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An Evaluation of 2,5-Norbornadiene as a Reversible Inhibitor of Ethylene Action in Deepwater Rice[1]

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ABSTRACT

Partial submergence of deepwater rice (*Oryza sativa* L. cv Habiganj Aman II) elicits three responses: enhancement of internodal elongation, inhibition of leaf growth, and promotion of adventitious root formation. All three responses can be induced in isolated stem sections by treatment with ethylene. Dose-response curves indicate that the responses are linearly related to the logarithm of the ethylene concentration over two orders of magnitude. Application of the cyclic olefin 2,5-norbornadiene (NBD) to ethylene-treated sections results in a parallel shift in dose-response curves to higher ethylene concentrations, indicating that NBD behaves as a competitive inhibitor of ethylene action. Internodal elongation of stem sections is promoted by gibberellic acid (GA_3) in the absence of exogenous ethylene. Endogenous ethylene levels do not increase in GA_3-treated sections, and application of NBD does not prevent GA_3-promoted elongation. To the contrary, NBD treatment results in increased growth at intermediate GA_3 concentrations. These results support the idea that ethylene acts through endogenous GA in promoting growth in deepwater rice. NBD applied to GA_3-treated stem sections results in increased ethylene production. This enhancement of ethylene formation is reversed by application of either ethylene or propylene, indicating that ethylene biosynthesis in rice internodes is under negative feedback control.

Deepwater rice, like a number of other semiaquatic plants (7), responds to partial submergence with a great increase in internodal elongation (23). This rapid growth response, which is based on an increased rate of cell division and elongation in the intercalary meristem of the internode (13), is mediated by an interaction of the plant hormones ethylene (C_2H_4) and GA[2] (12, 15). Regulation by altered hormone levels and changes in responsiveness to hormones are involved in this response. Submergence promotes ethylene biosynthesis in the internode (12). Ethylene alone stimulates internodal elongation in nonsubmerged plants (12) and stem sections (16), and rapid elongation is reversibly inhibited by inhibitors of C_2H_4 biosynthesis (12, 16). Thus, growth in partially submerged deepwater rice clearly is a response to environmental stress and is mediated by an increase in the level of a plant hormone.

Ethylene does not appear to promote growth directly but through the action of GA. Raskin and Kende (15) showed that C_2H_4 failed to promote rapid elongation in rice stem sections pretreated with tetcyclacis, an inhibitor of GA biosynthesis. On the other hand, application of 10 μM GA_3 elicited the full growth response in the absence of added C_2H_4. Since 1 μl/L C_2H_4 enhanced the effectiveness of low GA_3 concentrations in promoting the growth of tetcyclacis-treated sections, it appears that C_2H_4 acts, at least in part, by increasing the sensitivity of the internodal tissue to endogenous GA.

To extend the work described above and to define more clearly the qualitative and quantitative role of C_2H_4 in the submergence response of deepwater rice, a series of experiments were performed using the reversible inhibitor of C_2H_4 action, NBD. The inhibitory effects of NBD on C_2H_4 action in plants was first described by Sisler and Pian (20) and more recently characterized by Sisler and Yang (21). A number of physiological studies with NBD confirm the ability of this cyclic olefin to block C_2H_4 action in a number of plants (19–21). It is generally accepted that NBD competes with ethylene for binding at the ethylene receptor site. Thus, high concentrations of C_2H_4 can reverse the inhibitory effect of NBD (9, 21).

The work reported here examines the effectiveness of NBD as a reversible inhibitor of C_2H_4 action in three C_2H_4-responsive tissues of rice stems. In addition to the promotion of internodal elongation, C_2H_4 inhibits leaf growth (16), and promotes the development of adventitious roots at the nodes (2, 22). NBD was also used to evaluate the role of endogenous C_2H_4 in GA_3-promoted internodal elongation of stem sections.

MATERIALS AND METHODS

Chemicals. GA_3 was a gift from Merck and Co., Inc. (Rahway, NJ). 2,5-Norbornadine (bicyclo[2.2.1]hepta-2,5-diene) was purchased from Aldrich Chemical Co., and L-[3,4-^{14}C]methionine (45 mCi/mmol) was purchased from Research Products International Corp. (Mount Prospect, IL).

Plant Material. Seeds of deepwater rice (*Oryza sativa* L., cv "Habiganj Aman II") were obtained from the Bangladesh Rice Research Institute (Dacca).

Growth Conditions. Rice was germinated and grown as described by Métraux and Kende (12). Stem sections containing the youngest elongating internode were prepared from 10- to 12-week-old plants according to Raskin and Kende (16). Sections (15 per treatment) were incubated in sealed 2.5 L plexiglass cylinders under continuous light with the basal 2 cm of the sections standing in 30 ml of either distilled water or a GA_3 solution. The appropriate volumes of C_2H_4, propylene (C_3H_6) and/or NBD were injected through serum caps into each cylinder to yield the required gaseous concentration of each compound. The cylinders were purged with air for 5 min every 12 h, resealed, and C_2H_4 and/or NBD was injected again.

Analysis of Growth. Growth of internodes and leaves was determined after 3 d as previously described (16). The number of adventitious roots at the lower node was determined by counting all roots > 2 mm in length.

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[2] Abbreviations: GA, gibberellin; NBD, 2,5-norbornadiene; ACC, 1-aminocyclopropane-1-carboxylic acid.

Determination of Ethylene Concentration. At the end of the incubation period, gas samples were collected from the internodal lacunae as described (18), and the ethylene concentration was determined by gas chromatography (10). In the radioisotope labeling experiment, sections treated with 5 μM GA$_3$ were incubated in cylinders as above for 48 h at which time 5 μCi of L-[3,4-^{14}C]methionine was added to the GA$_3$ solution. Sections were returned to cylinders and incubated for an additional 24 h. Gas from the internodal lacunae was collected and transferred to a 40-ml syringe. Unlabeled C$_2$H$_4$ was added to give a final concentration of 100 μl/L. The C$_2$H$_4$ was trapped by addition of HgClO$_4$ to the syringe (1). Syringes were shaken overnight at 4°C. The HgClO$_4$ solutions were then transfered to 18 ml of Safety Solve scintillation fluid (Research Products International, Mount Prospect, IL). The radioactivity was determined by scintillation counting and expressed as cpm above background.

RESULTS

Effects of C$_2$H$_4$ and NBD Dosage on Rice Stem Sections. Internodal elongation of stem sections increased with increasing levels of applied C$_2$H$_4$ (Fig. 1A). The extent of growth was linearly related to the logarithm of the C$_2$H$_4$ concentration over a range of two orders of magnitude with maximum growth occurring between 5 and 10 μl/L C$_2$H$_4$. In the presence of increasing concentrations of NBD, both the threshold level of C$_2$H$_4$ needed for the growth response and the level of C$_2$H$_4$ required to saturate it were shifted to higher C$_2$H$_4$ concentrations, while the maximum growth attained and the slopes of the dose-response curves were essentially unaffected.

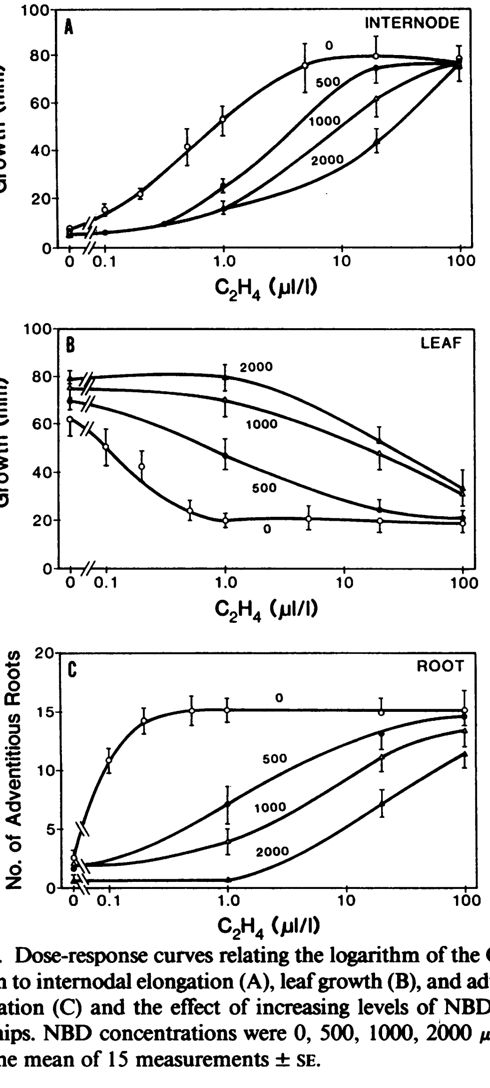

FIG. 1. Dose-response curves relating the logarithm of the C$_2$H$_4$ concentration to internodal elongation (A), leaf growth (B), and adventitious root initiation (C) and the effect of increasing levels of NBD on these relationships. NBD concentrations were 0, 500, 1000, 2000 μl/L. Each point is the mean of 15 measurements ± SE.

Ethylene inhibited leaf growth in isolated stem sections. Figure 1B shows the dose-response curves obtained in the presence of increasing concentrations of NBD. The curve generated in the absence of NBD indicates that the C$_2$H$_4$ response is saturated between 0.5 and 1 μl/L C$_2$H$_4$, a value substantially lower than that obtained for internodal elongation. Increasing concentrations of NBD again shifted the dose-response curve to higher C$_2$H$_4$ values.

In the course of these experiments, it was observed that C$_2$H$_4$ also increased the number of adventitious roots developing at the lower node of the stem section. Figure 1C shows that root development is saturated at 0.2 μl/L C$_2$H$_4$ and that inhibition of this process by NBD was reversed with increasing C$_2$H$_4$ concentrations.

Effect of NBD on GA$_3$-Promoted Internodal Elongation. Since GA$_3$ elicits the full growth response in stem sections that had not been treated with C$_2$H$_4$, we used NBD to examine the possible involvement of endogenous C$_2$H$_4$ in GA$_3$-promoted growth. The dose-response curves for GA$_3$-promoted growth in the presence or absence of 2000 μl/L NBD are shown in Figure 2. NBD did not inhibit the promotion of growth by GA$_3$. To the contrary, NBD-treated sections showed a small but consistent enhancement of growth over air controls at intermediate GA$_3$ concentrations.

Two possible explanations were considered for the NBD-stimulated growth of GA$_3$-treated sections: the enhanced growth was either due to some intrinsic growth-promoting activity of NBD, or resulted from the NBD-stimulated increase in endogenous C$_2$H$_4$ levels (Fig. 2). To distinguish between these alternatives, sections were incubated in 0.5 μM GA$_3$, 2000 μl/L NBD; and 0, 10, or 100 μl/L C$_2$H$_4$. The effects of these treatments on internodal growth are presented in Table I. Treatment with NBD enhanced growth by 26% over controls (Table I) and increased the C$_2$H$_4$ concentration in the lacunar space to 0.18 μl/L (Fig.

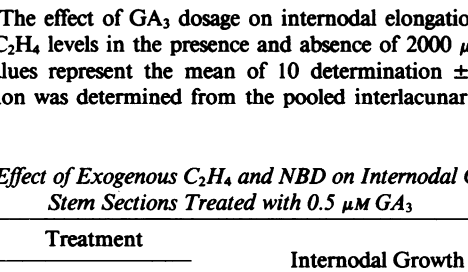

FIG. 2. The effect of GA$_3$ dosage on internodal elongation and endogenous C$_2$H$_4$ levels in the presence and absence of 2000 μl/L NBD. Growth values represent the mean of 10 determination ± SE. C$_2$H$_4$ concentration was determined from the pooled interlacunar gas of 10 samples.

Table I. *Effect of Exogenous C$_2$H$_4$ and NBD on Internodal Growth of Stem Sections Treated with 0.5 μM GA$_3$*

Treatment		Internodal Growth
C$_2$H$_4$	NBD	
μl/L		*mm ± SE*
0	0	54 ± 6
0	2000	68 ± 7
10	2000	69 ± 8
100	2000	85 ± 8

2). Addition of 10 µl/L C_2H_4 to this system did not result in additional enhancement of growth, indicating that the endogenous C_2H_4 concentration was not sufficient to account for the NBD-promoted enhancement of growth. Addition of 100 µl/L C_2H_4 did enhance growth showing that the sections were capable of responding.

The Effect of NBD on Endogenous Ethylene Levels. The concentration of ethylene in the lacunar space was measured in stem sections treated with GA_3 (Fig. 2). In the absence of NBD, C_2H_4 levels remained very low (0.05 µl/L) even at high (5 µM) concentrations of GA_3. However, in the presence of 2000 µl/L NBD, increasing amounts of C_2H_4 were correlated with increasing GA_3 concentrations, such that at 5 µM GA_3 C_2H_4 levels were 10-fold higher in NBD-treated sections than in air-grown controls. If one interprets these results in terms of the inhibitory properties of NBD on C_2H_4 action, the data indicate a negative feedback effect of C_2H_4 on C_2H_4 biosynthesis. To investigate this possibility, two kinds of experiments were performed. Sections were treated with 5 µM GA_3, 2000 µl/L NBD, and either no or 100 µl/L C_2H_4. To determine endogenous C_2H_4 synthesis, [3,4-^{14}C]methionine was fed to the plants during the 3rd d of growth. Table II shows the effect of NBD and NBD plus C_2H_4 on C_2H_4 synthesis. NBD-treated plants incorporated three times more radioactivity into C_2H_4 than did air-grown controls. Addition of 100 µl/L C_2H_4 to NBD-treated plants substantially reduced the amount of radioactivity in C_2H_4. To allow direct measurement of endogenous ethylene levels, C_3H_6 was used to reverse the effects of NBD on C_2H_4 synthesis. Propylene, a biologically active analog of C_2H_4 (3), is capable of stimulating the growth response in deepwater rice and of reversing the inhibitory effect of NBD (data not shown). The results in Table II show that C_3H_6 reversed the NBD-stimulated synthesis of C_2H_4 in GA_3-treated sections. These results are consistent with a negative feedback model of the regulation of C_2H_4 biosynthesis in internodes of deepwater rice.

DISCUSSION

The three C_2H_4-dependent responses examined in this study occurred over C_2H_4 concentration ranges of about two orders of magnitude. This is a relatively narrow range for plant hormone interactions (9) but is typical for such animal hormones as estrogen (8). Interestingly, the three responses differed widely in their sensitivities to applied C_2H_4. Initiation of adventitious roots was 80% saturated at 0.1 µl/L C_2H_4, which was near the threshold concentration for the internodal elongation response. We use the term sensitivity in the broadest sense (5) since the three responses may involve different biochemical mechanisms. At this point, we cannot say where in the signal transduction chain the limiting factors for each response may occur.

The inhibition of all three C_2H_4 responses by NBD could be overcome by increasing C_2H_4 concentrations, indicating the competitive nature of the interaction of NBD with C_2H_4 at the receptor site. A competitive interaction of NBD and C_2H_4 has been demonstrated in other plant systems. Using Lineweaver-Burke plots, Sisler and Yang (21) and Sisler *et al.* (19) found an NBD-dependent shift in the apparent K_m of C_2H_4 responses in pea seedlings (growth) and citrus leaves (abscission). These results were interpreted as indicating a decrease in the affinity of the receptor for C_2H_4 with increasing NBD concentrations, *i.e.* a competitive mode of inhibition.

Table II. *Reversible Inhibition of NBD-stimulated C_2H_4 Production in Stem Sections by C_2H_4 and C_3H_6*

Treatment[a]	Radioactivity in C_2H_4	Total C_2H_4
	cpm[b]	*nl/L*
Air	8.3	
NBD	28.6	
NBD + C_2H_4	14.8	
Air		95
NBD		470
NBD + C_3H_6		165

[a] All stem sections were treated with 5 µM GA_3, and some with 2000 µl/L NBD, 100 µl/L C_2H_4, and 2000 µl/L C_3H_6 as indicated. [b] Stem sections were fed L-[3,4-^{14}C]methionine.

The role of endogenous C_2H_4 in GA_3-promoted growth is shown in Figure 2. Even growth-saturating concentrations of GA_3 did not alter the low basal level of C_2H_4 present in the lacunae of air-grown sections, demonstrating that GA action does not involve alterations in C_2H_4 biosynthesis. When we attempted to show that the basal level of C_2H_4 present in GA_3-treated sections was not required for growth by applying NBD to such sections, we actually observed an increase in growth. Since this growth-promoting effect of NBD could be attributed to a weak C_2H_4-like activity of NBD, we cannot positively conclude that some low level of C_2H_4 activity is not required for the growth response in deepwater rice.

The idea that NBD may have both anti-ethylene and ethylene-like activity is not a contradictory one if one considers two probable components of C_2H_4 action: binding of C_2H_4 (or an analog) to the receptor and a biochemical (*e.g.* conformational) change of the receptor which initiates the chain of events leading to the physiological responses. Borrowing terminology from the field of pharmacology, the above components of hormone action would be called affinity and intrinsic activity, respectively (6). A compound which shows both affinity and intrinsic activity is termed an agonist, while a compound which shows affinity but no intrinsic activity is termed an antagonist (6). Partial agonists are drugs which show some intrinsic activity but block the action of other compounds with higher intrinsic activity. A good example of a partial agonist in C_2H_4 biology is C_3H_6, which competitively inhibits C_2H_4 action at lower concentration (4) but also shows intrinsic C_2H_4-like activity at higher doses (3). Whether NBD behaves as a pure antagonist or a very weak partial agonist may not be relevant to many physiological systems where, in practice, it acts as a very effective inhibitor of C_2H_4 action. However, in studies which call for the complete elimination of C_2H_4-like activity, the question whether NBD is an antagonist or partial agonist becomes important. In their study on the effects of NBD on pea seedlings, where C_2H_4 inhibits epicotyl elongation, Sisler and Yang (21) found that supraoptimal NBD concentrations led to a reduction in growth. They attributed this result to nonspecific, toxic effects of NBD since similar concentrations of cyclohexane also showed inhibitory effects. However, the effect of cyclohexane was much less pronounced than that of NBD. Therefore, the possibility that NBD exerted some C_2H_4-like activity cannot be ruled out in this system.

An alternative explanation for the growth-promoting activity of NBD observed in these experiments would be that NBD enhances growth independent of C_2H_4 action. NBD caused a rather large increase in pea epicotyl elongation in seedlings grown in air (21). This effect was attributed to an inhibition of endogenous C_2H_4 activity in the seedlings; however, no evaluation was made whether endogenous levels of C_2H_4 were sufficient to create the observed difference in growth. In our own experiments with leaf growth (Fig. 1B), we found that NBD promoted leaf growth over air-grown controls. Again, we cannot say how much of the growth promoted by NBD can be attributed to an anti-C_2H_4 effect alone.

The NBD-promoted increase in C_2H_4 production in GA_3-treated stem sections can be interpreted in terms of feedback inhibition of C_2H_4 synthesis. Such a regulatory system has been described in a number of plant systems (24). Feedback or autoinhibition has been noted in fruit and vegetative tissues and has been ascribed to inhibition of ACC synthase (17, 25) and/or increased conjugation of ACC (11, 14). Ethylene biosynthesis increases substantially in the internodes of deepwater rice when plants are submerged (12). Evidence indicates that low O_2 in the internode acts as a signal for this increase (16). By whatever mechanism low O_2 stimulates ethylene biosynthesis, it must somehow circumvent the feedback regulatory system observed here.

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