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Quinclorac-induced cell death is accompanied by generation of reactive oxygen species in maize root tissue

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ABSTRACT

The importance of reactive oxygen species for herbicide quinclorac (3,7-dichloro-8-quinolinecarboxylic acid)-induced cell death in roots was investigated. This was in order to understand its mode of action in grass species grown in the dark. Under these dark conditions, quinclorac suppressed the shoot and root growth of maize (*Zea mays* L. cv. Honey Bantam) in a concentration-dependent manner ($\leqslant$50 μM), although the inhibition level was less than that observed under growth conditions in the light. Analysis of cell viability using Evans blue or fluorescein diacetate–propidium iodide (FDA–PI) staining showed that the maize root cells significantly lost their viability after 14 h root treatment with 10 μM quinclorac, but not 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Determination of reactive oxygen species (ROS) in maize roots using a superoxide anion (O_2^-)-specific indicator, dihydroethidium (DHE), indicated that 50 μM quinclorac induced a high level of O_2^- production in maize roots after 14 h root treatment than that of either the control (non-treated) or with 50 μM 2,4-D. Moreover, either cell death or ethane evolution, an indicator of lipid peroxide formation, in maize root segments was significantly enhanced by 50 μM quinclorac, but not by 50 μM 2,4-D. On the other hand, the 50 μM 2,4-D treatment induced much higher ethylene and cyanide production in the root segments than with the 50 μM quinclorac. These results suggest that quinclorac-induced cell death in maize roots may be caused by ROS and lipid peroxidation, but not by ethylene and its biosynthetic pathway-related substances including cyanide, which have been thought to be the causative factor of quinclorac-induced phytotoxicity in susceptible grass weeds such as *Echinochloa*, *Digitaria*, and *Setaria*.

1. Introduction

Quinclorac (**1**, Fig. 1) controls some broad-leaved weeds and major grass weeds such as *Echinochloa*, *Digitaria*, and *Setaria* species in rice and turf fields (Menck et al., 1985; Wuerzer and Berghaus, 1985; Chism et al., 1991; Grossmann and Kwiatkowski, 2000). Its mode of action was originally proposed to be auxin-like, largely based on morphological response (Berghaus and Wuerzer, 1987). However, several studies reported that quinclorac-susceptible grasses showed different symptoms from those caused by 2,4-dichlorophenoxyacetic acid (2,4-D, **2**, Fig. 1): chlorotic discolouration and necrosis of newly emerged leaves, and finally, wilting and desiccation of the entire plant (Berghaus and Wuerzer, 1987; Koo et al., 1991a; Sunohara and Matsumoto, 1997), although many quinclorac-susceptible broadleaves showed auxin-like symptoms (Berghaus and Wuerzer, 1987; Koo et al., 1991a). Koo et al. (1991a) reported that quinclorac **(1)** did not exhibit auxin-like activities in barnyard grass based on a mesocotyl elongation assay. In addition, it was shown that the effects of quinclorac **(1)** on respiration and RNA and protein contents in shoot tissues of barnyard grass were different from those by 2,4-D **(2)** (Koo et al., 1991b). In the coleoptile elongation and binding assays with a membrane-bound auxin-binding protein (Sunohara and Matsumoto, 1997), quinclorac **(1)** did not exhibit auxin-like activities in maize (*Zea mays* L. cv. Honey Bantam), another susceptible grass, although 2,4-D **(2)**, indole-3-acetic acid (IAA), and α-naphthyl-1-acetic acid (NAA) exhibited auxinic activities. Moreover, root-applied quinclorac **(1)** reduced chlorophyll content in maize leaves, whereas 2,4-D **(2)** did not (Sunohara et al., 2003a,b). From these results, we proposed a hypothesis that quinclorac **(1)** does not entirely act by the same mechanism as 2,4-D **(2)** and other auxins, and has additional modes of action in susceptible grasses.

There have been many reports concerning the mode of action of quinclorac **(1)** in grass species (Abdallah et al., 2006; Grossmann and Kwiatkowski, 1995, 2000; Koo et al., 1996; Grossmann and Scheltrup, 1997; Sunohara and Matsumoto, 1997, 2004; Sunohara et al., 2003a,b; Tresch and Grossmann, 2003a,b). Tresch and Grossmann (2003a,b) reported that quinclorac **(1)** does not inhibit cellulose biosynthesis in sensitive barnyard grass and maize roots. Grossmann and Scheltrup (1997) instead showed that quinclorac enhances ethylene production by stimulating 1-aminocyclopro-

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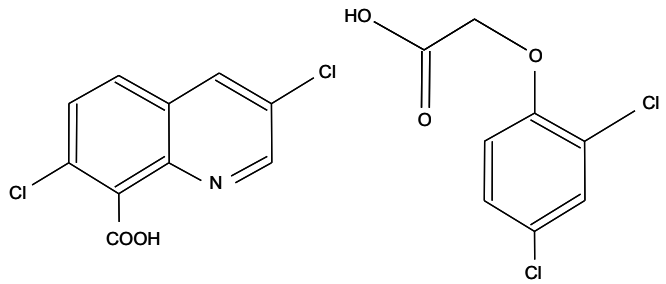

Quinclorac, **1**
3,7-Dichloro-8-quinolinecarboxylic acid

2,4-D, **2**
2,4-Dichlorophenoxyacetic acid

Fig. 1. Chemical structures of quinclorac **(1)** and 2,4-D **(2)**.

pane-1-carboxylic acid (ACC) synthase (EC 4.4.1.14) activity, and the levels of endogenous ACC in a concentration-dependent manner in barnyard grass. Since quinclorac **(1)** enhances the accumulation of cyanide, an ethylene co-product formed during the oxidation of ACC, they concluded that quinclorac-stimulated cyanide is the causative factor of leaf chlorosis in several grass species (Grossmann and Kwiatkowski, 1995). Therefore, the selective induction of ACC synthase activity, which ultimately leads to cyanide accumulation in the tissues of susceptible grasses, is considered to be the primary mechanism of quinclorac **(1)** action (Grossmann and Kwiatkowski, 2000). In addition, Abdallah et al. (2006) showed an evident relationship between cellular cyanide content and quinclorac **(1)** phytotoxicity, suggesting that its toxicity to smooth crabgrass (*Digitaria ischaemum*) is mainly caused by an accumulation of cyanide in shoot tissues.

We reported previously on the enhancement of quinclorac **(1)** activity under illumination in maize seedlings by showing a strong correlation between light intensity and the reduction in total chlorophyll content and rate of ethylene production (Sunohara and Matsumoto, 1997; Sunohara et al., 2003a,b). Moreover, quinclorac **(1)** was shown to induce oxidative damage in early watergrass (*Echinochloa oryzicola* Vasing.), another susceptible grass, and suggested that the difference in inherent antioxidative ability is one of factors that results in the high selectivity of quinclorac **(1)** between rice and early watergrass (Sunohara and Matsumoto, 2004). To our knowledge, these were the first reports to suggest a relationship between reactive oxygen species (ROS) and the herbicidal activity of quinclorac **(1)** in grass species. As described above, light enhances quinclorac-induced phytotoxicity in maize and early watergrass; however, higher concentrations of quinclorac **(1)** also caused wilting and growth inhibition even in the dark (unpublished observations). In our previous studies (Sunohara and Matsumoto, 1997, 2004; Sunohara et al., 2003a,b), ROS seem to relate to the herbicidal activity of quinclorac **(1)** in shoots of susceptible grasses, but it is still unknown whether it induces ROS production in roots and that results in root growth inhibition in grass species in the dark. In the present study, we focused on ROS production in quinclorac-treated roots of maize, as a test species of susceptible grasses, and examined the importance of ROS for quinclorac-induced cell death in maize roots.

2. Results and discussion

2.1. Effects of quinclorac *(1)* and 2,4-D *(2)* on shoot and root growth

Quinclorac **(1)** suppressed shoot and root growth in early watergrass (*Echinochloa oryzicola* Vasing.) under light and dark conditions in a dose-dependent manner (⩽10 μM), while the herbicide showed no effect on the growth of rice (*Oryza sativa* L. cv. Nipponbare) under light and dark conditions at the concentrations tested (data not shown). Under light conditions, quinclorac **(1)** inhibited shoot and root growth of early watergrass more strongly than in the dark (data not shown). In maize (*Zea mays* L. cv. Honey Bantam) seedlings, quinclorac **(1)** also suppressed shoot and root growth in the dark in a concentration-dependent manner (Fig. 2a) and inhibited the development of lateral roots (data not shown). 2,4-D **(2)** also caused root growth suppression in the dark, but at 10 and 50 μM, it showed no inhibitory effect on the lateral root development (data not shown) or shoot growth in maize seedlings (Fig. 2b). Moreover, 2,4-D **(2)** at 10 and 50 μM tended to promote shoot elongation in maize seedlings that were grown in the dark for 4 days after treatment, but quinclorac **(1)** did not (data not shown). In our previous study (Sunohara and Matsumoto, 1997), 2,4-D **(2)**, IAA, and NAA at 1–100 μM promoted maize coleoptile elongation in darkness, but quinclorac **(1)** (100 μM) inhibited elongation. Thus, quinclorac **(1)** seems to have a different action from other auxins, including 2,4-D **(2)**, on maize shoot elongation in the dark.

2.2. Effects of quinclorac *(1)* and 2,4-D *(2)* on cell viability in maize roots

The viability of maize root cells was determined by a double-staining method using fluorescein diacetate (FDA) and propidium iodide (PI). PI readily enters the cells with injured membranes, but not intact cells. PI can be detected by its red fluorescence, and it serves as a marker of permeabilized cells (Umebayashi

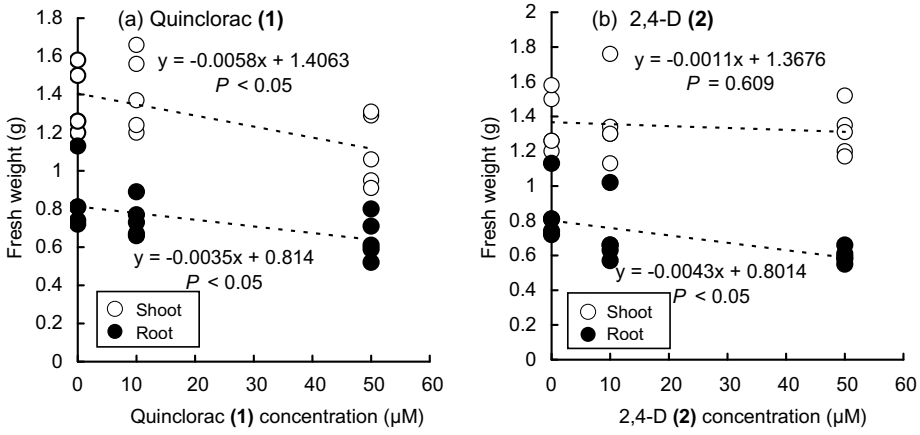

Fig. 2. Effects of (a) quinclorac **(1)** and (b) 2,4-D **(2)** on the growth of shoots (○) and roots (●) tissues in maize seedlings grown in the dark for 4 days after 14 h root treatment.

et al., 2003). FDA is a non-fluorescent compound, and readily enters intact cells and then undergoes hydrolysis by endogenous esterase, which releases free fluorescein that can be detected by its green fluorescence. Fluorescein can not be excluded from the intact cells, but is not retained in the cells with damaged membranes; therefore, it is used as a marker for cells with intact membranes (Umebayashi et al., 2003). Hence, red and green fluorescence indicate dead and viable cells, respectively. Ten μM quinclorac **(1)**-induced cell death in maize root tip cells except for the root-cap after 14 h root treatment, and the viability in root-cap cells was lost following 50 μM quinclorac **(1)** treatment (Fig. 3b and c). In 2,4-D **(2)** treatment, root-cap cells were not affected even at 50 μM, but faint red fluorescence due to staining with PI was observed in root tip cells other than the root-cap after 14 h root treatment with 10 and 50 μM 2,4-D (Fig. 3d and e).

Cell viability in maize roots was also determined by Evans blue staining to quantify the rates of dead cells. Evans blue is a dye unable to pass through intact membranes and is used to assess the integrity of cells (Gaff and Okong'o-Ogola, 1971). Fig. 4c shows maize roots stained with Evans blue after 14 h root treatment with 50 μM quinclorac **(1)** or 2,4-D **(2)**. The uptake of Evans blue in 50 μM quinclorac-treated root cells was much higher than that in 2,4-D (50 μM)-treated root cells (Fig. 4c). Ten μM quinclorac **(1)** also enhanced significantly Evans blue uptake in maize roots after 14 h root treatment, but 10 μM 2,4-D **(2)** did not (Fig. 4a and b). In 50 μM 2,4-D-treated maize roots, Evans blue uptake increased significantly; however, the level was much lower than that in 10 or 50 μM quinclorac-treated roots (Fig. 4a and b). This indicates that quinclorac **(1)** has higher activity to induce cell death in maize roots than 2,4-D **(2)** in this concentration range (⩽50 μM).

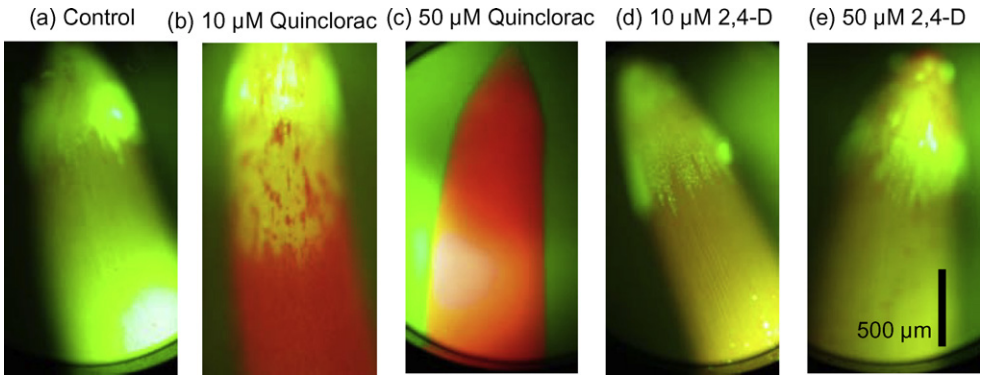

Fig. 3. Effects of quinclorac **(1)** and 2,4-D **(2)** on viability of maize root tips 0 h after 14 h root treatment. Roots were stained with FDA-PI. Fluorescence images were obtained using a Nikon E600 fluorescence microscope with a B-2A filter (excitation 450–490 nm, emission ⩾ 520 nm). Green and red fluorescence indicate viable and dead cells, respectively. Scale bar indicates 500 μm.

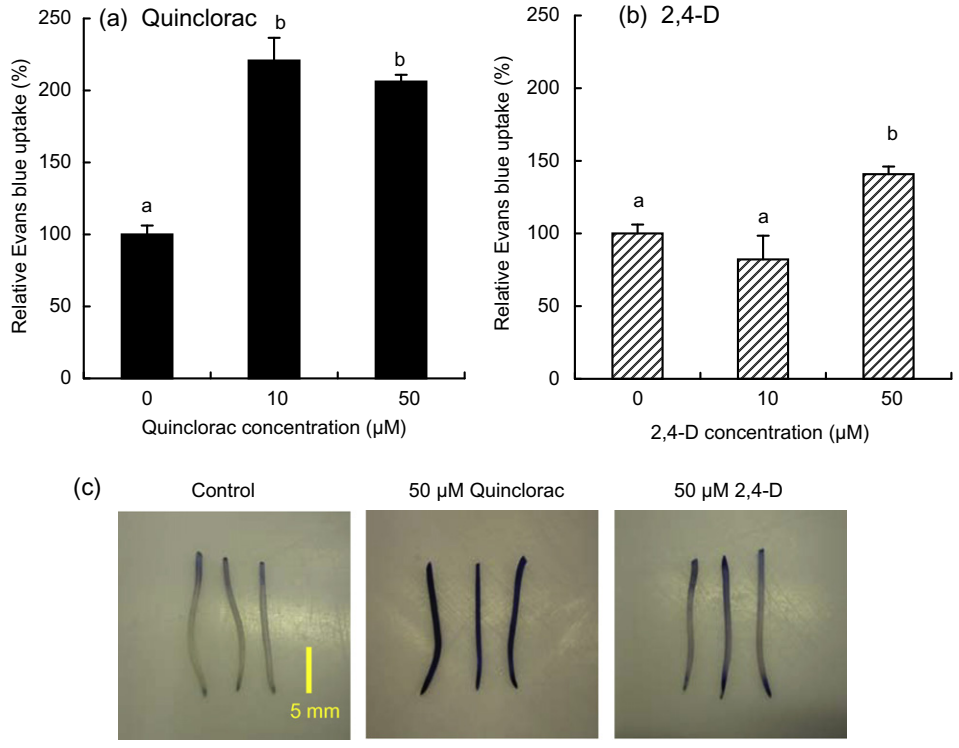

Fig. 4. Effects of (a) quinclorac **(1)** and (b) 2,4-D **(2)** on Evans blue uptake on maize roots 0 h after 14 h root treatment. Bars with the same letter are not significantly different, $P \geqslant 0.05$. (c) Maize roots which were stained with Evans blue after 14 h root treatment with 50 μM quinclorac **(1)** or 2,4-D. Cells stained by Evans blue indicate nonviable cells. Scale bar indicates 5 mm.

2.3. Effects of quinclorac **(1)** and 2,4-D **(2)** on ROS production in maize roots

Production of reactive oxygen species (ROS) was investigated by staining maize roots with dihydroethidium (DHE). DHE enters the cell and is oxidized by ROS to yield fluorescent ethidium. Ethidium binds to DNA (Eth-DNA), further amplifying its fluorescence (Carter et al., 1994). Fig. 5 shows ROS production in maize root tip cells after 14 h root treatment with 50 μM quinclorac **(1)** or 2,4-D **(2)**. ROS production in 50 μM 2,4-D-treated maize roots was slightly stimulated compared to the control roots, but quinclorac **(1)** (50 μM)-induced ROS more strongly after 14 h root application. DHE is known to be relatively specific for superoxide anion, O_2^- (Becker et al., 1999). Becker et al. (1999) reported that, whereas O_2^- readily oxidized DHE in concentrations below 100 μM, H_2O_2 had almost no effect in concentrations in excess of 1 mM. In addition, no DHE oxidation was shown by application of superoxide dismutase (SOD, known to generate approximately equimolar H_2O_2). Therefore, it can be considered that the quinclorac-induced strong increase in the fluorescence is mainly attributable to O_2^- generation. To our knowledge, this is the first report showing that quinclorac **(1)** stimulates O_2^- generation in the roots of a susceptible grass species. Excess ROS formation is known to induce serious disruption of normal metabolism through oxidative damage to lipids, protein, and nucleic acids (Fridovich, 1986a; Davies, 1987; Imlay and Linn, 1988). As previously reported (Sunohara and Matsumoto, 2004), quinclorac **(1)** induced membrane lipid peroxidation in susceptible early watergrass shoots, suggesting that the herbicide caused overproduction of ROS in shoots. Moreover, there was a positive correlation between constitutive antioxidative enzyme activities and quinclorac **(1)** sensitivity (Sunohara and Matsumoto, 2004). In maize seedlings, constitutive levels of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbic acid peroxidase (APX, EC 1.11.1.11), and glutathione reductase (GR, EC 1.6.4.2) were significantly lower than those in tolerant rice, and slightly higher than those in more susceptible *Echinochloa* spp. Although these antioxidant enzymes play protective roles against ROS, the antioxidative ability of maize may not be sufficient to eliminate quinclorac-induced ROS. In particular, the inherent activity of SOD, a major scavenger of O_2^-, in maize was much lower (about 14-fold) than in rice (Sunohara and Matsumoto, 2004), suggesting a low scavenging ability for quinclorac-induced O_2^- overproduction. However, the enzyme activity was measured in total extract in the previous study (Sunohara and Matsumoto, 2004). It is well known that SODs are distributed in different cell compartments, mainly chloroplasts, cytosol, mitochondria, and peroxisomes (Fridovich, 1986b; del Río et al., 1992; Bowler et al., 1994). Therefore, more detail investigation between quinclorac **(1)** sensitivity and the compartment-specific responses or the enzyme activities in the compartments may be useful to clarify the mechanism of the ROS production.

2.4. Effects of quinclorac **(1)** and 2,4-D **(2)** on ethylene production and cyanide accumulation in maize roots

Ethylene is a plant hormone known to regulate many important events in plant growth and development (Yip and Yang, 1998). The biosynthesis of ethylene has been well-characterized at the biochemical level. The first dedicated step in ethylene synthesis is the production of 1-aminocyclopropane-1-carboxylic acid (ACC)

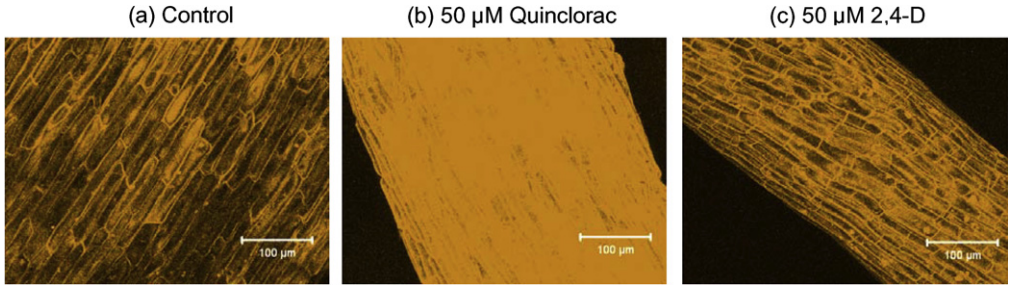

Fig. 5. Effects of (a) quinclorac **(1)** and (b) 2,4-D **(2)** on ROS production in maize roots 0 h after 14 h root treatment. Orange fluorescence shows ROS production (presumably O_2^-). Scale bars indicate 100 μm. Fluorescence images were obtained using a LSM 510 confocal laser scanning microscope with filter set No. 9 (Carl Zeiss; excitation 488 nm, emission ⩾ 505 nm).

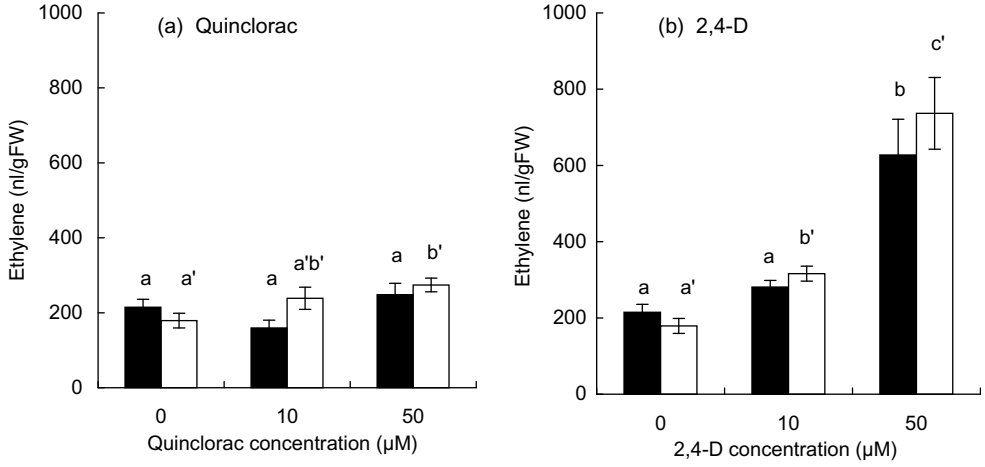

Fig. 6. Effect of (a) quinclorac **(1)** and (b) 2,4-D **(2)** on ethylene production in maize root segments soaked with the test solution for 24 (black bar) or 48 h (white bar) in the dark. Bars with the same letter are not significantly different, $P \geqslant 0.05$.

via ACC synthase (ACS; EC 4.4.1.14), and this reaction is an important regulatory step in ethylene synthesis (Yang and Hoffman, 1984). Auxins are known to induce ethylene production by activating ACS in many plant tissues. The subsequent oxidation of ACC by ACC oxidase (ACO; EC 1.14.17.4) produces ethylene and cyanide, and cyanide is produced in stoichiometrically equal amounts to ethylene (Peiser et al., 1984). This process is the main source of cyanide in the tissues of many plants (Goudey et al., 1989). At high concentrations (5–20 μM), cyanide is toxic to enzymes in many important metabolic pathways (Solomanson, 1981) and phytotoxic effects of quinclorac **(1)** on several susceptible grasses have been considered to be due to the induction of ACC-dependent cyanide (Grossmann and Kwiatkowski, 1995; Abdallah et al., 2006).

Fig. 6 shows the effects of quinclorac **(1)** and 2,4-D **(2)** on ethylene production in maize root segments that were soaked in the test solution for 24 or 48 h in the dark. Quinclorac **(1)** (10 and 50 μM) did not enhance ethylene production in maize roots for 24 h treatment, while 50 μM quinclorac **(1)** slightly enhanced production for a 48 h treatment. On the other hand, 50 μM 2,4-D **(2)** markedly enhanced the ethylene production for 24 or 48 h treatment, and the amount of ethylene synthesized was approximately three times higher than that by the 10 and 50 μM quinclorac **(1)** treatments. Cyanide formation, in both 50 μM quinclorac (1) or 2,4-D **(2)**, was not affected in maize roots at 0 h after 14 h root treatment, but both treatments significantly induced cyanide accumulation 48 h after treatment (Fig. 7a and b). However, the amount of cyanide accumulated in the roots after 2,4-D **(2)** treatment was much higher than that after quinclorac **(1)** treatment (Fig. 7b). In this study, 2,4-D **(2)** showed no inhibitory effect on shoot growth, but induced root growth suppression in maize seedlings (Fig. 2b). In maize roots, 2,4-D **(2)** remarkably enhanced ethylene production (Figs. 6b and 8c) and cyanide accumulation (Fig. 7). Therefore, 2,4-D-induced root growth inhibition may be due to the excessive production of ethylene and the subsequent accumulation of cyanide.

To investigate the relationships between cell death, lipid peroxidation, and ethylene biosynthesis in quinclorac- and 2,4-D-treated maize roots in more details, measurements were made using the same roots after herbicide treatment. Fig. 8 shows cell death (a), ethane (b), an index of lipid peroxidation, and ethylene production (c) in 50 μM quinclorac- and 2,4-D-treated maize root segments that were soaked for 24 h in the dark. In 50 μM quinclorac-treated

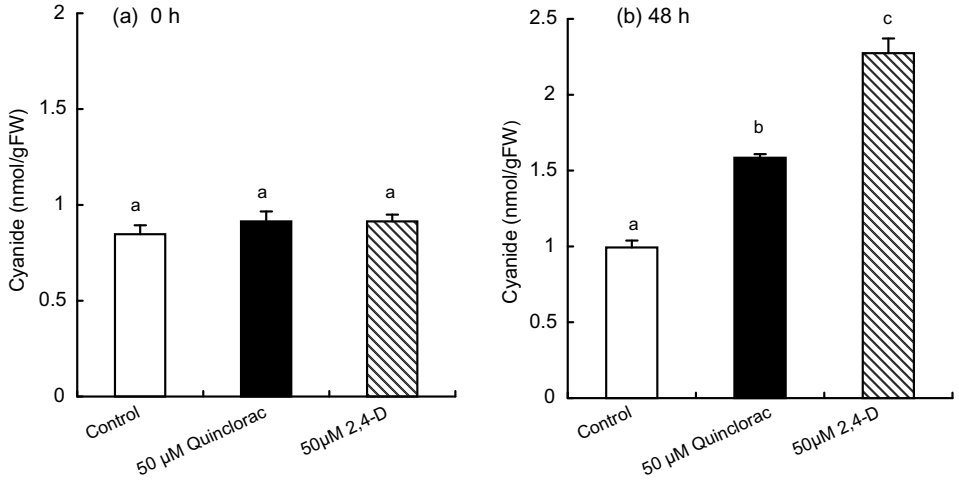

Fig. 7. Effects of 50 μM quinclorac **(1)** and 2,4-D **(2)** on cyanide formation in maize roots (a) 0 h or (b) 48 h after 14 h root treatment. Bars with the same letter are not significantly different, $P \geqslant 0.05$.

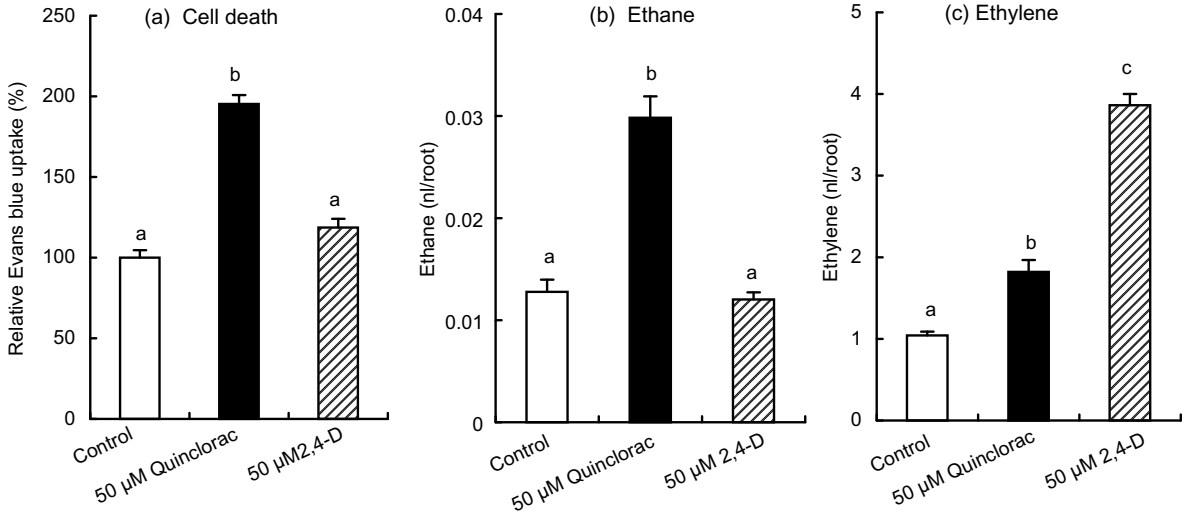

Fig. 8. Effects of quinclorac **(1)** and 2,4-D **(2)** on (a) cell death, (b) ethane evolution, and (c) ethylene production in maize root segments soaked with the test solution for 24 h in the dark. Maize root segments of 15 mm were obtained by cutting from root tip. Bars with the same letter are not significantly different, $P \geqslant 0.05$.

maize roots, cell death and ethane evolution were enhanced markedly, and ethylene production was also induced; however, the amount of ethylene was much lower than that in 50 μM 2,4-D-treated maize roots. On the other hand, in 50 μM-2,4-D-treated maize roots, cell death and ethane were not enhanced but only ethylene production was markedly induced. These results imply that quinclorac-induced ethylene did not enhance cell death or lipid peroxidation in maize roots, because 2,4-D **(2)** induced formation of more ethylene than quinclorac but did not enhance cell death and lipid peroxidation. Moreover, because cyanide is produced in stoichiometrically equal amounts to ethylene in its biosynthetic pathway (Peiser et al., 1984) and there were linear relationships between cyanide content and ethylene production both for quinclorac ($P < 0.05$) and 2,4-D ($P < 0.01$) in maize in this study, quinclorac-induced cell death or lipid peroxidation in maize roots can not be explained by cyanide. These results suggest that ethylene biosynthesis-related products including ethylene and cyanide are not primary factors for quinclorac-induced cell death in maize roots. Quinclorac-induced cell death in maize roots in darkness is likely to be caused by ROS and the oxidative damage, such as lipid peroxidation. There are many potential sources of ROS in plants, including intracellular sources such as chloroplasts, mitochondria and peroxisomes, plasma membrane NADPH oxidase, cell wall peroxidase, and apoplastic oxalate oxidase and amine oxidases (Grant and Loake, 2000; Mittler, 2002; Neill et al., 2002; Vranová et al., 2002). Recently, Grossmann et al. (2001) found that auxinic herbicides including quinclorac **(1)** and quinmerac (7-chloro-3-methylquinoline-8-carboxylic acid) stimulated H_2O_2 generation in the shoots of cleavers (*Galium aparine* L.), a susceptible dicot species, and proposed that the decline of photosynthetic activity, due to abscisic acid (ABA)-mediated stomatal closure, leads to an overproduction of ROS. This effect seems to be involved in the induction of tissue damage and cell death in cleavers leaves by auxinic herbicides including quinclorac **(1)**. However, in our present study, quinclorac-induced O_2^- overproduction in roots (Fig. 5) strongly suggesting the existence of another phytotoxic action mechanism unrelated to the decline of photosynthetic activity. Cyanide is well-known harmful ion which forms a very stable complex with the active site metal (e.g., Fe and Mg) in enzymes, thereby inhibiting vital functions in cells such as respiration, carbon fixation, and nitrate reduction (Yip and Yang, 1998). However, quinclorac-induced cyanide, a co-product of ethylene biosynthesis, is unlikely to link with the herbicide-induced ROS generation in maize roots, because 2,4-D **(2)** induced formation of more cyanide and ethylene than quinclorac **(1)** (Figs. 6, 7 and 8c) but enhanced less O_2^- generation than quinclorac **(1)** (Fig. 5). Further studies are needed to clarify the mechanism of the ROS production.

Grossmann and Kwiatkowski (1995) showed that quinclorac-induced ethylene biosynthesis via the ACC pathway, and cyanide concentrations in the shoot tissue reached a maximum of 40 μM in susceptible barnyard grass. As described above, cyanide is known to be toxic to enzymes in many important metabolic pathways at high concentrations of 20 μM or more (Solomanson, 1981). Exogenously applied cyanide caused phytotoxic symptoms in shoots similar to those induced by quinclorac **(1)** (Grossmann and Kwiatkowski, 1995; Abdallah et al., 2006). In addition, the addition of an ethylene biosynthesis inhibitor decreased the quinclorac **(1)** phytotoxicity in sensitive grasses such as *Echinochloa* spp. and *Digitaria ischaemum* (Lopez-Martinez et al., 1998; Abdallah et al., 2006). Abdallah et al. (2006) also showed an evident relationship between cellular cyanide content and quinclorac **(1)** phytotoxicity, suggesting that quinclorac **(1)** toxicity to *Digitaria ischaemum* is mainly caused by an accumulation of cyanide in shoot tissues. From these reports, the phytotoxic effect of quinclorac **(1)** on the susceptible grasses is probably via the induction of ACC-dependent cyanide. Concerning the inter-specific difference in susceptibility to quinclorac **(1)**, the selective induction of ACC synthase activity, which ultimately leads to cyanide accumulation in the tissues of susceptible grasses, is considered to be the primary mechanism underlying selective action of quinclorac **(1)** (Grossmann and Kwiatkowski, 2000). Grossmann and Kwiatkowski (1993, 2000) also indicated lower β-cyanoalanine synthase (EC 4.4.1.9) activity, a major HCN-detoxifying enzyme, in quinclorac-susceptible barnyard grass than in quinclorac-tolerant rice (*Oryza sativa*); barnyard grass accumulated more cyanide and suffered more damage after treatment with KCN. From these studies, the capacity to metabolize HCN seems to differ considerably among plant species, and the capacity may influence the degree of phytotoxicity of quinclorac **(1)** in susceptible glass species. However, our present study suggests that ROS is more important for quinclorac-induced cell death in maize roots than cyanide. Maize might have a higher β-cyanoalanine synthase activity, and be more easily affected by ROS than cyanide due to the high cyanide detoxification capacity. In other words, the difference of the capacity for cyanide detoxification might determine which factor, cyanide or ROS, is primarily responsible for quinclorac-induced phytotoxicity in susceptible grass species.

3. Concluding remarks

From the results presented above, we conclude that the primary factor for quinclorac-induced cell death in maize roots may be ROS and the subsequent oxidative damage, such as lipid peroxidation. Ethylene biosynthesis pathway-related substances including cyanide are probably not the causative factor in maize roots. To our knowledge, this is the first report that ROS may be relatively important for quinclorac-induced phytotoxicity in a grass species. It should be further examined if the primary mode of action of quinclorac **(1)** differ between plant species depending the capacity of cyanide detoxification.

4. Experimental

4.1. Plant materials

Germinated seeds of rice (*Oryza sativa* L. cv. Nipponbare), maize (*Zea mays* L. cv. Honey Bantam), and early watergrass (*Echinochloa oryzicola* Vasing.), were grown hydroponically in Kasugai nutrient solution (Ohta et al., 1970) in a growth chamber at 25/20 °C with 12 h of light (250 μmol m^{-2} s^{-1}) per day. Seedlings of rice and early watergrass at the 2.5–3.5 leaf stage, and maize at the 1.5–3.0 leaf stage were used for all experiments.

4.2. Herbicide treatment and growth measurement

Roots of intact seedlings of each species were immersed in 0.1, 1, 10, or 50 μM quinclorac **(1)** (Wako Pure Chemical Indus., Osaka, Japan) or 2,4-dichlorophenoxyacetic acid, 2,4-D **(2)** solution containing 0.4% acetone and 0.1% DMSO at 25 °C for 14 h in the dark. Roots of untreated control seedlings were immersed in distilled H_2O containing 0.4% acetone and 0.1% DMSO. After herbicide treatment, roots were washed with distilled H_2O and transplanted to herbicide-free nutrient solution under continuous light (250 μmol m^{-2} s^{-1}) or darkness for 3 or 4 days. After treatment, seedlings were washed with distilled H_2O and then fresh weights (FW) of the shoots and roots were measured.

4.3. Determination of cell viability

Root cell viability was evaluated by staining with fluorescein diacetate and propidium iodide (Pan et al., 2001). Root segments

(1.0 cm length from the tip) were excised from the intact maize seedlings treated with 0, 10, or 50 μM quinclorac **(1)** or 2,4-D **(2)** for 14 h, respectively. The roots were stained with a mixture of 12.5 μg/ml FDA (Aldrich Chemical Co., Milwaukee, USA) and 5 μg/ml PI for 10 min, and then washed with distilled H_2O. The fluorescence images of the stained roots were observed using a fluorescence microscope (Nikon E600 with a B-2A filter (excitation 450–490 nm, emission ≥ 520 nm); Nikon Corp., Tokyo, Japan).

Root cell viability was also evaluated by Evans blue staining (Tamás et al., 2004). The intact maize seedlings were treated with either 0 or 50 μM quinclorac **(1)** or 2,4-D **(2)** for 14 h. After the roots were washed with distilled H_2O, root segments (1.5 cm length from the tip) were taken from the treated maize seedlings. The root segments were stained with a 0.25% (w/v) aqueous solution of Evans blue (Sigma Chemical Co., St. Louis, USA) for 1 h at room temperature. The stained roots were then washed with distilled H_2O for 15 min. The Evans blue that was taken into the maize root segments was then extracted using *N,N*-dimethylformamide solution (200 μl) without grinding. After the root tips were allowed to soak in the *N,N*-dimethylformamide solution overnight 30 °C in the dark, absorbance of the released Evans blue was measured at 600 nm spectrophotometrically.

4.4. Detection of ROS

ROS production was estimated by using a superoxide anion (O_2^-)-specific indicator, DHE, according to the procedures of Yamamoto et al. (2002) with minor modification. Root segments (1.5 cm length from the tip) were excised from the intact maize seedlings treated with 0, 10, or 50 μM quinclorac **(1)** or 2,4-D **(2)** for 14 h, and then stained with 10 μM dihydroethidium in 100 μM $CaCl_2$, pH 4.75, by shaking gently for 30 min at room temperature in the dark. After soaking in 100 μM $CaCl_2$, for 5 min to remove the residual dye, roots were observed using a confocal laser scanning microscope (model LSM510 with filter set No. 9 (excitation 488 nm, emission ⩾ 505 nm); Carl Zeiss, Jena, Germany). Fluorescence of ethidium derived from DHE oxidation by ROS (presumably O_2^-) was observed under the microscope.

4.5. Ethylene production

Ethylene production was quantified as described previously (Sunohara and Ikeda, 2003; Sunohara and Matsumoto, 2004; Sunohara et al., 2002). Ten root segments (1.5 cm length from the tip) were excised from intact maize seedlings at the 1.5–2.0 leaf stage, and then transferred to a 5 ml glass vial containing 500 μl of 0, 10, or 50 μM quinclorac **(1)** or 2,4-D **(2)** solution, respectively. The glass vials were sealed tightly with a rubber plug and an aluminum stopper using a crimper, and incubated at 27 °C for 24 h in the dark. After incubation, gas samples (1 ml) were taken from each glass vial with a syringe. The ethylene content in the vial was quantified using a gas chromatograph (GC-17A; Shimadzu Corp., Kyoto, Japan) equipped with a glass column packed with Unipak S (GL Sciences Inc., Tokyo, Japan). The chromatograph oven temperature, injection temperature, and detector temperature were maintained at 60, 120, and 120 °C, respectively. N_2 carrier gas was applied at 50 ml min^{-1}, and H_2 gas and air pressure were 60 and 50 kPa, respectively. The ethylene retention time was 0.98–1.0 min under these conditions. The ethylene concentration was calculated from a calibration curve using standard ethylene.

4.6. Cyanide content

The cyanide content in tissue samples was measured using the methods of Grossmann et al. (2001) with some modifications. Root samples were taken from the intact maize seedlings treated with either 0 or 50 μM quinclorac **(1)** or 2,4-D **(2)** 0 h or 48 h after 14 h root treatment. Root material (approximately 1.5 g FW) was ground to a fine powder in liquid N_2 and transferred to a glass vial (30 ml). A disc of filter paper (Cat No. 1851-032, QMA; Whatman Japan K.K., Tokyo, Japan) was used to cover the top of the glass vial, and then 1.5 N NaOH (250 μl) was applied to the disc. Then, of 5% H_2SO_4 (2.5 ml) was injected onto the root material through the disc, and immediately screwed a lid onto the vial. The vial was incubated at 22 °C for 5 h to allow evolved HCN be trapped by the filter paper disc. The filter paper disc was then eluted with 0.1 N NaOH for 1 h and cyanide content was determined colorimetrically by a modified method of Lambert et al. (1975). Acidification of the eluted cyanide sample (200 μl) with acetic acid (1 M) (100 μl). One ml of succinimide-*N*-chlorosuccinimide, made up in the proportion 2.5 g succinimide to 0.25 g *N*-chlorosuccinimide per liter of distilled H_2O was then added. Finally, the barbituric acid-pyridine reagent (200 μl) was added and the mixture was allowed to incubate for 10 min. The absorbance at 580 nm was recorded by a spectrophotometer. The recovery of this assay was 86.4%. Four replications were assayed for cyanide content.

4.7. Lipid peroxidation

Lipid peroxidation was estimated by measuring the formation of ethane, which is a product of lipid peroxide decomposition. Ethane evolution from maize roots was quantified in the same way as ethylene measurement. The ethane retention time was 0.70–0.72 min, and the ethane concentration was calculated from a calibration curve using standard ethane.

4.8. Relationship of cell death to lipid peroxidation and ethylene production

Twenty root segments (1.5 cm length from the tip) were excised from intact maize seedlings at the 1.5–2.0 leaf stage, and then transferred to a glass vial (5 ml) containing either 500 μl of 0 or 50 μM quinclorac **(1)** or 2,4-D **(2)** solution. The glass vials were incubated for 24 h at 27 °C in the dark, and then the ethylene or ethane content in the vial was determined. After the ethylene and ethane measurements, root cell viability of the root segments in the each vial was determined by Evans blue staining.

4.9. Statistical analyses

All results were represented as the means ± S.E. of at least three replicates, and all experiments were repeated at least once. For statistical analyses, relationships were considered to be significant when $P < 0.05$. Relationships between either quinclorac **(1)** or 2,4-D **(2)** concentration and shoot or root growth were analyzed by linear regression analysis. The effects of quinclorac **(1)** and 2,4-D **(2)** on the Evans blue uptake, ethylene production, cyanide content, and ethane formation in maize roots were analyzed using Tukey's HSD test.

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