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Effect of Oryzalin on the Gravitropic Response and Ethylene Production in Maize Roots

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Oryzalin is a dinitroaniline herbicide, which disrupts the arrangement of microtubules. Microtubules and microfilaments are cytoskeletal components that are thought to play a role in the sedimentation of statoliths and the formation of cell walls. Statoliths regulate the perception of gravity by columella cells in the root tip. To determine the effect of oryzalin on the gravitropic response, ethylene production in primary roots of maize was investigated. Treatment with 10^{-4} M oryzalin to the root tip inhibited the growth and gravitropic response of the roots. However, the treatment had no effect on the elongation zone of the roots. An application of 10^{-4} M oryzalin for 15 hr to the root tip caused root tip swelling. The application of 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, to the root tip also inhibited the gravitropic response. To understand the role of oryzalin in the regulation of the growth and gravitropic response of roots, ethylene production in the primary roots of maize was measured following treatment with oryzalin. Oryzalin stimulated ethylene production via the activation of ACC oxidase (ACO) and ACC synthase (ACS), and it increased the expression of *ACO* and *ACS* genes. Indole-3-acetic acid (IAA) played a key role in the asymmetric elongation rates observed during gravitropism. The results suggest that oryzalin alters the gravitropic response of maize roots through modification of the arrangement of microtubules. This might reduce the distribution of IAA in the upper and lower sides of the elongation zone and increase ethylene production, thereby inhibiting growth and gravitropic responses.

Key words : ACC, ethylene, gravitropic response, microtubule, oryzalin

Introduction

It has been known that oryzalin is one of the dinitroaniline families, disrupting the microtubule arrangement by binding with tubulin, and acting in herbicide in plants [9, 15]. Microtubule, one of the cytoskeleton, plays important roles in the development of plant, the formation of cell wall, and the sedimentation of amyloplast in the columella cell of root tip [4]. The sedimentation of amyloplast in columella cell of the root tip is correlated with the sensing of gravity, resulting in the downward growth into the soil permits absorption of water and minerals [3]. The orientation of microtubule is changed according to the level of ethylene in plant cells [21].

Ethylene, a gaseous plant hormone, is one of the stress hormones and participates in various plant development and differentiation reactions including seed germination, fruit ripening and senescence [1]. Ethylene synthesis begins from methionine via two major intermediates, S-adenosylmethionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC), in sequence. The enzyme of ACC synthase (ACS) and ACC oxidase (ACO) regulate the steps from AdoMet to ACC and from ACC to ethylene, respectively. Several factors regulate these two enzymes, especially auxin which stimulates the ethylene production through increasing the expression level of the ACS gene [21]. There are findings that ethylene regulates the root growth and gravitropism via alteration of auxin transport [6, 13], and ethylene is required for root penetration in soil with auxin [14].

In this research, we examine the effect of oryzalin on the gravitropic response since microtubule is involved in sedimentation of amyloplast, which move downward of the cell

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according to the gravity. According to Schwuchow and Sack [16], amiprophos-methyl and cytochalasin D, inhibitors of plant microtubule polymerization, increase the plastid sedimentation in the protonema of Ceratodon. Blancaflor and Masson [3] suggested that the sedimentation of amyloplast is a key role of sensing the gravity, and the polymerization of cytoskeleton such as microtubule affects the gravitropism. And ethylene also inhibits the growth of root and gravitropism. However, there are not many evidences of involving the oryzalin and ethylene in the gravitropism. Therefore, we examined the role of oryzalin on the gravitropic response in maize root. An agar block system was used to apply oryzalin to specific regions such as root tip or elongation zone in maize root. Further, we investigated the possibility that oryzalin plays a role in gravitropic response via ethylene production.

Material and Methods

Plant material

Maize seeds (*Zea mays* L., Golden × Bantam 70) were soaked in aerated tap water for 10 hr and then placed on wet paper towels held between opaque plastics trays in a vertical position. The trays were kept in the incubator at 27℃. The seedlings were used when the primary roots were approximately 1.5 cm long (40 hr after planting).

Measurement of ethylene production

Ethylene production was measured in 10 mm root segments excised from the tips of maize roots. The root tips were placed in 10 ml, silicon-capped vials containing 1 ml of potassium phosphate buffer (50 mM, pH 6.8) containing the test compounds. The vials were shaken in the dark at 27℃ in an incubator. After incubation, 1 ml of gas sample was withdrawn from the vial with a syringe and injected to the gas chromatograph (HP5890 Series II; Hewlett-Packard, USA) equipped with an alumina column (80/100 Porapak-Q; 1.8-m x 2.1-mm).

Determination of root growth and gravitropic curvature

Chemicals to be tested were incorporated into agar blocks (0.6% agar; 1 mm^3). The agar blocks were applied to the root tip and the seedling oriented horizontal or vertical in a chamber with near saturating humidity (>98%). Growth rate and curvature were monitored using a camera (Rexsa, DS-400 PC-camera) and time-interval software (SupervisionCam ver. 3.2.2.4; http://supervisioncam.com). Images were recorded at 15 min intervals. The images were analyzed using UTHSCSA Image Tool Program (ver. 3.0; http://comdent.uthscsa.edu/dig/itdes.html).

Assay of *in vivo* ACC oxidase (ACO) activity

The analysis of ACO activity was performed *in vivo* as described by Wang and Woodson [18]. Root segments were incubated in a 50 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM aminoethoxyvinylglycine (AVG). After 1 hr incubation, the root segments were washed with distilled water, and infiltrated for 2 hr with 1 mM ACC at 27℃ in the dark. After the infiltration of ACC, roots are completely washed with distilled water, and ethylene production was measured using the prior procedure in potassium phosphate buffer, without ACC or other chemicals. The ethylene production is regarded as *in vivo* ACO activity.

Assay of *in vitro* ACC synthase (ACS) activity

ACS activity was determined by the modified method of Woester et al [19]. After incubation, root segments were ground on ice with potassium phosphate buffer (pH 8.0) containing 10 μM pyridoxal phosphate, 1 mM EDTA, 2 mM PMSF and 5 mM DTT. Samples were centrifuged at 15,000 rpm for 15 min. The supernatant (1 ml) was incubated for 1 hr with 5 mM AdoMet (0.1 ml) at 22℃. Ethylene production measured from this mixture was used for calculating ACS activity, using a blend of 0.1 ml of 20 mm $HgCl_2$ and 0.1 ml of NaOH/NaOCl (saturated NaOH : 5% NaOCl = 1:1 [v/v]) that was incubated on ice for 10 min.

Extraction of total RNA and RT-PCR

Tissue samples were ground into a powder with a mortar and pestle under liquid nitrogen. The powder was suspended with an RNA extraction buffer [0.1 M Tris-HCl (pH 9.0), 0.1 M NaCl, and 1% SDS]. A volume of phenol: chloroform: isoamylalcohol (25:24:1; v:v:v) equal to the volume of the buffer was added and the mixture incubated for 10 min at 50℃. The reaction mixture was centrifuged at 13,000 rpm for 15 min at 4℃; the supernatant was transferred into new tubes and incubated with 2.5 M LiCl for 30 min at -20℃. The mixture was centrifuged for 15 min at 13,000 rpm (4℃); the pellet was washed with 70% ethanol and dissolved in DEPC-treated water. Total RNA was quantified at 260/280 using a microplate reader (Microplate Reader Infinite® 200, Tecan Group Ltd, Morrisville, NC, USA).

Table 1. The gene specific primers used for RT-PCR experiments

Gene (accession no.)	Forward (5′ to 3′)	Reverse (5′ to 3′)
ZmACO20 (NM_001111765)	TGGTGAACATGGAGAAGCTG	ATCACTTGCCGGTAGTGGTC
ZmACS7 (NM_001152929)	GTTGGACCTGATCGAGCAAT	AGCTTCACTCCTGACCTCCA
GAPDH	CTGGTTTCTACCGACTTCCTTC	CGGCATACACAAGCAGCAAC

ZmACO20 and *ZmACS7* are primers for the ACC oxidase and ACC synthase gene from maize (*Zea mays* L.), respectively.

The purified total RNA was used for first-strand complementary DNA synthesis in a AccuPower® RT Premix (Bioneer, Korea). Polymerase chain reaction (PCR) conditions included 30 cycles of denaturing at 95℃ for 5 min, annealing at 55℃ for 20 s, and extension at 72℃ for 5 min; and, a final elongation step at 72℃ for 10 min. The gene specific primers were used as listed in Table 1. ZmACO20 and ZmACS7 are primers for ACC oxidase and ACC synthase gene in maize, respectively. The gels were analyzed using Gel Image Analysis System (i-MAX-D500, Core, Korea).

Statistical analysis

All experiments were conducted at least three times, with no fewer than 40 primary roots each. To test for significance at p values of <0.05, the data mean values were calculated according to Student's t-test.

Results and Discussion

Effects of oryzalin on the root growth

Agar blocks (0.6% agar; 1 mm^3) containing each concentration of oryzalin provided point application of the compound to the maize roots. To examine the effect of oryzalin on the root growth, the point applications of agar blocks containing oryzalin were made to the elongation zone or tip of root separately. When oryzalin was applied to the root tip using an agar block, root growth was inhibited at 10^{-4} M oryzalin application, whereas it was not inhibited at 10^{-6} M oryzalin (Fig. 1A). The treatment of 10^{-4} M oryzalin inhibited root growth within 2 hr, and finally inhibited 37% of control at 8 hr. However, the application of oryzalin to the elongation zone did not inhibit the root growth at all (Fig. 1B).

The application of oryzalin for 15 hr caused root tip swelling (Fig. 2). This result suggested that oryzalin may cause the accumulation of auxin in the root tip via inhibiting the distribution on the plasma membrane of the auxin efflux carrier such as PIN complex, and oryzalin might reduce the difference of indole-3-acetic acid (IAA) distribution between upper and lower side of root resulting in the inhibition of gravitropic response. The gravity signal is passed from root cap to the elongation zone where asymmetric auxin distribution is established which induces curvature as a result of differential growth in horizontally-oriented root [2]. To examine the possibility of the role of oryzalin in the gravi-

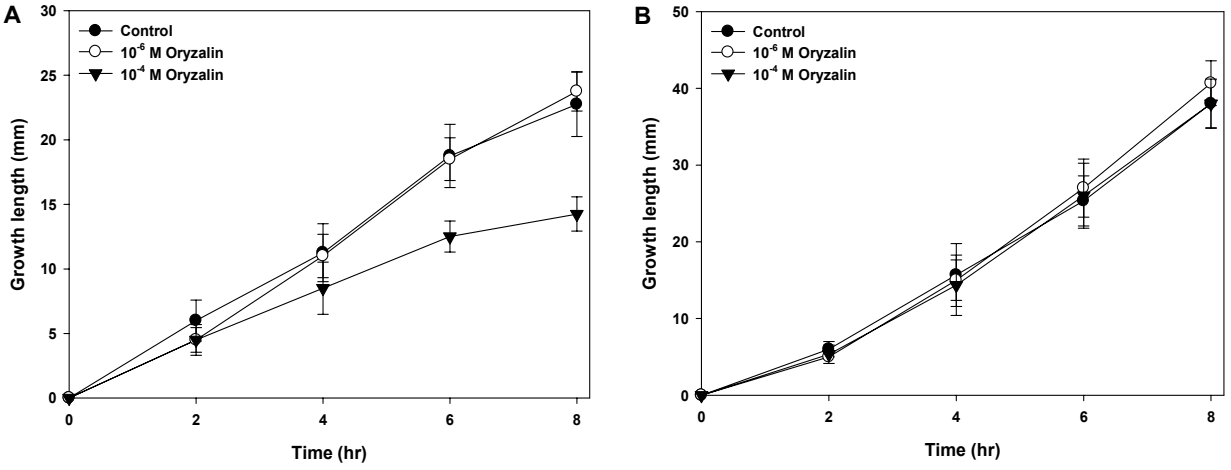

Fig. 1. Effect of oryzalin on root growth in the root tip (A) and elongation zone (B) for 8 hr. Oryzalin was applied to the root tip or elongation zone using an agar block (0.6%, 1 mm^3). The growth was measured for 8 hr after treatment. Bars are mean values ± SE from 4 independent experiments.

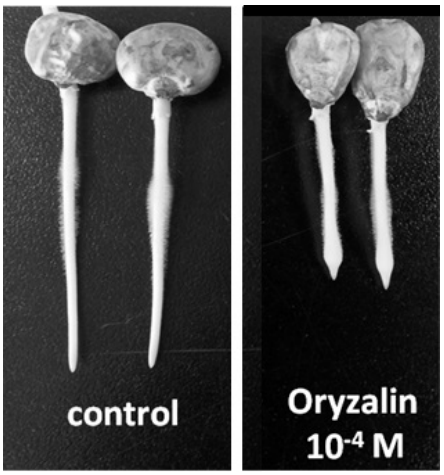

Fig. 2. Morphology of vertically-grown roots treated for 15 hr with 10^{-4} M oryzalin. Swelling in oryzalin-treated roots occurred in the root tip.

tropic response, we applied the oryzalin to the root tip which may affect the IAA redistribution in the elongation zone when roots perceive gravity.

Effects of oryzalin on the gravitropic response

Application of oryzalin to the tip region inhibited the gravitropic response at concentrations of 10^{-6} M and 10^{-4} M after 4 hr while inhibiting root growth (Fig. 3A). Especially, the gravitropic response was inhibited 17% and 42% of control by the treatment of 10^{-6} M and 10^{-4} M oryzalin respectively. However, the treatment of oryzalin to the elongation zone did not affect the gravitropic response (Fig. 3B). According to the Fig. 3B, 10^{-4} M oryzalin inhibited 14% of control in gravitropic response at 4 hr, and this inhibition

Table 2. Effect of oryzalin on the gravitropic response in the 10^{-4} M IAA-pretreated roots at 4 hr and 8 hr

(unit: degree)

	4 hr		8 hr	
	+	-	+	-
control	40±5.93	68±2.94	53±1.96	68±1.52
10^{-4} M oryzalin	28±5.50	43±3.63	32±4.16	46±2.00

(+: pretreated with IAA; - without IAA)

became disappeared at 8 hr. These results showed that both gravitropic response and root growth were inhibited by the application of oryzalin to the root tip rather than to the elongation zone.

Gravitropic response in roots results from differential growth between upper and lower side of roots in horizontal position. The gravity is transduced form the root tip to the elongation zone resulting in differential growth. It has been known that different distribution of IAA cause the differential growth in horizontal root [12]. Roots, unlike stem, are sensitive to IAA, so higher concentration of IAA in the lower side results in inhibition of elongation [11]. Therefore, IAA is one of the major factors to induce the differential elongation during the gravitropism curvature [10]. According to the Fig. 2 and Fig. 3A, oryzalin might reduce the difference of IAA amount between upper and lower side of root.

In order to examine the relationship between oryzalin and IAA, roots were pre-treated with 10^{-4} M IAA solution for 1 hr vertically, and then oryzalin applied to the root tip using an agar block. In Table 2, oryzalin inhibited 30% and 37% of control in gravitropic response in roots pre-treated with IAA and roots without IAA at 4 hr, respectively. At

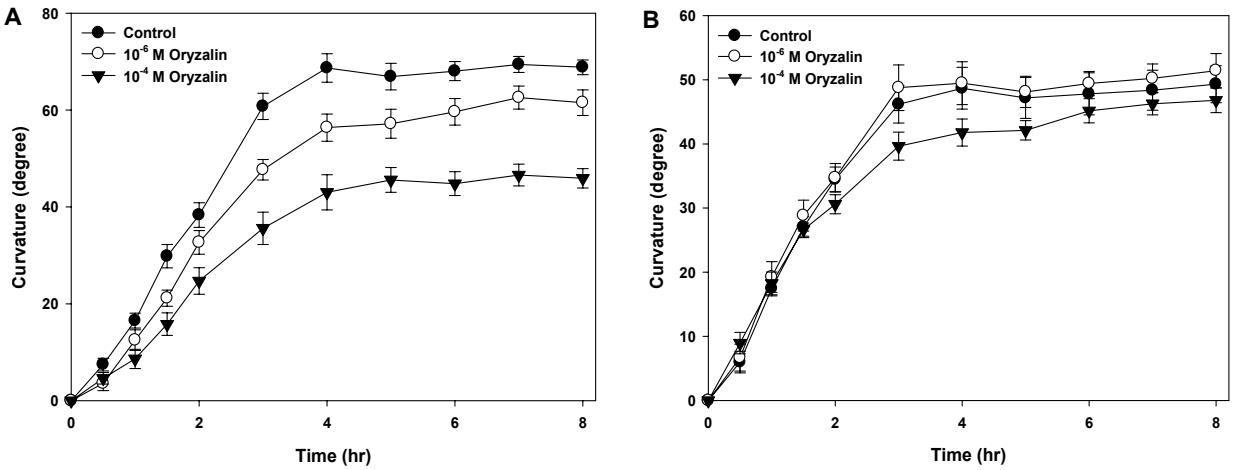

Fig. 3. Effect of oryzalin on the gravitropic response in the root tip (A) and elongation zone (B) for 8 hr. Oryzalin was applied to the root tip or elongation zone using an agar block (0.6%, 1 mm^3). The gravitropic response was measured for 8 hr after treatment. Bars are mean values ± SE from 4 independent experiments.

8 hr, oryzalin showed more inhibition in IAA pre-treated roots such as 40% of control. This result means that oryzalin in the root tip might reduce the difference of IAA amount in the elongation zone between upper and lower side of root, resulting in the inhibition of gravitropic response. This data suggests that oryzalin might play a role on the IAA redistribution in the elongation zone when the root perceived the gravity in the root tip.

It has been reported that ethylene inhibits the elongation of stem and roots [17]. Inhibition of root elongation by IAA can be reversed by the treatment of the tissue with ethylene antagonists [5]. Lee et al. [7] suggested that ethylene may modify positive curvature in the primary roots of maize by affecting gravity induced-lateral auxin transport. Kim et al. [6] suggested that an optimal concentration of ethylene might be required for the regulation of gravitropism in maize roots. Yuan et al. [20] reported that cortical microtubule reoriented in wounding stem in pea from transverse to vertical orientation. It is well known that wounding in plants induce the ethylene production to protect them [1].

To examine the effect of ethylene in the gravitropism in roots, we measured the curvature in application of 1-aminocycloprpane-1-carboxylic acid (ACC), a precursor of ethylene, to the root tip using an agar block (Fig. 4). The curvature was inhibited about 30% of control in roots treated with 10^{-4} M ACC at 4 hr. However, ACC application to the elongation zone did not inhibit the gravitropic curvature (data not shown). And we measured modification of ethylene production in the presence of oryzalin. These data may provide insight into the inhibition of growth and gravicurvature in the elongation zone by oryzalin.

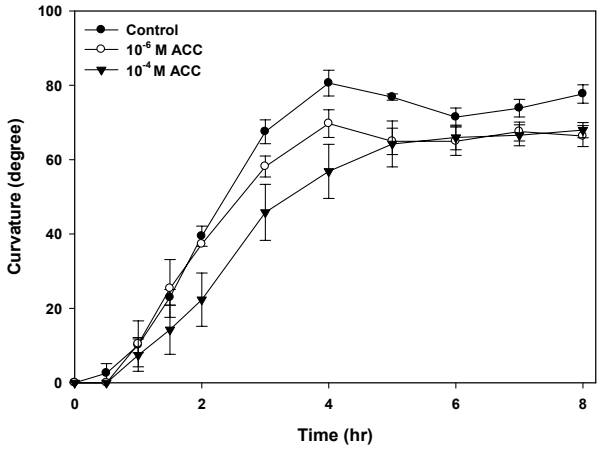

Fig. 4. Effect of ACC on the gravitropic response in the root tip for 8 hr. ACC was applied to the root tip using an agar block (0.6%, 1 mm^3). The gravitropic response was measured for 8 hr after treatment. Bars are mean values ± SE from 4 independent experiments.

Effect of oryzalin on the ethylene production

External application of oryzalin stimulated the ethylene production in the concentration of 10^{-6} M and 10^{-4} M oryzalin for 8 hr (Fig. 5). The treatment of 10^{-4} M oryzalin increased about 60% of control at 8 hr. Typically, ethylene affects the growth pattern of plants by reducing the rate of elongation and increasing lateral expansion, leading to swelling of the tissue and modifying the gravitropic response [1]. Thus, the inhibition of growth and gravicurvature by oryzalin applied to the root tip may be due to the reorientation of microtubule in elongation zone. Additionally, the more inhibition of curvature by in combination with IAA (Table 2) could be related to the modification of gravity-induced lateral auxin transport in the root by ethylene. Ruzicka et al. [13] reported that ethylene stimulated auxin biosynthesis and increased the capacity of auxin transport by regulating the transcription of auxin transport components such as AUX1 and PIN2. The increase in auxin production due to ethylene could alter redistribution of auxin, by polar auxin transport, from root cap to the elongation zone via outer cell layer, to the elongation zone where auxin could inhibit cell elongation. Recently, Ma and Ren [8] reported during the germination of flax, the gravitropic response of the flax root becomes weaker due to a decrease in auxin sensitivity and decrease in auxin transport regulated by ethylene. Therefore, stim-

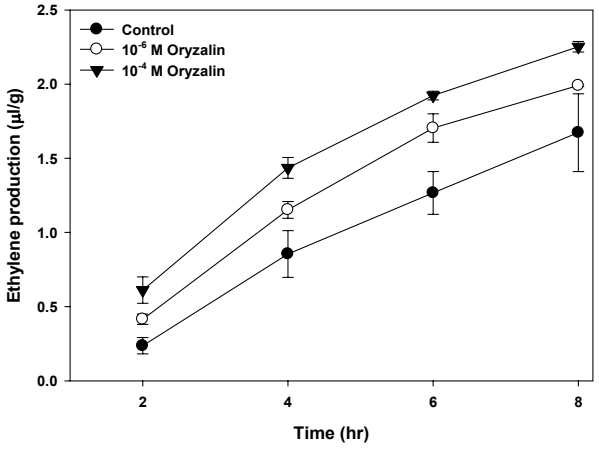

Fig. 5. Effect of oryzalin on the ethylene production in the root segments for 8 hr. Root segments were incubated for 4 hr in solution containing 10^{-6} M and 10^{-4} M oryzalin. At every 2 hr, 1 ml of gas sample was withdrawn from the vials for measuring ethylene production. Symbols are mean values ± SE from 3 independent experiments.

ulation of ethylene production by oryzalin treatment could regulate auxin movement and/or lateral auxin transport in horizontal roots. To confirm the action of oryzalin on ethylene production (Fig. 5), we measured the activity of ACC synthase (ACS) and ACC oxidase (ACO). ACO and ACS activities were increased depending on the oryzalin concentrations at 4 hr in the same pattern as the ethylene production (Fig. 6). Therefore in this system, oryzalin stimulates ethylene production via the activation of ACS and ACO.

To confirm this result, we determined whether oryzalin activates the expression of the *ACO* and *ACS* gene through conducting RT-PCR. The expression level of *ACS* and *ACO* was increased by 10^{-6} M and 10^{-4} M oryzalin, respectively

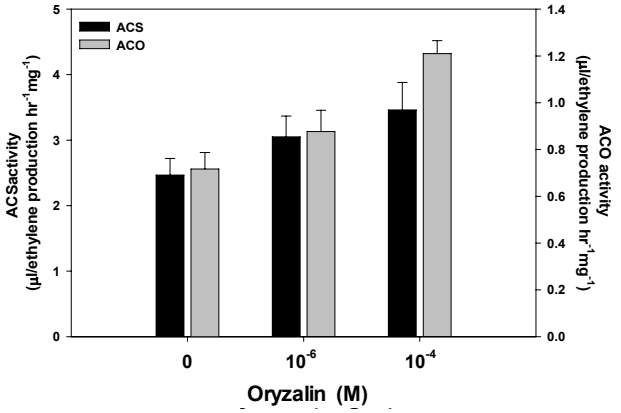

Fig. 6. Effect of oryzalin on *in vitro* ACS and *in vivo* ACO activities. To measure ACS activity, roots were incubated for 4 hr in solution containing 0.1 mM AVG with 10^{-6} M and 10^{-4} M oryzalin. To measure ACO activity, roots were incubated with oryzalin for 4 hr. ACS and ACO activities were measured as described in Material and Methods. Symbols are mean values ± SE from 3 independent experiments.

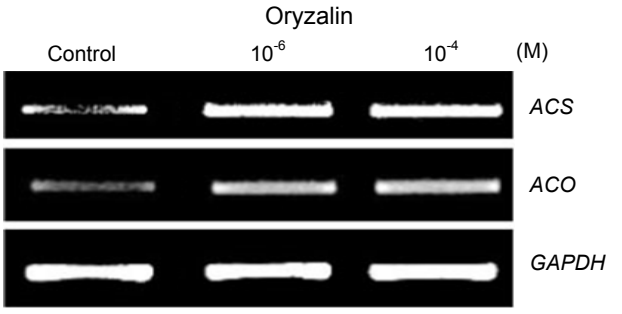

Fig. 7. Effect of oryzalin on gene expression of *ACO* and *ACS*. Root segments were incubated for 4 hr in solution containing 10^{-6} M and 10^{-4} M oryzalin. Total RNAs were extracted and used for RT-PCR as described in Material and Methods. Gel image shows results with consistent patterns from 3 independent experiments.

(Fig. 7).

In these studies, oryzalin inhibited both gravitropic curvature and growth of maize roots when oryzalin was applied to the root tip, and increased the ethylene production via activation of ACO and ACS. However, oryzalin applied to the elongation zone of the root did not affect the gravitropic response and root growth at all. These data suggested that oryzalin might play a key role in gravity sensing in the root tip. Ethylene production was stimulated by the treatment of oryzalin via increasing activities of ACS and ACO. The increased ethylene may regulate the orientation of microtubule in elongation zone, resulting in inhibition of root growth. The application of oryzalin to the root tip might regulate the auxin transport from root tip to elongation zone, which reduced the difference of auxin amounts between upper and lower side of elongation zone.

Acknowledgement

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초록 : 옥수수 일차뿌리에서 oryzalin이 굴중성 반응과 에틸렌 생성에 미치는 효과

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Oryzalin은 미세소관을 분열시키는 dinitroaniline계의 제초제이다. 미세소관과 미세섬유는 평형석 침강과 세포벽을 구성하는 세포골격들이다. 평형석은 뿌리 끝에 있는 columella 세포에서 중력 인지 조절을 한다. 본 연구는 oryzalin이 옥수수 일차 뿌리에서 ethylene 생성을 통하여 굴중성 반응에 미치는 영향을 연구하였다. 뿌리 끝 부분에 10^{-4} M oryzalin의 처리는 뿌리 성장과 굴중성 반응을 저해하였으나, 신장대에 처리하게 되면 저해현상은 관찰되지 않았다. 10^{-4} M oryzalin을 뿌리 끝에 15시간 처리하면 뿌리 끝의 생장이 억제되고 둥근 형태로 부풀었다. 에틸렌의 전구물질인 ACC를 뿌리 끝에 처리하여도 굴중성 반응이 억제되었다. Oryzalin의 작용과 에틸렌 생성에 대한 관련성을 연구하기 위하여 oryzalin 처리 후 에틸렌 생성을 측정하였다. Oryzalin 처리에 의해 ACC oxidase와 ACC synthase의 활성이 증가되어 에틸렌 생성이 촉진되었다. Oryzalin은 ACO와 ACS의 유전자의 발현도 증가 시켰다. Indole-3-acetic acid (IAA)는 굴중성 반응 동안 관찰되는 비 대칭적 신장에 중요한 역할을 한다. 이러한 연구 결과는 oryzalin이 뿌리 끝에서 IAA transport를 억제하여 뿌리 신장대의 윗면과 아랫면의 IAA 양의 차이를 감소시키고, 또한 에틸렌 생성을 촉진하며 미세소관의 배열을 방해하여 뿌리 글중성과 생장을 억제할 가능성을 제시하고 있다.