J Chem Ecol (2006) 32: 1101–1109
DOI 10.1007/s10886-006-9041-z

Possible Mechanism of Inhibition of 6-Methoxy-Benzoxazolin-2(3*H*)-One on Germination of Cress (*Lepidium sativum* L.)

Hisashi Kato-Noguchi · Francisco A. Macías

Received: 7 February 2005 / Revised: 9 June 2005 /
Accepted: 1 November 2005 / Published online: 19 May 2006

Abstract 6-Methoxy-benzoxazolin-2(3*H*)-one (MBOA) inhibited the germination of cress (*Lepidium sativum* L.) seeds at concentrations greater than 0.03 mM. Inhibition was overcome by sucrose, suggesting that MBOA may inhibit sugar metabolism in cress seeds. Induction of α-amylase activity in seeds was also inhibited by MBOA at concentrations greater than 0.03 mM. Inhibition of both germination and induction of α-amylase activity increased with increasing concentrations of MBOA, and the extent of germination correlated positively with the activity of α-amylase in the seeds. MBOA added to a reaction mixture for α-amylase assay did not affect enzyme activity, indicating that MBOA does not inhibit *in vitro* α-amylase activity. Cress seeds germinated approximately 16 hr after incubation, and inhibition of α-amylase by MBOA occurred within 6 hr after incubation. These results suggest that MBOA may inhibit the germination of cress seeds by inhibiting the induction of α-amylase activity, because α-amylase plays a key role in the conversion of reserve carbohydrate into soluble sugars, a prerequisite for seed germination.

Keywords α-Amylase · Allelopathy · Germination inhibitor · *Lepidium sativum* · 6-Methoxy-benzoxazolin-2(3*H*)-one

Introduction

6-Methoxy-benzoxazolin-2(3*H*)-one (MBOA) was first found in *Croix lacryma-jobi* L. (Koyama, 1955) and later in several graminaceous plant species such as wheat, corn,

H. Kato-Noguchi (✉)
Department of Biochemistry and Food Science, Faculty of Agriculture,
Kagawa University, Miki, Kagawa 761-0795, Japan
e-mail: hisashi@ag.kagawa-u.ac.jp

F. A. Macías
Department of Organic Chemistry, Faculty of Science,
University of Cádiz, Apdo 40,
11520 Puerto Real, Spain

and maize (Niemeyer, 1988). Benzoxazolinones, including MBOA and their precursor hydroxamic acids, have been recognized as resistance agents in plants because of their phytotoxic activity on insects (Dowd and Vega, 1996; Bravo and Copaja, 2002), fungi, and bacteria (Frey et al., 1997; Yue et al., 1998; Glenn et al., 2002).

MBOA and related compounds have also attracted attention because of their allelopathic effects (Barnes and Putnam, 1987; Inderjit and Duke, 2003; Belz and Hurle, 2004). MBOA inhibits the germination and growth of several plant species (Pérez, 1990; Hayashi et al., 1994; Kato-Noguchi et al., 1998; Kato-Noguchi, 2000). At concentrations greater than 0.03 mM, MBOA inhibits the germination of *Digitaria sanguinalis* (L.) SCOP, *Lolium multiflorum* LAM, and *Pheleum pretense* L. (Kato-Noguchi, 2000). However, the physiological mechanism of MBOA on germination inhibition is not understood.

This study focused on the mechanism of inhibition of seed germination by MBOA. α-Amylase is considered to be essential for seed germination because this enzyme triggers starch degradation in the endosperm and enables seeds to germinate and grow (Perata et al., 1992, 1997; Vartapetian and Jackson, 1997). It is possible that MBOA inhibits germination because of its inhibition of α-amylase activity in seeds. Thus, the effects of MBOA on germination and α-amylase activity in cress seeds were investigated.

Methods and Materials

Plant Material

Seeds of cress (*Lepidium sativum* L.) were sterilized in a 2% (w/v) solution of sodium hypochlorite for 15 min and rinsed (4 ×'s) in sterile distilled water. All further manipulations were carried out under sterile conditions. MBOA (purchased from Sigma; 0.001, 0.03, 0.1, 0.3, 1, or 3 mM, final concentrations in Tween 20 solution described below) was dissolved in a small volume of methanol and added to two sheets of filter paper (No. 1, Merck) in a 9-cm Petri dish. The solvent was allowed to evaporate in a draft chamber for 1 hr. The filter paper in the Petri dish was then moistened with 4 ml of 0.05% (v/v) aqueous Tween 20. Sucrose (1 mM) was added into the medium in the Petri dish. Fifty seeds of cress were arranged on the filter paper and the Petri dishes were sealed. After incubation in the dark at 25°C for 6–48 hr, the lengths of emerged radicles of cress seeds were measured with a ruler. Seeds were considered to have germinated when their radicles emerged. Experiments were repeated three times, and the mean, standard error (SE), *t*-test value, and regression coefficient were calculated. For determination of α-amylase activity, cress seeds were harvested, frozen immediately in liquid N_2, and freeze-dried.

Pulse Treatment of MBOA

Cress seeds were incubated with 0 or 3 mM MBOA in 0.05% (v/v) aqueous Tween 20 solution in the dark at 25°C for 24 hr as described above. Then, the seeds were rinsed (5 ×'s) with sterile distilled water, transferred onto two sheets of fresh filter paper moistened with fresh Tween 20 solution containing 0 or 3 mM MBOA, and grown under the same conditions for 24 hr.

Extraction and Assay of α-Amylase

Freeze-dried cress seeds (10 seeds for each determination) were ground to a fine powder in a mortar by use of a pestle. The powder was homogenized with 1.5 ml of ice-cold 100 mM HEPES–KOH (pH 7.5) solution containing 1 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM $NaHSO_3$, and 50 mM bovine serum albumin. The homogenate was centrifuged at 30,000 × *g* for 30 min; the supernatant was heated with 3 mM $CaCl_2$ at 75°C for 15 min to inactivate β-amylase and α-glucosidase (Sun and Henson, 1991; Guglielminetti et al., 1995), then used for α-amylase assay.

α-Amylase was assayed by measuring the rate of generation of reducing sugars from soluble starch. Appropriate dilutions of enzyme preparations were made, and 0.2 ml of the diluted enzyme was added to 100 mM sodium acetate (0.5 ml; pH 6.0) containing 10 mM $CaCl_2$. The reaction was initiated with 2.5% (w/v) soluble starch (0.5 ml). After incubation at 37°C for 15 min, the reaction was terminated by adding 40 mM dinitrosalicylic acid (0.5 ml) containing 400 mM NaOH and 1 M K–Na tartrate and then placing immediately into a boiling water bath for 5 min. After dilution with distilled water, the absorbance at 530 nm (A_{530}) of the reaction mixture was measured and reducing power evaluated using a standard curve obtained with glucose (Guglielminetti et al., 1995). Before MBOA was added to the assay, it was first dissolved in differing concentrations of dimethyl sulfoxide, then diluted to 0.001, 0.03, 0.1, 0.3, 1, or 3 mM with the reaction mixture. Experiments were repeated five times, with four assays for each determination.

Results and Discussion

Effects of MBOA on Germination

Radicles of control seeds emerged about 16 hr after sowing. MBOA inhibited germination, with germination completely inhibited by 3 mM MBOA (Fig. 1). When the length of cress radicles was plotted against the logarithm of MBOA concentration, there was a good logistic concentration–response curve (Fig. 2). At concentrations greater than 0.03 mM, MBOA inhibited the growth of cress radicles significantly. The concentration required for 50% inhibition of radicle growth was 0.18 mM (calculated from the regression equation of the concentration–response curve). These results suggest that MBOA inhibited the cress germination process and that the inhibition was increased with increasing MBOA concentrations (Figs. 1

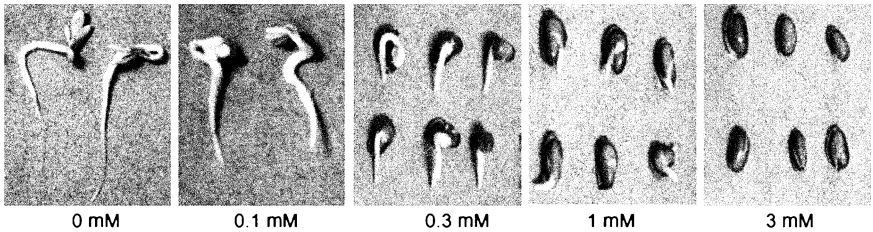

Fig. 1 Effects of MBOA on the germination of cress seeds. Cress seeds were incubated with MBOA in the dark at 25°C for 48 hr

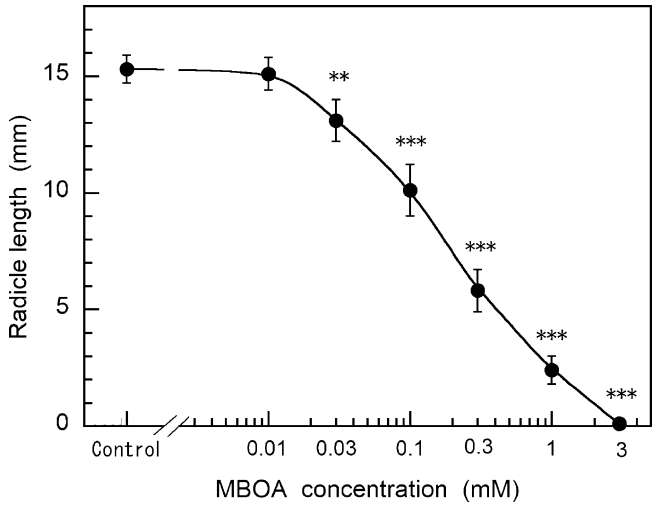

Fig. 2 Effects of MBOA on radicle length of cress seeds. Cress seeds were incubated with MBOA in the dark at 25°C for 48 hr. Means ± SE from three independent experiments with 50 plants for each determination are shown. **Significant at $P < 0.01$, *** Significant at $P < 0.001$ level as compared with the control seedlings

and 2). MBOA also inhibits germination in several other plant species (Pérez, 1990; Kato-Noguchi, 2000), and uptake of MBOA by plant seeds is significantly faster than uptake of its precursor hydroxamic acid, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (Pérez, 1990).

Germination inhibition by MBOA was overcome by addition of sucrose to the medium (Fig. 3), which suggests that inhibition may not be due to a direct

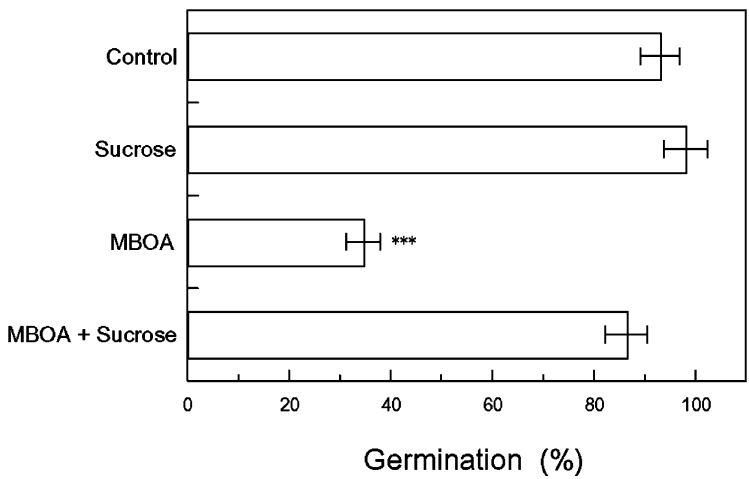

Fig. 3 Effect of sucrose on MBOA-induced inhibition of cress germination. Seedlings were incubated with 0.3 mM MBOA in the presence and absence of 1 mM sucrose in the dark at 25°C for 24 hr. Control seeds were incubated without MBOA and sucrose. Seeds were considered as germinated if their radicles emerged. Other details as for Fig. 2

influence of MBOA molecules themselves. Considering this observation, MBOA may inhibit sugar metabolism in the seeds because soluble sugars such as maltose and sucrose must be produced continuously from reserve starch to maintain the germination process (Ricard et al., 1998; Saglio et al., 1999; Guglielminetti et al., 2000).

Germination was inhibited when cress seeds were transferred into a solution containing 3 mM MBOA from one without MBOA (0 to 3 mM MBOA; Fig. 4). This suggests that MBOA is able to inhibit the germination process even after radicle emergence. In contrast, after the transfer of seeds into a solution without MBOA from one containing 3 mM MBOA, germination was initiated and radicles began to grow (3 to 0 mM MBOA; Fig. 4). This result indicates that 3 mM MBOA did not kill seeds, and that MBOA absorbed by seeds may be metabolized and detoxified in seeds (Inderjit and Duke, 2003; Glenn et al., 2003; Sicker et al., 2003). However, cress seeds did not germinate at concentrations greater than 300 mM, even after transfer into a solution without MBOA (data not shown).

Effects of MBOA on α-Amylase Activity

MBOA inhibited α-amylase activity in cress seeds at concentrations greater than 0.03 mM (Fig. 5). α-Amylase activity could be fitted to a logistic concentration-response curve when plotted against the logarithm of MBOA concentration. The concentration required for 50% inhibition of activity was 0.16 mM. This value was almost the same as the concentration required for 50% inhibition of radicle growth (Fig. 2).

MBOA was added into the α-amylase reaction mixture, but the enzyme, activity was not affected by MBOA in the assay mixture (data not shown), indicating that MBOA does not directly inhibit *in vitro* α-amylase activity. Evaluating α-amylase

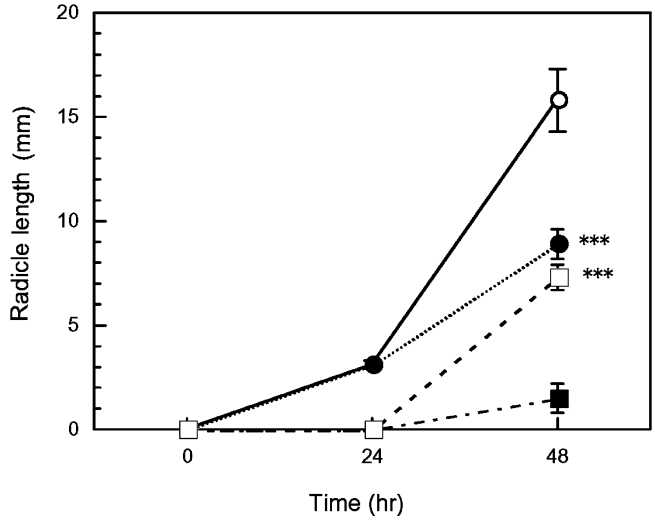

Fig. 4 Effects of pulse treatment of MBOA on cress germination. Cress seeds were incubated with 3 mM MBOA (or 0 mM) in the dark at 25°C for 24 hr, and then the seeds were transferred to medium of 0 mM MBOA (or 3 mM MBOA), and grown for 24 hr. ○, 0 to 0 mM MBOA; ●, 0 to 3 mM MBOA; □, 3 to 0 mM MBOA; ■, 3 to 3 mM MBOA. *** Significant at $P < 0.001$ level as compared between ● and ○, and □ and ■. Other details as for Fig. 2

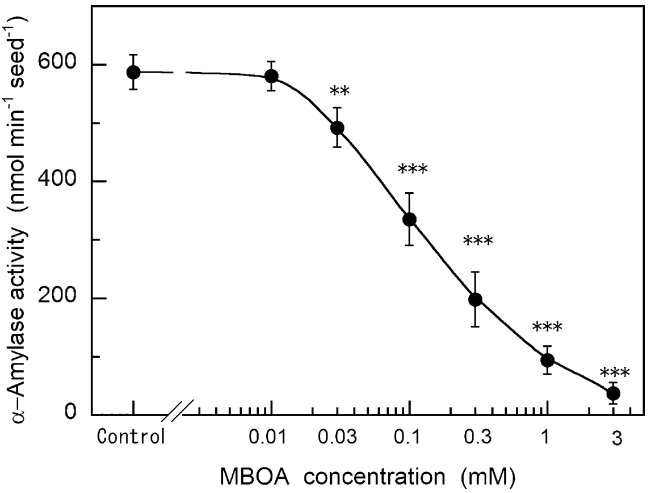

Fig. 5 Effects of MBOA on α-amylase activity in cress seeds. Cress seeds were incubated with MBOA in the dark at 25°C for 48 hr. Means ± SE from five independent experiments with four assays for each determination are shown. Other details as for Fig. 2

induction at the protein level, as influenced by MBOA showed an increase in inhibition with increasing MBOA concentrations. In addition, both concentration–response curves (Figs. 2 and 5) indicate that length of cress radicles is positively correlated with α-amylase activity in seeds.

Figure 6 shows changes in α-amylase activity in cress seeds after sowing. The activity in control seeds (0 mM MBOA) was low at time 0 and increased as germination proceeded. Radicles emerged about 16 hr after sowing. MBOA

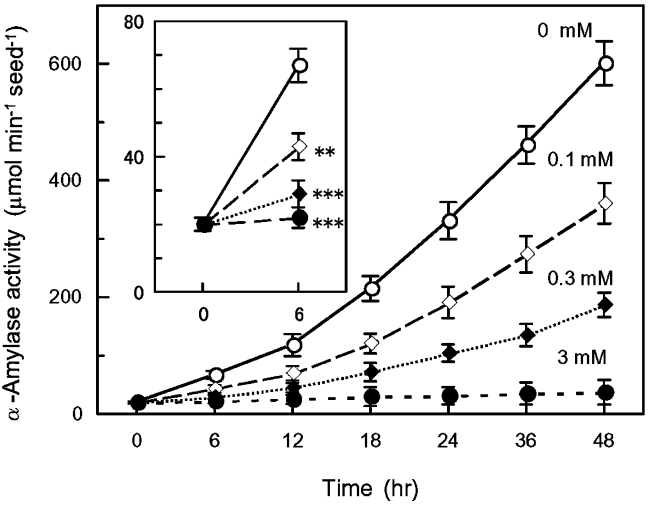

Fig. 6 Changes in α-amylase activity in cress seeds. Cress seeds were incubated with MBOA in the dark at 25°C. Means ± SE from five independent experiments with four assays for each determination are shown. Other details as for Fig. 2

inhibited the induction of α-amylase activity within 6 hr after sowing, and the inhibition was greater with increasing MBOA concentrations. At 48 hr, the activities in seeds treated with 0.1 and 0.3 mM MBOA were 58 and 32% of that in control seeds, respectively, whereas the activity in seeds treated with 3 mM MBOA remained almost unchanged.

Although the α-amylase activity in control cress seeds increased continually (0 to 0 mM MBOA; Fig. 7), the rate of increase was reduced when cress seeds were transferred into 3 mM MBOA from a solution without MBOA (0 to 3 mM MBOA; Fig. 7), suggesting that MBOA may slow down the increase in α-amylase activity even after radicle emergence. In contrast, enzyme activity increased after transfer to 0 mM MBOA from a solution containing 3 mM MBOA (3 to 0 mM MBOA; Fig. 7) and radicles of these seedlings resumed a growth rate similar to that of untreated seedlings (Fig. 4). This suggests that α-amylase activity in cress seeds correlates well with the germination process (Figs. 4 and 7), and the induction of α-amylase may occur after MBOA is metabolized and detoxified, as suggested above.

Starch breakdown during seed germination is triggered by α-amylase that catalyzes the hydrolysis of α-1,4 glucan linkages to produce maltose and larger oligosaccharides (Beck and Ziegler, 1989; Vartapetian and Jackson, 1997). α-Amylase induction in many seeds is regulated by gibberellin at the transcriptional level (Ritchie and Gilroy, 1998). Inhibition of α-amylase activity by MBOA occurred within 6 hr after sowing (Fig. 6). Considering that radicles emerged around 16 hr after sowing, this inhibition may not be too late to inhibit the translation process of α-amylase.

Although it is possible that MBOA inhibits gibberellin biosynthesis, inhibitors of gibberellin biosynthesis do not inhibit the increase in α-amylase production during germination (Groselindemann et al., 1991). During germination, gibberellin precursors stored in seeds are mobilized, and the gibberellin produced triggers α-amylase induction (Groselindemann et al., 1991; Ritchie and Gilroy, 1998). Thus,

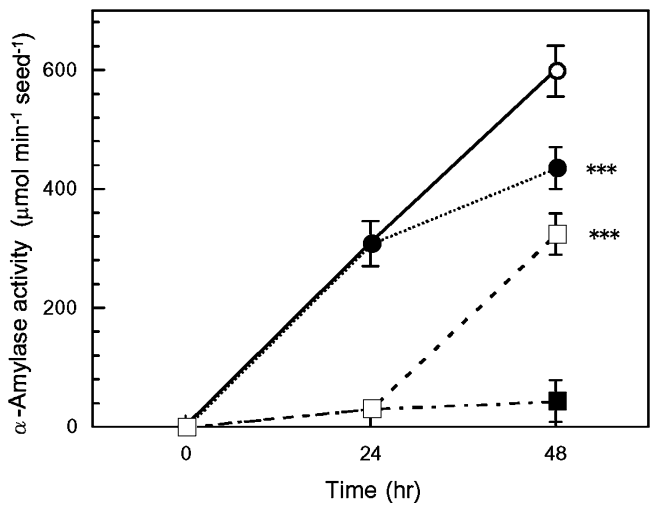

Fig. 7 Effects of pulse treatment of MBOA on α-amylase activity in cress seeds. Means ± SE from five independent experiments with four assays for each determination are shown. Other details as for Fig. 4

MBOA may inhibit α-amylase induction in antagonism with gibberellin-induced events by affecting the α-amylase translation process rather than inhibition of gibberellin biosynthesis. Abscisic acid inhibits α-amylase induction in antagonism with gibberellin-induced events in this manner (Ritchie and Gilroy, 1998).

Germination Inhibition and α-Amylase Activity

Plant germination is a complex phenomenon involving many genes and enzymes. However, amylolytic breakdown of reserve starch in seeds is thought to be a prerequisite for seed germination and subsequent seedling growth. Starch breakdown during seed germination is principally triggered by α-amylase (Beck and Ziegler, 1989; Thomas, 1993; Conley et al., 1999).

During germination, respiration accelerates to produce metabolic energy and biosynthetic precursors for constructing cell structures (Perata et al., 1997). Therefore, soluble sugars that are readily used in respiration must be continuously supplied to maintain respiratory metabolism. However, the amount of utilizable soluble sugars in seeds is usually very limited (Ricard et al., 1998; Saglio et al., 1999; Guglielminetti et al., 2000). α-Amylase plays a major role in the conversion of reserve starch into soluble sugars during germination (Beck and Ziegler, 1989; Vartapetian and Jackson, 1997). Induction of α-amylase is essential to maintain active respiratory metabolism, which produces energy and carbon skeletons for the biosynthesis of new cellular components. Therefore, induction of α-amylase is a prerequisite not only for seed germination, but also subsequent seedling growth until photosynthesis is sufficient to support growth (Beck and Ziegler, 1989; Thomas, 1993; Conley et al., 1999).

MBOA inhibited seed germination and induction of α-amylase in cress seeds. Furthermore, α-amylase activity in the seeds reflected the extent to which they had completed the germination process. These results suggest that MBOA inhibits the induction of α-amylase, which is one possible mechanism of MBOA inhibition of the germination process. The observation that exogenously applied sucrose overcame the inhibitory effect of MBOA supports this hypothesis.

References

Barnes, J. P. and Putnam, A. R. 1987. Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). *J. Chem. Ecol.* 13:889–906.

Beck, E. and Ziegler, P. 1989. Biosynthesis and degradation of starch in higher plants. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* 40:95–117.

Belz, R. G. and Hurle, K. 2004. A novel laboratory screening bioassay for crop seedling allelopathy. *J. Chem. Ecol.* 30:175–198.

Bravo, H. R. and Copaja, S. V. 2002. Contents and morphological distribution of 2,4-dihydroxy-1,4-benzoxazin-3-one and 2-benzoxazolinone in *Acanthus mollis* in relation to protection from larvae of *Pseudaletia impuncta*. *Ann. Appl. Biol.* 140:129–132.

Conley, T. R., Peng, H.-P., and Mingh, C. S. 1999. Mutations affecting induction of glycolytic and fermentative genes during germination and environmental stresses in *Arabidopsis*. *Plant Physiol.* 119:599–608.

Dowd, P. F. and Vega, F. E. 1996. Enzymatic oxidation products of allelochemicals as a basis for resistance against insects: effects on the corn leafhopper *Dalubulus maidis*. *Nat. Toxins* 4:85–91.

Frey, M., Chomet, P., Glawischnig, E., Stettner, C., Grün, S., Winklmair, A., Eisenreich, W., Bacher, A., Meeley, R. B., Briggs, S. P., Simcox, K., and Gierl, A. 1997. Analysis of a chemical plant defense mechanism in grasses. *Science* 277:696–699.

Glenn, A. E., Gold, S. E., and Bacon, C. W. 2002. Fdb1 and Fdb2, *Fusarium verticillioides* loci necessary for detoxification of preformed antimicrobials from cone. *Mol. Plant. Microb. Interact.* 15:91–101.

Glenn, A. E., Meredith, F. I., Morrison, W. H., and Bacon, C. W. 2003. Identification of intermediate and branch metabolites resulting from biotransformation of 2-benzoxazolinone by *Fusarium verticillioides*. *Appl. Environ. Microbiol*. 69:3165–3169.

Groselindemann, E., Graebe, J. E., Stockl, D., and Hedden, P. 1991. *ent*-Kaurene biosynthesis in germinating barley (*Hordeum vulgare* L. cv. Himalaya). Caryopses and its relation to α-amylase production. *Plant Physiol.* 96:1099–1104.

Guglielminetti, L., Yamaguchi, J., Perata, P., and Alpi, A. 1995. Amylolytic activities in cereal seeds under aerobic and anaerobic conditions. *Plant Physiol.* 109:1069–1076.

Guglielminetti, L., Busilacchi, H. A., and Alpi, A. 2000. Effect of anoxia on α-amylase induction in maize caryopsis. *J. Plant Res.* 113:185–192.

Hayashi, T., Gotoh, K., Ohnishi, K., Okamura, K., and Asamizu, T. 1994. 6-Methoxy-2-benzoxazolinone in *Scoparia dulcis* and its production by cultured tissues. *Phytochemistry* 37:1611–1614.

Inderjit and Duke, S. O. 2003. Ecophysiological aspects of allelopathy. *Planta* 217:529–539.

Kato-Noguchi, H. 2000. Allelopathy in maize. II. Allelopathic potential of a new benzoxazolinone, 5-chloro-6-methoxy-2-benzoxazolinone and its analogues. *Plant Prod. Sci.* 3:47–50.

Kato-Noguchi, H., Kosemura, S., and Yamamura, S. 1998. Allelopathic potential of 5-chloro-6-methoxy-2-benzoxazolinone. *Phytochemistry* 48:433–435.

Koyama, T. 1955. Studies on the constituents of *Coix* species. II. On the chemical structure of Coixol. *J. Pharm. Soc. Jpn.* 75:702–704.

Niemeyer, H. M. 1988. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the Gramineae. *Phytochemistry* 27:3349–3358.

Perata, P., Pozueta-Romero, J., Akazawa, T., and Yamaguchi, Y. 1992. Effect of anoxia on starch breakdown in rice and wheat seeds. *Planta* 188:611–618.

Perata, P., Guglielminetti, L., and Alpi, A. 1997. Mobilization of endosperm reserves in cereal seeds under anoxia. *Ann. Bot.* 79(Suppl):49–56.

Pérez, F. J. 1990. Allelopathic effect of hydroxamic acids from cereals on *Avena sativa* and *A. fatua*. *Phytochemistry* 29:773–776.

Ricard, B., VanToai, T., Chourey, P., and Saglio, P. 1998. Evidence for the critical role of sucrose synthase for anoxic tolerance of maize roots using a double mutant. *Plant Physiol.* 116:1323–1331.

Ritchie, S. and Gilroy, S. 1998. Gibberellins: regulating genes and germination. *New Phytol.* 140:363–383.

Saglio, P., Germain, V., and Ricard, B. 1999. The response of plants to oxygen deprivation. Role of enzyme induction in the improvement of tolerance to anoxia, pp. 373–393, *in* H. R. Lerner (ed.). Plant Responses to Environmental Stresses, from Phytohormones to Genome Reorganization. Marcel Dekker, New York.

Sicker, D., Hao, H., and Schulz, M. 2003. Benzoxazolin-2(3*H*)-ones; generation, effects and detoxification in the competition among plants, pp. 77–102, *in* F. A. Macías, J. C. G. Galindo, J. M. G. Molinillo, and H. G. Cutler (eds.). Allelopathy, Chemistry and Mode of Action of Allelochemicals. CRC Press, New York.

Sun, Z. and Henson, C. A. 1991. A quantitative assessment of importance of barley seed α-amylase, β-amylase, debranching enzyme, and α-glucosidase in starch degradation. *Arch. Biochem. Biophys.* 284:298–305.

Thomas, T. L. 1993. Gene expression during plant embryogenesis and germination: an overview. *Plant Cell* 5:1401–1410.

Vartapetian, B. B. and Jackson, M. B. 1997. Plant adaptations to anaerobic stress. *Ann. Bot.* 79(Suppl):3–20.

Yue, Q., Bacon, C. W., and Richardson, M. D. 1998. Biotransformation of 2-benzoxazolinone and 6-methoxy-benzoxazolinone by *Fusarium moniliforme*. *Phytochemistry* 48:451–454.