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Corn metabolites affect growth and virulence of *Agrobacterium tumefaciens*

[acetosyringone/corn transformation/2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one/growth inhibition/virulence gene induction]

SHIVENDRA V. SAHI*, MARY-DELL CHILTON†, AND WILLIAM SCOTT CHILTON*‡

*Department of Botany, North Carolina State University, Raleigh, NC 27695-7612; and †CIBA-Geigy Agricultural Biotechnology Research Unit, P.O. Box 12257, Research Triangle Park, NC 27709-2257

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ABSTRACT **Homogenates of corn seedlings inhibit both growth of *Agrobacterium tumefaciens* and induction of its Ti plasmid virulence (*vir*) genes by acetosyringone (AS). The heat-labile inhibitor has been identified as 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), present in 2-week-old seedlings (B73) at a concentration of 1.5 mM or greater. A concentration of 0.3 mM DIMBOA is sufficient to block growth of *A. tumefaciens* completely for 220 hr. DIMBOA at 0.1 mM concentration completely inhibited *vir* gene induction by 100 μM AS and reduced growth rate by 50%. Thus, DIMBOA can be expected to have a significant effect on attempts to transform corn by using *A. tumefaciens* as a vector.**

Agrobacterium tumefaciens induces crown gall tumors in wounded dicotyledonous plants by excision of a segment of DNA (T-DNA) from its tumor-inducing (Ti) plasmid, transfer of the T-DNA into the host plant cell, and integration of the T-DNA into plant chromosomes (1, 2). The T-DNA contains several genes, including genes for cytokinin and auxin synthesis, whose products cause cell proliferation to form a gall (3).

Expression of virulence genes (*virA*, *virB*, *virC*, *virD*, *virE*, and *virG*), located on the Ti plasmid outside of the T-DNA, is required for T-DNA transfer. The constitutively expressed *virA* gene produces a protein located in the inner membrane, which acts as a receptor for acetosyringone (AS) and related syringyl and vanillyl plant wound metabolites (4, 5). The autophosphorylation of virA protein activates the intracellular, signal-transducing virG protein, presumably by phosphorylation (6, 7). The virG protein then induces expression of the remaining virulence genes. Products of these genes are responsible for precise excision of single-stranded T-DNA (8), protection of T-DNA from bacterial and plant DNase (8), and probably formation of a bacterial–plant conjugative structure for passage of the T-DNA (8). Elucidation of the virulence mechanism has relied heavily on virulence gene mutants prepared by insertion of the Tn*3*HoHo1 transposon containing the *Escherichia coli* β-galactosidase gene (*lacZ*) (9). Expression of the inducible *vir*::*lacZ* genes can be quantitated by β-galactosidase enzyme assay [measuring the release of *o*-nitrophenoxide from *o*-nitrophenyl β-D-galactopyranoside (ONPG)] (10).

Disarmed Ti plasmids have been used to transfer genes for herbicide resistance, viral resistance, and insect resistance into economically important plants (11). Many dicot plants and some gymnosperms are susceptible to crown gall disease (12) and thus are potentially transformable by *A. tumefaciens*. However, most monocots are resistant to *A. tumefaciens*-induced gall formation (13). Transformation without gall formation may be achieved in some species of the *Liliales* (14, 15). Although the *Agrobacterium* Ti plasmid has been used to deliver the maize streak virus into corn (16), and similar viral agroinfection of other members of the *Graminae* have been reported (17–19), stable transformation of the economically important cereals by *A. tumefaciens* has not been achieved.

We have studied the effect of corn metabolites on growth and virulence gene induction of *A. tumefaciens* to obtain a better understanding of their effects on attempts to transform corn with this vector.

MATERIALS AND METHODS

Biological Materials. Corn variety B73 was obtained from CIBA–Geigy. Inbred *bxbx*, a 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA)-deficient mutant, selected from an open pollinated Gehu Yellow Dent population (20) and maintained through 11 generations of selfing, was provided by Kevin Simcox (Purdue University). The *bxbx* seedlings contain no DIMBOA (Fig. 1) but do contain a small amount of a metabolite identified as 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one by gas chromatography (K. Simcox, personal communication).

A. tumefaciens A277 is a transconjugant strain carrying pTi B6-806 in the cured C58 genetic background (21). *A. tumefaciens* A348 (pSM358), provided by E. W. Nester (University of Washington), contains a *virE*::*lacZ* gene fusion (22), permitting measurement of induction of the *virE* gene by a β-galactosidase assay (10).

Chemicals. DIMBOA was isolated from 2-week-old greenhouse-grown corn seedlings (B73). The homogenate resulting from blending 200 g of whole seedlings in 400 ml of water was allowed to stand at room temperature for 1 hr to allow time for enzymatic hydrolysis of DIMBOA glucoside. Particulates were removed by centrifugation, and the supernatant was extracted three times with ethyl acetate. Ethyl acetate was removed *in vacuo*, and the residual oil was redissolved in a small volume of ethyl acetate. After several days, crystals of DIMBOA separated from the solution. DIMBOA recrystallized from ethyl acetate had a m.p. of 156°C; UV in methanol: 262 nm (16,200), shoulder 286; mass spectrum: *m/e* 211 (M+, 1%), 195 (3%), 193 (7%), 165 (100%), 150 (68%), 122 (13%), 109 (16%), 106 (37%). These physical constants are consistent with those previously reported (23, 24). DIMBOA (R_f 0.4), containing no contaminants, was detected on Merck silica gel 60 thin-layer chromatograms (benzene/acetone, 70:30), developed with either the general visualizing reagent phosphomolybdic acid or acidic/alcoholic ferric chloride reagent for hydroxamic acids (24).

6-Methoxy-2(3*H*)-benzoxazolone (MBOA; Fig. 1) was

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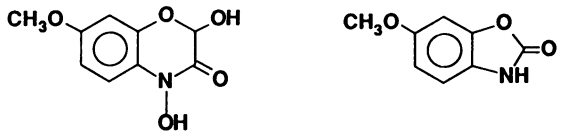
Abbreviations: AS, acetosyringone; DIMBOA, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one; MBOA, 6-methoxy-2(3*H*)-benzoxazolone; ONPG, *o*-nitrophenyl β-D-galactopyranoside.

‡To whom reprint requests should be addressed.



1: DIMBOA 2: MBOA

FIG. 1. Structure of DIMBOA and MBOA.

synthesized by nitrosation of *m*-methoxyphenol and reduction to 2-amino-5-methoxyphenol, followed by cyclization to MBOA using carbonyldiimidazole (25, 26). MBOA recrystallized from dichloromethane had a m.p. of 149°C; UV in ethanol: 230 nm (6000), 287 (3200), consistent with physical constants reported previously (26). Synthetic MBOA gave a single spot (R_f 0.6) detectable with phosphomolybdic acid after silica gel TLC in benzene/acetone (70:30).

Dilute aqueous solutions of DIMBOA and MBOA were prepared for growth and virulence inhibition experiments by dissolving the metabolite in a small volume of methanol and diluting into a larger volume of rapidly stirred water to prepare a 10 mM solution. This aqueous solution was further diluted into the appropriate aqueous medium for each experiment. Final methanol concentration in aqueous solutions was <0.5%. Aqueous solutions of DIMBOA were freshly prepared immediately before use because DIMBOA is unstable in water. In all biological experiments, the control was treated with an aqueous solution containing the same concentration of methanol.

AS, ONPG, and other reagents were purchased from Sigma.

Bioassays. *Bacterial media.* Low phosphate AB glucose medium, optimized for virulence induction assays (27), but also used for growth rate measurements and for preparation of corn homogenates, contained 1% glucose, AB salts, 20 mM morpholineethanesulfonic acid (Mes), and 2 mM phosphate buffer. This medium was adjusted to pH 5.5 for virulence induction assays and to pH 7.0 for growth rate measurements.

Corn homogenates for growth studies were prepared by homogenizing 100 g of 2-week-old greenhouse-grown corn seedlings (B73) in 200 ml of low phosphate AB glucose medium. The resulting extract was filtered through cheesecloth and centrifuged 10 min at 10,000 rpm (Sorvall RC2B). The clarified corn extracts were adjusted to pH 7.0. For some growth experiments, the corn homogenate was then heated by immersing in a large boiling water bath for the specified time. Corn homogenates were filter-sterilized and inoculated with *A. tumefaciens* immediately after preparation.

Growth rate. A preculture for each set of growth experiments was prepared by growing the appropriate bacterial strain in AB medium on a roller drum at 28°C for 20 hr. For cultivation with corn homogenate, 50 μl of the preculture was inoculated into 2.5 ml of test medium and cultured on a roller drum at 28°C. Optical density of growing cultures was measured periodically at 600 nm on a Bausch and Lomb Spectronic 20 until maximum growth was obtained.

Virulence induction. Precultures of *A. tumefaciens* strain A348 (pSM358) were grown in AB medium (pH 7.0) on a roller drum for 20 hr. Bacteria were harvested by centrifugation, washed with AB medium, and finally inoculated into induction medium. Induction medium contained 2 ml of low phosphate AB glucose medium (pH 5.5) and 100 μM AS, with or without DIMBOA or MBOA. Sufficient inoculum was added to make the initial optical density 0.1 at 600 nm. Cultures were incubated on the roller drum for 20 hr. Induction of the *virE::lacZ* fusion gene was measured by the *o*-nitrophenyl galactoside assay (10, 28). The concentration of bacteria was adjusted to A_{600} between 0.1 and 0.5. Bacteria were lysed by sodium dodecyl sulfate and chloroform and ONPG was added. After 15 min at 28°C, enzyme hydrolysis of ONPG was stopped by addition of sodium carbonate. Bacteria were removed by centrifugation and optical density was measured at 420 nm. Miller units of β-galactosidase activity were calculated by the equation $[(A_{420} \times 1000)/(t\ (\text{min}) \times A_{600})]$.

DIMBOA treatment of wounded Kalanchoe. The two uppermost leaves (4 cm long) of *Kalanchoe daigremontiana* were wounded by slicing with a razor blade. A 25-μl aqueous solution of 0.5 mM DIMBOA was distributed uniformly along 3-cm wounds on one leaf, and 25 μl of distilled water was distributed on the control leaf. All wound sites were inoculated 20 min later with 25 μl of a 20-hr-old culture of *A. tumefaciens* A277 in exponential growth phase on AB medium. Wounds were observed daily for appearance of galls.

RESULTS

Growth of *Agrobacterium* in Corn Homogenate. The rate of growth of *A. tumefaciens* in glucose medium with and without added corn extract was measured to determine whether corn metabolites have any effect on growth of the bacterium. *A. tumefaciens* A348 (pSM358) grew rapidly in AB glucose medium after an 8-hr lag phase (Fig. 2). In contrast, when the medium consisted of corn seedling extracted with AB glucose medium (1:2, wt/vol), the onset of the exponential growth phase was delayed to 35 hr (Fig. 2). The growth inhibitory effect of corn seedling homogenate was reduced by brief boiling of the homogenate before inoculation. Twenty minutes of boiling reduced the lag phase to ≈10 hr. This indicated the presence of a heat-labile, bacteriostatic metabolite in corn homogenate. We then explored whether DIMBOA (Fig. 1), which is degraded by heating, principally to MBOA (29) (Fig. 1) could be responsible for this effect.

Presumptive evidence that DIMBOA is responsible for poor growth of *A. tumefaciens* was obtained by using a homogenate of seedlings of the inbred corn line *bxbx*, known to contain only low levels of DIMBOA-related metabolites (20). *A. tumefaciens* grows better in AB glucose medium containing *bxbx* corn homogenate than in medium prepared with homogenate of corn variety B73, although not as well as in AB glucose medium alone (Fig. 3).

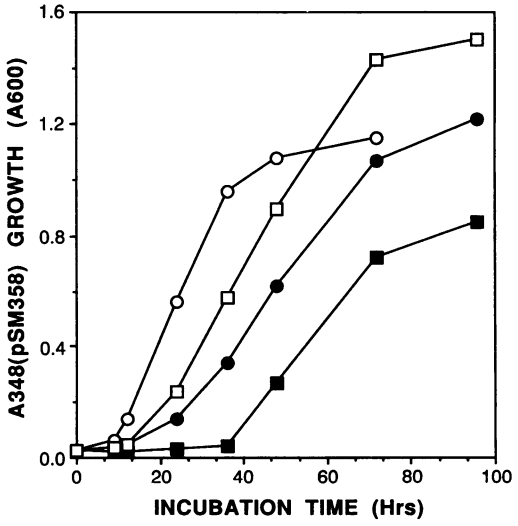


FIG. 2. Growth of *A. tumefaciens* A348 (pSM358) in AB glucose medium (○), corn homogenate in AB glucose medium (■), corn homogenate in AB glucose medium boiled for 10 min (●), and boiled for 20 min (□).

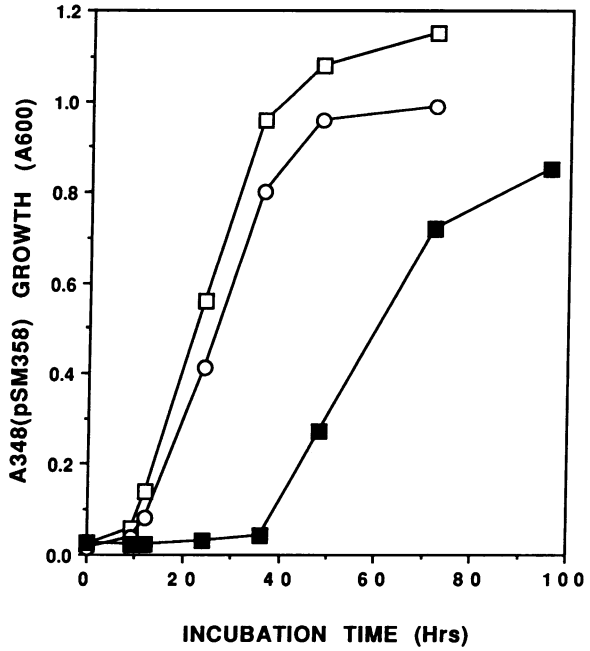


FIG. 3. Growth of *A. tumefaciens* A348 (pSM358) in AB glucose medium (□), *bxbx* corn homogenate in AB glucose medium (○), and B73 corn homogenate in AB glucose medium (■).

Growth inhibitors were also detected by observing zones of *Agrobacterium* growth inhibition in soft agar overlays of thin-layer chromatographic plates loaded with the ethyl acetate extract of corn seedling homogenates (unpublished data). An extremely broad zone of growth inhibition was centered at the R_f of authentic DIMBOA. A weaker, high R_f inhibition zone was also present.

Growth of *Agrobacterium* in the Presence of Pure DIMBOA and MBOA. Studies on corn homogenates strongly suggested that the major inhibitory metabolite was DIMBOA. Therefore, we isolated DIMBOA from corn and investigated the effect of the purified metabolite on *A. tumefaciens* growth.

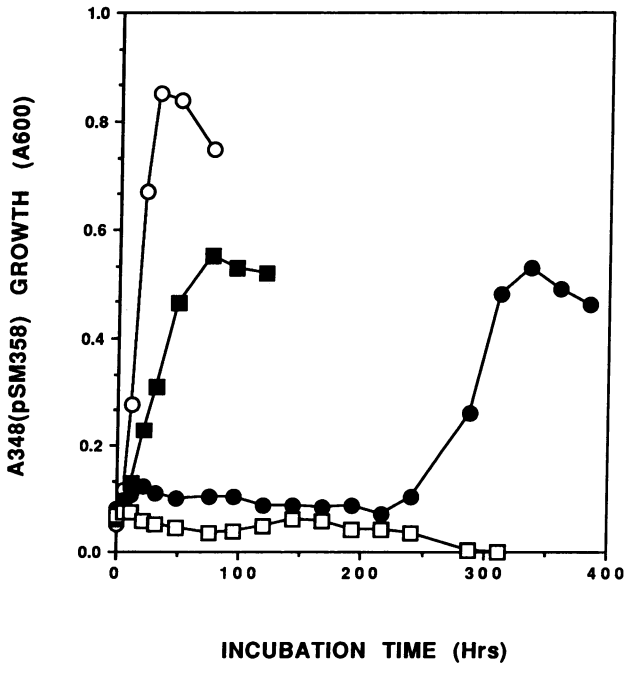


FIG. 4. Growth of *A. tumefaciens* A348 (pSM358) in AB glucose medium containing no DIMBOA (○), 0.1 mM DIMBOA (■), 0.3 mM DIMBOA (●), and 0.5 mM DIMBOA (□).

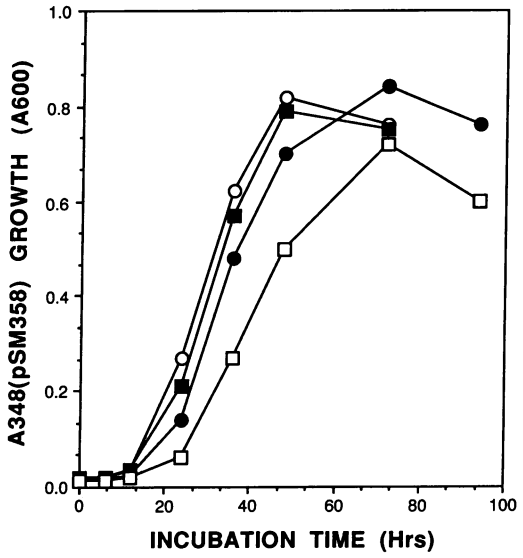


FIG. 5. Growth of *A. tumefaciens* A348 (pSM358) in AB glucose medium containing no MBOA (○), 0.1 mM MBOA (■), 0.3 mM MBOA (●), and 0.5 mM MBOA (□).

AB glucose medium containing low levels of DIMBOA was highly bacteriostatic to *A. tumefaciens*. A concentration of 0.5 mM DIMBOA was totally inhibitory to *Agrobacterium* growth throughout a 2-week experiment (Fig. 4), while 0.3 mM DIMBOA was sufficient to block growth for 220 hr; 0.1 mM DIMBOA did not cause any growth lag but did slow the rate of exponential growth by ≈50%.

Since DIMBOA degrades rapidly in water to MBOA (29), we prepared synthetic MBOA and studied its effect on growth of *A. tumefaciens* also. MBOA was only very weakly inhibitory to *Agrobacterium* growth and did not cause significant prolongation of the lag phase, even at 0.5 mM concentration (Fig. 5).

Effect of DIMBOA and MBOA on Virulence Gene Induction. Strain A348 (pSM358), containing a *virE::lacZ* fusion gene, was used in the Miller assay of β-galactosidase activity to assess the effect of DIMBOA and MBOA on virulence gene induction in *Agrobacterium*. Half maximal induction of the *virE* gene of *A. tumefaciens* A348 (pSM358) was caused by 15 μM AS and 80% of maximal induction was obtained with 100 μM AS (data not shown). A concentration of 100 μM AS was selected as a standard inducing concentration to use when testing for inhibition of virulence induction. A concentration of 0.1 mM DIMBOA completely blocked induction by 100 μM AS; 50% inhibition was caused by ≈0.04 mM DIMBOA (Fig. 6). This concentration range of DIMBOA did not cause a detectable increase in lag phase in growth experiments. However, 0.1 mM DIMBOA did slow the growth rate to ≈50% of the control containing no DIMBOA. Concentrations of MBOA between 0.05 and 0.3 mM had no significant effect on virulence induction by 100 μM AS.

Effect of DIMBOA on Crown Gall Formation. If DIMBOA is both bacteriostatic and an inhibitor of virulence gene induction, then it might be predicted to interfere with transformation and gall formation on an otherwise sensitive host. When wounds on the host plant *K. daigremontiana* were painted with 0.5 mM DIMBOA just before inoculation with *A. tumefaciens* A277, gall formation was almost completely suppressed (Fig. 7). Since DIMBOA has a half-life of ≈1 day at pH 5.5, one wounded leaf was painted daily with 0.5 mM DIMBOA. Daily application of DIMBOA was no more effective in suppressing gall formation than a single dose just before inoculation.

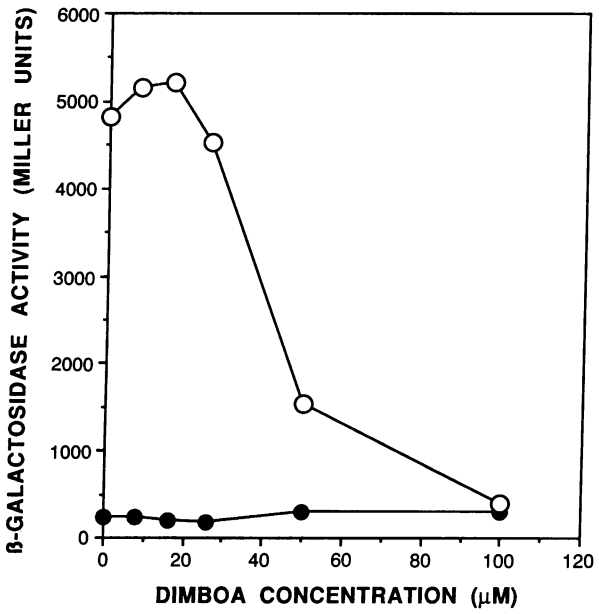


FIG. 6. Effect of DIMBOA concentration on expression of *virE::lacZ* fusion gene of *A. tumefaciens* A348 (pSM358) in the presence of 0.1 mM AS (○) and in the absence of AS (●).

DISCUSSION

In experiments intended to determine whether there were metabolites in a variety of dicotyledons, monocotyledons, and gymnosperms that could induce the virulence genes of the Ti plasmid, we encountered great difficulty in either growing *Agrobacterium* or performing virulence gene induction assays in any medium containing corn extract. Both inhibition of virulence induction by 100 μM AS (data not shown) and growth inhibition were greatly reduced if the corn extract was boiled for 20 min before inoculation with bacteria (Fig. 2). The lability of the growth inhibitor suggested that it was DIMBOA, a known antimicrobial metabolite of corn. The observation that homogenates of seedlings of the low DIMBOA corn mutant, *bxbx*, were much less inhibitory to growth of *A. tumefaciens* contributed further evidence that DIMBOA is the major growth inhibitor present in corn seedlings.

Purified DIMBOA completely blocks growth of *Agrobacterium* at 0.5 mM (Fig. 4). Many varieties of corn have been found to have a DIMBOA content between 1 and 20 mM (30, 31), depending on variety, location (32), and developmental stage (33). Our recovery of crystalline DIMBOA from corn extracts corresponded to a concentration of 1.5 mM DIMBOA in 2-week-old seedlings of variety B73. Allowing for mechanical losses in manipulating solutions and crystals as well as degradative losses to MBOA during purification, we conclude that the DIMBOA level in variety B73 is >1.5 mM. A concentration of 0.1 mM DIMBOA halves the growth rate of *Agrobacterium* but completely blocks virulence induction in the presence of AS (Fig. 6). Thus, there is ample DIMBOA in corn to account for both the growth inhibitory and virulence induction inhibitory effects of corn homogenate on *A. tumefaciens*.

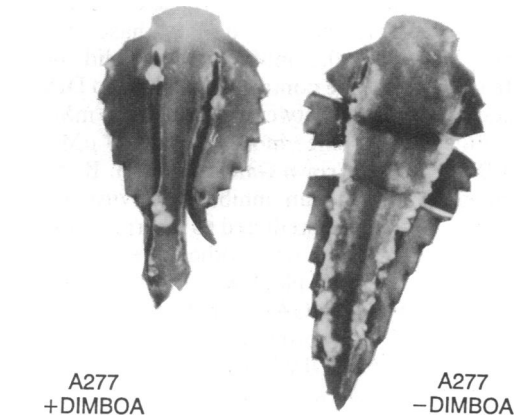


FIG. 7. *Kalanchoe* leaves inoculated with *A. tumefaciens* A277 in the presence of 25 μl of 0.5 mM DIMBOA.

DIMBOA is present in intact cells as a glucoside (34). Rupture of the cells brings the glucoside into contact with a glucosidase, which releases DIMBOA. Enzymatic hydrolysis is complete within 1 hr of homogenization (32). DIMBOA decomposes in aqueous solution in a nonenzymatic reaction, principally to MBOA (35). The half-life of DIMBOA at pH 5.5, approximately the pH of corn homogenate, is 24 hr (29). Corcuera *et al.* (35) have shown that DIMBOA is bacteriostatic, not bacteriocidal, toward *Erwinia*, while MBOA has little effect on *Erwinia*. These investigators observed that after several half-lives the DIMBOA level fell below the threshold necessary for bacteriostasis, after which *Erwinia* grow rapidly to the density supportable by the medium. We find that *Agrobacterium* behaves similarly, although the lag phase is longer for a given DIMBOA concentration than was observed for *Erwinia*, indicating that *A. tumefaciens* has a lower threshold tolerance for DIMBOA.

There may be several problematic steps in transformation of corn by *Agrobacterium* gene vectors. We have shown that concentrations of DIMBOA found in corn have a significant adverse effect on the *Agrobacterium* population and the induction of virulence genes. The finding that tumor induction in the dicotyledonous plant *Kalanchoe* is greatly reduced by DIMBOA supports the view that DIMBOA toxicity is worth further study in future attempts to transform corn by *Agrobacterium*.

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