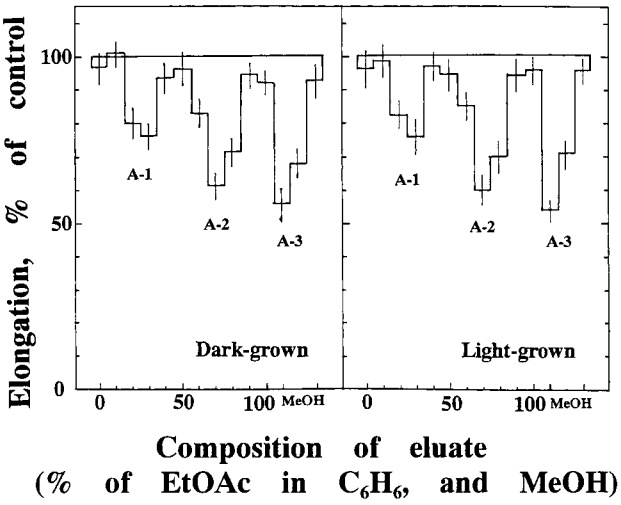

Fig. 2. Bioassay of acidic fractions isolated from dark-grown and light-grown maize seedlings, seperated by column chromatography. Conditions are as described in legend of Fig. 1.

2.3. Biological activity and quantification of DIBOA

DIBOA inhibited the growth of roots and hypocotyls of lettuce seedlings at concentrations greater than 0.03 mM, and increasing the dose increased the inhibition (Fig. 3). When percentage elongation was plotted against logarithm of dose, the dose-response curves for both roots and hypocotyls were linear between 20–90% elongation. The concentrations of DIBOA for 50% growth inhibition of the roots and the shoots were 0.64 and 1.9 mM, respectively (Fig. 3). Using the 50% growth inhibition concentration, the effectiveness of DIBOA on the roots was approximately four-fold greater than on the hypocotyls in the seedlings.

Table 1
Level of DIBOA in maize seedlings and its root exudates[a]

Treatment	Seedling (μmol kg^{-1} fr. wt.)	Exudate (μmol kg^{-1} fr. wt.)
Dark-grown	19 ± 3	0.17 ± 0.02
Light-grown	43 ± 4	0.38 ± 0.03

[a] Light-grown seedlings were irradiated for 24 h. Mean ± s.e. from three replicate experiments with three injections to HPLC for each determination are shown.

Table 1 shows the levels of DIBOA in dark-grown and light-grown maize seedlings and in their root exudates. Levels in the light-grown seedlings were ca. 1.5-fold higher than those of the dark-grown ones, and the levels of DIBOA secreted by the roots are proportional to its concentration in the seedlings. It has been reported that some secondary metabolites are exuded from living plant roots, and that stress and other factors such as light, moisture, and plant age could increase root exudation (Rovira, 1969; Niemeyer, 1988; Putnam, 1988). The detection of DIBOA in the root exudate and its effect on the growth (Fig. 3 and Table 1) suggest that DIBOA may act as an allelochemical for germinating maize against other plants (Rice, 1984; Putnam, 1988; Einhellig, 1996).

2.4. Effect of light irradiation on the level of DIBOA

Fig. 4 shows the change in the level of DIBOA in dark-grown maize seedlings after the onset of continuous light irradiation. The level of DIBOA increased sharply up to 3 h and then decreased gradually. In 3 h, the level of DIBOA in the seedlings had increased to ca. five-fold higher than in the initial seedlings. In

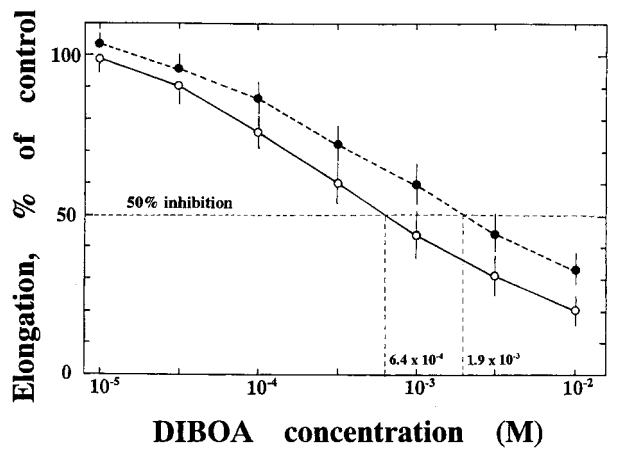

Fig. 3. Effect of DIBOA on growth of roots (○) and hypocotyls (●) of lettuce seedlings. Mean ± s.e. from three replicate experiments with 15 plants each are shown. Elongation of control plants was 18.5 ± 1.3 and 13.7 ± 0.9 mm for roots and hypocotyls, respectively.

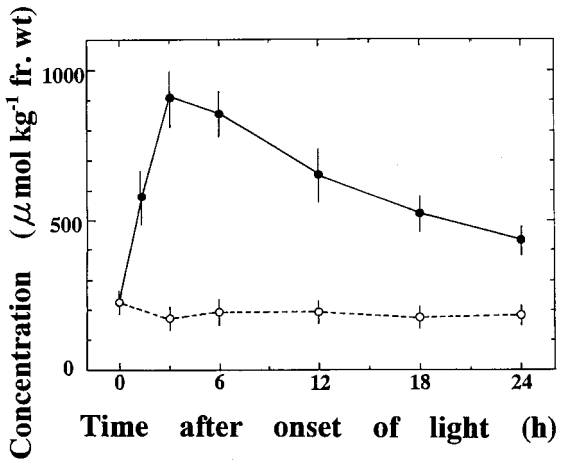

Fig. 4. Changes in levels of DIBOA in dark-grown maize seedlings after onset of light-irradiation (●). Control plants were grown in darkness (○). Mean ± s.e. from three replicate experiments with three injections to HPLC for each determination are shown.

contrast, the level remained almost unchanged during 24 h at this plant development stage in darkness, although the DIBOA level was reported to fluctuate with plant age in further development stages (Dowd & Vega, 1996; Iwamura et al., 1996).

High light intensity (Thompson, Slife & Butler, 1970; Manuwoto & Scriber, 1985), long photoperiod (Epstein, Rowsemitt, Berger & Negus, 1986) and elevated growth temperature (Thompson et al., 1970; Epstein et al., 1986) decrease the levels of cyclic hydroxamic acid in plants, whereas water stress increases the level (Richardson & Bacon, 1993). However, information related to environmental effects on DIBOA levels is limited (Niemeyer, 1988; Einhellig, 1996). The present experiments demonstrate that visible light irradiation causes an increase in the level of DIBOA in the germinating maize. The mechanism of light enhancement of DIBOA levels in the germinating maize is under investigation.

In conclusion, germinating maize contains DIBOA, and its roots secrete DIBOA to the medium (Table 1). The level of DIBOA in the dark-grown seedlings increased rapidly upon visible light irradiation (Fig. 4). These data coupled with the effect of DIBOA on growth (Fig. 3) suggest that visible light could effect the allelopathic potential of germinating maize through an increase in the level of DIBOA.

3. Experimental

3.1. Plant material

Seeds of maize (*Zea mays* L. cv. Popcorn; Takii, Kyoto, Japan) were sterilized in a 2% (w/v) solution of NaOCl for 10 min, rinsed in distilled H_2O for 20 min (3×), and germinated on two sheets of moist filter paper (No. 1, Toyo, Tokyo) in darkness at 25°C for 4 days. Uniform dark-grown, four-day-old seedlings were divided into two groups: one group was left in darkness at 25°C (dark-grown seedlings), and the other group was irradiated continuously from above with two white fluorescent lamps (3.2 W m^{-2} at plant level; Fl-20S, 20 W, National, Tokyo) at 25°C (light-grown seedlings). After one day, the seedlings were harvested, frozen immediately in liquid N_2 and stored at −80°C until extraction. All manipulations after sowing were performed under a dim green safe-light (0.03 W m^{-2}).

3.2. Extraction and column chromatography

Light-grown and dark-grown maize seedlings (10 g fw. eq.) were separately homogenized with 100 ml of cold Me_2CO and the homogenate was filtered through filter paper (No. 1, Toyo). The residue was homogenized again with 100 ml of 80% (v/v) cold aqueous Me_2CO and filtered. The two filtrates were combined and concentrated at 35°C in vacuo to give an aqueous residue. The residue was adjusted to pH 7.5 with 1 M K–Pi buffer and partitioned (3×) against an equal volume of EtOAc. The EtOAc phase was evaporated to dryness in vacuo at 35°C after drying over anhydrous Na_2SO_4 (neutral fraction). The aqueous phase was adjusted to pH 2.5 with 2 M H_3PO_4 and partitioned (3×) against an equal volume of EtOAc. The EtOAc phase was evaporated to dryness after drying over anhydrous Na_2SO_4 (acidic fraction).

Each fraction was separately chromatographed on a column (2 × 60 cm) of silica gel (100 g, Silica gel 60, 70–230 mesh; Merck) and eluted stepwise with C_6H_6–EtOAc containing increasing amounts of EtOAc (10% per step, v/v, 200 ml per step). After the eluting solvent had reach 100% EtOAc, the elution was completed with 600 ml MeOH (a total of 200 ml was collected at each step).

3.3. Bioassay

Each eluted fraction from the column was evaporated to dryness and dissolved in a mixture of EtOAc and MeOH, added to a sheet of filter paper (No. 2, Toyo Ltd.) in a 3-cm Petri dish and dried. The filter paper in the Petri dish was moistened with 1 ml of 0.05% (v/v) aqueous Tween 20, and 15 seeds of lettuce (*Lactuca sativa* cv. Grand Rapids L.) were sown on it. The length of roots and hypocotyls of the lettuce seedlings was determined after two and four days of incubation in the darkness at 25°C, respectively, and percentage elongation of growth was calculated by reference to the elongation of control plants.

3.4. Isolation of N-3

N-3 obtained from light-grown maize seedlings (200 g fr. wt.) was further purified by a TLC plate (Silica gel 60 GF254, 20 × 20 cm; Merck) with $CHCl_3$–HOAc (19 : 1, v/v). Biological activity was determined using a lettuce bioassay as described above and the active zone (Rf 0.0–0.1) was excised and eluted with MeOH. After evaporation, the residue was dissolved in 10 ml of 20% (v/v) aqueous MeOH, passed through a C_{18} Sep-Pak cartridge (Waters) and evaporated. The residue was finally purified by HPLC (0.46 × 15 cm, TSK Gel ODS-80TM, Toso Tokyo; 1 ml min^{-1}, 0.5% HOAc, v/v; detected at 254 nm), yielding an active component (9 mg) that eluted with Rt of 56–59 min.

3.5. Quantification of DIBOA

Maize seeds were germinated and grown for 3 days as described above, and uniform seedlings were transferred in groups of 50–24 cm Petri dishes each contain-

ing two sheets of filter paper moistened with 50 ml sterilized H_2O. After being kept in the dark at 25°C for one additional day, the dark-grown seedlings were irradiated continuously with white fluorescent lamps as described above. For quantification of DIBOA in the maize seedlings, three Petri dishes for each determination were removed at intervals after time zero in Fig. 4, and the seedlings were harvested, immediately frozen in liquid N_2 and stored at −80°C. For quantification measurements of DIBOA in the root exudates, the seedlings were incubated in Petri dishes as described above. After 24 h light irradiation, the aqueous medium was separated, filtered with No. 1 filter paper and stored at −80°C. All instruments used in these experiments were sterilized.

Frozen plant materials were extracted and purified according to the methods of Lyons, Hipskind, Wood & Nicholson (1988), and the samples were subjected to HPLC (0.46×15 cm, TSK Gel ODS-80TM; 2 ml min^{-1}, 0.5% HOAc, v/v; detected at 280 nm). The Rt of DIBOA was 27.3 min under these conditions. Quantitative measurements was performed by measuring the peak areas on chromatograms of HPLC and compared to those of standard DIBOA.

The media of the seedlings were evaporated to dryness and the level of DIBOA in the residue was determined as described above.

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