Induction and Regulation of Ethylene Biosynthesis by Pectic Oligomers in Cultured Pear Cells

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ABSTRACT

Pectic oligomers induced a rapid, transient increase in ethylene biosynthesis when added to pear cells in suspension culture. The rate of ethylene biosynthesis increased within 30 to 40 minutes after oligomer addition, reached a maximum between 90 and 120 minutes after addition, and then decreased to basal rates of synthesis. Both the rapid increase and decrease in biosynthesis appear to be precisely regulated components of the ethylene response to oligomers. Induction of ethylene biosynthesis by pectic oligomers resulted in a reduced sensitivity of cells to further ethylene induction. This reduction in sensitivity occurred within 90 minutes after an oligomer treatment, slightly preceding the decline in ethylene synthesis. The degree of insensitivity induced was proportional to the concentration of oligomer in the first treatment. Induced insensitivity to elicitors appears to represent a novel mechanism which may limit continued ethylene biosynthesis after ethylene induction. Ethylene was produced by pear cells throughout the cell growth cycle, as cells increased in density over a 6 day period. Endogenous ethylene biosynthesis was at a maximum during the first 4 days of rapid cell growth, then declined to half the peak rate through day 10. Pectic oligomers could induce an increase in ethylene biosynthesis above this background rate only after day 5, as endogenous biosynthesis declined. Changes in sensitivity to added oligomer during the growth cycle may result from insensitivity to elicitors induced by growth processes.

Many environmental responses and developmental processes in plants are accompanied by changes in the rate of ethylene biosynthesis. Ethylene synthesis is rapidly induced by a variety of environmental events, including wounding, mechanical perturbation, pathogen attack, elevated UV and CO₂, and reduced oxygen (2, 9, 12, 22, 28). The rate of ethylene biosynthesis also changes rapidly during many developmental processes, such as germination, growth, elongation, abscission, and ripening (3, 10, 12, 28). Little is known of the physiological or molecular mechanisms by which ethylene synthesis is regulated in these instances (6, 9, 22). Less yet is known of the means or the extent to which evolved ethylene acts to regulate other temporally associated processes (7, 8, 14, 26). However, ethylene’s rapid induction, movement, and dissipation would make it an ideal signal to coordinate cellular responses to significant changes in a plant’s internal or external environment.

Of the various aspects of ethylene induction and action, regulation of the pathway of ethylene biosynthesis is best understood, and is best characterized in response to wounding and climacteric ripening. Ethylene is produced via a short biosynthetic pathway: SAM3 (or AdoMet) is converted to ACC by ACC synthase, and ACC to EFE (28).

Ethylene biosynthesis in response to wounding follows a consistent pattern in most plants. The local rate of ethylene biosynthesis begins to increase rapidly 20 to 30 minutes after wounding, reaches a peak at 40 to 60 min, then declines (2, 9, 21). The rise in wound-induced ethylene biosynthesis is limited by ACC synthase activity, rather than EFE activity; the pattern of biosynthesis reflects the de novo synthesis, accumulation, and activity of ACC synthase in the induced cells (2, 9).

Several mechanisms have been identified which may limit continued ethylene synthesis after induction (10, 28). For example, ethylene is known to act as a feedback regulator of its own biosynthesis. In response to wounding, this feedback is negative, and ethylene biosynthesis is self-limiting and transient (10). In climacteric ripening, this feedback is apparently positive, and ethylene biosynthesis is autocatalytic and persistent (15). McMurchie et al. (15) proposed the existence of two systems for regulating ethylene biosynthesis: system I controlling most developmental and wound ethylene, system II responsible for climacteric ethylene.

It has been suggested that oligosaccharides released from cell walls by wounding may play a role in regulating various plant responses, including ethylene biosynthesis (19, 22, 27). Pectic oligomers, in particular, are known to induce wound and defense responses in many plants (16, 20, 27) and to induce transient ethylene biosynthesis in preclimacteric tomato pericarp discs (5). Oligosaccharides are also released from cell walls during fruit ripening and have been proposed as regulators of the ripening process (3, 14, 24). Pectic oligomers, in particular, have been shown to increase climacteric ethylene biosynthesis and tissue reddening in whole tomato fruit and tomato pericarp discs (4, 5).

Cells in suspension cultures have been used as model systems for the investigation of both wound responses and ripening.

1 Abbreviations: SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene-forming enzyme; PCV, packed cell volume; DP, degree of polymerization; G7, mixture of smaller pectic oligomers; G12, mixture of larger pectic oligomers.
ening processes (17, 18, 25, 27). Tong et al. (25) have recently shown that digestion products of pear cell walls induce an increase in ethylene production by pear cells in suspension culture. In this study, we further explore the induction of ethylene biosynthesis in pear cells by pectic fragments. We examine the change in response to pectic oligomers following an initial induction of ethylene biosynthesis, and suggest a novel mechanism which may limit ethylene biosynthesis after induction by pectic oligomers. We characterize the response of pear cells to added pectic oligomers throughout the suspension culture growth cycle.

**MATERIALS AND METHODS**

**Cell Culture**

Cells established in suspension culture from young 'Passe Crassane' pear (Pyrus communis L.) fruit were provided by Dr. Roger Romani (17, 18). Cells were grown in 600 mL of medium in 2 L flasks, sealed with a porous cotton plug and foil cap, on a rotary shaker (100–120 rpm) in constant light at 27°C. Cultures were maintained by weekly subculturing of a 10% inoculum into fresh medium with mineral nutrients of Murashige and Skoog and organic nutrients of Nitsch et al., as modified by Pech and Romani (17), including 10 μM 2,4-D and 4 mM CaCl₂.

For detailed studies of the dynamics of ethylene induction, cells were grown in nutrient medium without 2,4-D. Cell cultures were established from cells grown for 7 d on medium with 2,4-D. Seven day cells were washed three times by settling with gentle centrifugation (5 g for 1 min), decanting the supernatant, and adding an equal volume of fresh medium without 2,4-D. After the last wash, cell dry weight density was determined and cultures were diluted with fresh medium to an inoculum cell density of 2.5 mg/mL. After a 7 to 9 d incubation, cells were decanted, then washed and used for detailed study of ethylene induction.

For studies of ethylene induction by pectic oligomers throughout the suspension culture growth cycle, cells were grown in nutrient medium both with and without 2,4-D. Test cultures were established from cells grown on medium with 2,4-D. Seven day cells were washed three times, as described above, adding an equal volume of fresh medium either with or without 2,4-D. After the last wash, cell dry weight density was determined and cultures were diluted with fresh medium to an inoculum cell density of 1.25, 2.5, or 3.75 mg/mL. Duplicate flasks were established for each medium and concentration. After inoculation, a 40 mL sample of cell suspension was decanted daily from each culture flask through a low sidearm, then washed and tested for ethylene induction, as described below.

Cell density was measured as mg of cell dry weight per mL of cell suspension. 5 mL aliquots of cell suspension were filtered through preweighed filter paper (No. 1 Whatman, 5 cm), then cell-laden filters were dried in a microwave oven for 5 min, reweighed, and weight change calculated per mL of suspension. Dry weight was a more precise measure of cell density than PCV and a more rapid measure than number of cells per unit volume.

**Cell Treatment and Ethylene Measurement**

Decanted cells were washed three times with fresh medium, then diluted with fresh medium to a cell density of 5 mg/mL. Five milliliter aliquots of cells were transferred to 25 mL flasks on a rotary shaker and treatments were added. Elapsed time from decanting to first treatment was generally around 90 min. Treatments were added as solutions in 500 μL of fresh medium. Pectic oligomers were added at concentrations of 1000, 500, 100, or 25 μg in 500 μL. Monomeric galacturonic acid was added at a concentration of 500 μg in 500 μL to account for ethylene induction that might be due to changes in pH or metabolizable substrate. An aliquot of 500 μL of fresh medium was added to account for ethylene induction due to medium addition. ACC was added at a concentration of 100 μmol per 500 μL to account for changes in ethylene biosynthesis due to EFE activity.

Rates of ethylene biosynthesis were determined by capping individual flasks for 15 min, then sampling accumulated gases. Ethylene was analyzed by gas chromatography at 80°C with an alumina column and quantified by integration of the peak from a flame ionization detector. Ethylene biosynthesis is plotted as the average rate over the preceding 15 min interval. For detailed studies of the dynamics of ethylene induction, ethylene was measured at regular intervals after treatment. For studies of induced change in sensitivity to oligomers, second treatments with oligomers or ACC were added to flasks at 15 min intervals between 45 and 135 min after a first treatment with medium or oligomer. For studies of ethylene induction throughout the growth cycle, ethylene was measured 90 min after treatment, at the peak of ethylene biosynthesis.

**Pectic Oligomer Composition**

The pectic fragments used in this study were homooligomers of α-1,4-D-galacturonic acid prepared by Mike Saxton of the University of California, Davis, Plant Growth Laboratory (M Saxton, personal communication). Citrus pectin (Sigma P9135) was hydrolyzed as a 1% solution in 0.5 M HCl for 3 h with refluxing under a nitrogen atmosphere, then neutralized with NaOH, decolorized with charcoal, and dialyzed against H₂O. Resulting oligomers were separated into mixtures of smaller (G7) and larger (G12) oligomers by gel filtration chromatography. The G7 and G12 oligomer mixtures differed significantly in average oligomer size, but contained some oligomers in common, as shown by anion-exchange chromatography on QAE-Sephadex, with an imidazole gradient (Fig. 1). Preliminary HPLC gradient analysis indicated that G7 contained oligomers with a DP from 5 to 13, with 90% of carbohydrates in DP 7 to 9, and G12 contained oligomers from DP 6 to 19, with 90% in DP 10 to 12 (JM Labavitch, LC Greve, unpublished data) (1).

**RESULTS**

**Ethylene Induction by Pectic Oligomers**

Pectic oligomers consistently induced a rapid, transient increase in ethylene biosynthesis when added to pear cells from 7 d old suspension cultures grown without 2,4-D (Fig.
2). The rate of ethylene biosynthesis always began to increase within 30 to 40 min after oligomer addition, reached a maximum between 90 and 120 min after addition, and then decreased, slowly returning to basal rates of synthesis. The maximum rate of ethylene production increased with oligomer concentration up to 500 µg per flask, while the temporal pattern of increase and decrease remained the same at all concentrations (Fig. 2a).

Induction of ethylene was specific to the oligomeric form of galacturonic acid; monomeric galacturonic acid did not induce an increase in ethylene biosynthesis (Fig. 2a). Mixtures of smaller oligomers (G7) were more effective inducers of ethylene biosynthesis than mixtures of larger oligomers (G12) on an equal weight basis (Fig. 2b). Pectic oligomers did not apparently act by binding Ca²⁺; neither ethylene biosynthesis nor the ethylene response to oligomers was altered by pretreatment or cotreatment of cells with 11 to 550 mg CaCl₂ per flask (4–200 mM final concentration).

The timing of the ethylene response to pectic oligomers was constant among experiments, but the amplitude of the response varied. For example, the amount of oligomer necessary to induce a detectable response ranged from 10 to 50 µg, and the amount to induce a maximum ("saturated") response varied from 100 to over 500 µg. The increase in ethylene biosynthesis induced by a saturating treatment differed among experiments, from less than 100% to over 300%. All described experiments were repeated several times, and typical results are reported.

**Induced Changes in Sensitivity to Oligomers**

Several experiments examined whether the decline in ethylene biosynthesis following the peak might be due, in part, to a decrease in the sensitivity of pear cells to oligomer concentration. After a first treatment with 100 µg of G7, a second treatment with 500 µg of G7 was added to sets of flasks at 15 min intervals between 45 and 135 min after the first treatment. When the second oligomer treatment was added to cells within 1 h after a nonsaturating primary treatment, a normal ethylene response to the second treatment was superimposed over the first response. As the second oligomer treatment was delayed beyond 1½ h after the first, the ethylene response to the second treatment decreased. By 2 h after the first treatment, response to a second treatment was at a minimum. An example of responses to second treatments at 1 and 2 h is shown in Figure 3a.

The decline in response to a second treatment was not due to a time-dependent decrease in the capacity of cells to respond to changes in oligomer concentration; untreated cells remained capable of a normal ethylene response to a first treatment with oligomers for several hours after subculturing (Fig. 3b). The decline in response was not due to a decrease in the activity of EFE induced by a first treatment; both untreated and oligomer-treated cells produced similar amounts of ethylene following addition of ACC for several hours after subcloning (Fig. 3c).

**Concentration-Dependent Reduction in Sensitivity**

Several experiments tested whether the concentration of the first oligomer treatment affected the decrease in the ethylene response to a second treatment. Following a first treatment with 500, 100, 25, or 0 µg of G7, a second treatment with 500 µg was added after 1 or 2 h (Fig. 4). When the

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**Figure 1.** Chromatography of mixtures of smaller (G7) or larger (G12) pectic oligomers on a QAE-Sephadex ion-exchange column with an imidazole gradient from 0.2 to 0.9 M (pH 7.0). Fractions were assayed for uronic acids (1) and relative conductivity (+).

**Figure 2.** Ethylene biosynthesis by pear cells after addition of (a) 500, 100, or 25 µg of smaller pectic oligomers (G7), or (b) 500 µg of smaller (G7) or larger (G12) pectic oligomers. Treatments with culture medium (H₂O) or with 500 µg galacturonic acid (G1) were used to account for dilution or pH and carbohydrate effects.
second treatment was added 1 h after the first, ethylene biosynthesis did not peak at 1.5 h, but continued to rise for 90 min after the second treatment to a peak at 2.5 h (Fig. 4a). The responses to combined doses greater than 500 μg were apparently saturated and were similar in peak rate and pattern of decline. When the second treatment was added 2 h after the first, any induced second peak also occurred 90 min after the second treatment (Fig. 4b). However, the peak of the ethylene response to a second treatment varied and was usually inversely related to the rate of the first ethylene peak (Fig. 4b): the greater the ethylene response to the first treatment, the more reduced was the ethylene response to a second treatment. The timing of reduction in sensitivity to a second treatment induced by a first was independent of the amount of oligomer added and always preceded the first peak in ethylene biosynthesis (data not shown).

Reduction of the second ethylene response did not require that the first and second treatments be chemically identical. A first treatment with G7 or G12 oligomers, or with an ethylene-inducing mixture of alginates, all reduced the response to a second oligomer treatment (data not shown). In all cases, the reduction in the second ethylene response was

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**Figure 3.** Induction of insensitivity to change in oligomer concentration between 1 and 2 h after a first treatment with pectic oligomers: (a) ethylene biosynthesis after addition of pectic oligomers (G7) as a single treatment of 100 or 500 μg, or as a first treatment of 100 μg followed by a second treatment of 500 μg added 1 or 2 h after the first; (b) ethylene biosynthesis after addition of 500 μg of pectic oligomers (G7) either as a first treatment or as a second treatment added 1 or 2 h after a first treatment with culture medium. (c) Ethylene biosynthesis after addition of ACC either as a first treatment or as a second treatment added 2 h after a first treatment with culture medium or with 100 or 500 μg of pectic oligomers (G7).

**Figure 4.** Concentration-dependent induction of insensitivity to change in oligomer concentration: ethylene biosynthesis after a first treatment with culture medium (H2O), or with 25, 100, or 500 μg of pectic oligomers (G7) followed by a second treatment of 500 μg added (a) 1 h later or (b) 2 h later. Ethylene biosynthesis after a single treatment with culture medium (H2O) or 500 μg of pectic oligomers (G7) also shown.
related to the ethylene response to the first treatment, not to the amount of inducer used.

Ethylene Biosynthesis During the Cell Growth Cycle

In a typical growth cycle, pear cells inoculated into fresh medium increased in density from 2.5 mg/mL to 30 mg/mL over a 6 d period (Fig. 5a). After 6 d, cell dry weight stabilized and then slowly decreased, the decrease perhaps due to metabolism of starch. The growth curve of the culture depended on the concentration of the initial cell inoculation; initial cell densities of 1.25, 2.5, and 3.75 mg/mL usually resulted in stable cell weight after 8, 6, and 5 d, respectively (data not shown).

Ethylene was produced by pear cells isolated throughout the cell growth cycle (Fig. 5, b and c). Ethylene biosynthesis occurred at the highest rates during the first 4 d of growth, then typically declined to half the peak rate through d 10. The peak ethylene biosynthesis rate was usually twice as high in cells grown with 2,4-D (Fig. 5b), but the relative decline from the peak rate was greater in cells grown without 2,4-D (Fig. 5c).

The rate of ethylene biosynthesis appeared to depend on the stage in the growth curve, not simply the elapsed time in culture. When initial inoculum level was increased from 1.25 to 2.5 mg/mL, and from 2.5 to 3.75 mg/mL, the decrease in ethylene biosynthesis was usually advanced by 1 d and 2 d, respectively (data not shown).

Sensitivity to Oligomers during the Cell Growth Cycle

Pectic oligomers added to pear cells induced a significant increase in ethylene biosynthesis only during part of the cell growth cycle (Fig. 5, b and c). In the first 4 d after inoculation, when uninduced ethylene biosynthesis was at its highest rates, added pectic oligomers could not induce a consistent increase in ethylene biosynthesis in either medium. After d 5, as uninduced ethylene biosynthesis decreased, added oligomers induced a significant increase in ethylene biosynthesis in both media. For cells grown without 2,4-D, added pectic oligomers raised ethylene biosynthesis to the earlier rates of uninduced cells during rapid growth (Fig. 5c). For cells grown with 2,4-D, added oligomers increased ethylene biosynthesis half way to the earlier peak rate (Fig. 5b).

Ethylene induction by pectic oligomers appeared to depend on stage in the growth curve, not on elapsed time in culture. Pectic oligomers could induce a significant increase in ethylene biosynthesis only as uninduced ethylene biosynthesis decreased, whether the decrease was delayed or advanced by establishment of cultures with different inoculum levels (data not shown).

DISCUSSION

Temporal Pattern of Ethylene Biosynthesis

Pectic oligomers and other cell wall oligosaccharides are known to induce a wide range of responses typical of wounded tissues when added to cells in suspension culture (16, 19, 20, 27). Thus, induction of ethylene in pear cells may be part of a more general response to added pectic oligomers. The
Increase in ethylene induced by oligomers is similar in pattern to the local increase induced by mechanical wounding of herbaceous tissues reported by Saltveit and Dilley (21). In both cases, after a brief lag in ethylene biosynthesis following induction, the rate of ethylene biosynthesis increases rapidly to a peak and then, almost as rapidly, declines. The rapid increase and the rapid decrease in biosynthesis both appear to be precisely regulated components of the ethylene response to oligomers and to wounding.

Most researchers who have examined the ethylene response to wounding have reported a more extended synthesis of ethylene following wound induction than reported here, without an early peak and decline (2, 10). This difference in the observed patterns may be a consequence of the experimental systems that have been used to study ethylene induction. Progressive induction of a brief response in increasing numbers of cells, which might occur in complex tissues, would result in extended ethylene production by the tissue. This extended ethylene production might not reflect the pattern of synthesis in individual cells. If a sequentially regulated response is progressively induced, individual cells will always be at different stages and later stages in a response sequence will not be observable. Chemical induction of ethylene biosynthesis in suspension culture may allow the simultaneous induction of a response in a large population of cells, and observation of later regulatory steps.

Mechanisms Limiting Ethylene Biosynthesis

The rapid increase in ethylene biosynthesis following wounding has been shown to be caused by the rapid de novo synthesis, accumulation, and activity of ACC synthase (2, 10, 28). Tong et al. (25) demonstrated that the ethylene increase in pear cells induced by cell wall fragments was accompanied by an increase in intracellular ACC. Little is known about the possible causes of the subsequent, rapid decrease in ethylene biosynthesis observed in our experiments.

Several mechanisms have been identified which might limit continued synthesis of ethylene after its induction. Ethylene itself has been shown to suppress synthesis of ACC synthase (9, 28) and to induce the malonylation of ACC in certain tissues (28, 29). However, in these experiments, inhibition by evolved ethylene cannot explain the observed decrease in ethylene synthesis. In experiments with single treatments, the timing of the decrease was the same regardless of the amount of ethylene evolved after treatment (Fig. 2). In experiments with second treatments, neither the timing nor the extent of the decrease in ethylene synthesis after the second treatment was related to the rate of synthesis at the time of treatment (Figs. 3 and 4).

In some tissues, continued ethylene synthesis can be limited by a decline in EFE activity (28). However, EFE activity does not appear to limit ethylene biosynthesis following ethylene induction by pectic oligomers. The ability to convert added ACC to ethylene remained high throughout each experiment, and was unaffected by a first treatment with ethylene-inducing pectic oligomers (Fig. 3c).

Induced Insensitivity to Oligomers

In these experiments, induction of ethylene biosynthesis by oligomers is followed by a decrease in the sensitivity of cells to further ethylene induction (Figs. 3 and 4). This induced insensitivity occurs within 90 min after an oligomer treatment, slightly preceding the decline in ethylene synthesis. The temporal coincidence between the decrease in sensitivity and the decline in synthesis suggests the possibility of a common regulatory mechanism. A change in sensitivity of cells or tissues to ethylene elicitors following induction of ethylene has not been previously noted by other researchers. However, Saltveit and Dilley (21) reported that additional wounding could not induce further ethylene synthesis after an initial induction of ethylene by wounding had run its course. Induced insensitivity to elicitors appears to represent a novel mechanism which may limit continued ethylene biosynthesis after ethylene induction.

Mechanisms of Induced Insensitivity

As shown above, the degree of insensitivity induced by oligomers is proportional to the ethylene response to the first treatment (Fig. 4). The quantitative correlation between oligomer, ethylene, and sensitivity might arise from a variety of physiological processes. We propose that a suspension culture contains cells which differ in sensitivity to changes in oligomer concentration. Addition of a given amount of pectic oligomers induces ethylene biosynthesis only in the fraction of the cell population sensitive to the resulting oligomer concentration. For the individual cell, induction of ethylene by pectic oligomers induces insensitivity to further changes in oligomer concentration. Thus, a first treatment with increasing amounts of oligomer results in ethylene biosynthesis in a progressively larger fraction of the cell population, and consequently leaves a progressively smaller fraction of the population able to respond to a second oligomer addition (Fig. 4). Such a model would explain the precise quantitative relationship between a first response to oligomers and the reduction in culture response to a second treatment after insensitivity has developed.

The precise timing of the reduction in sensitivity to oligomers suggests that the reduction results from an internal cellular process which is temporally coupled to cell induction. We hypothesize that expression of the oligomer-induced ACC synthase gene is a temporally determinate process. Once expression of the gene has ceased, approximately 60 min after its induction, we suggest that expression cannot be reinduced for some undetermined interval of time. Insensitivity to oligomers would indicate the end of gene expression, and the decline in ethylene synthesis would reflect the subsequent decline in ACC synthase activity.

Ethylene Biosynthesis and Induction during the Cell Growth Cycle

Although ethylene biosynthesis occurred throughout the pear cell growth cycle, it was at a maximum during the period of rapid cell growth, then declined as cell growth ceased. This relationship between ethylene biosynthesis and cell growth has also been observed for apple fruit cells in suspension...
culture (13). Pear cells in suspension culture appear to have a maximum rate of ethylene biosynthesis. Only as endogenous ethylene biosynthesis declined below this maximum could pectic oligomers induce an increase in ethylene biosynthesis and then only back to the previous endogenous maximum (Fig. 5). This maximum rate is apparently determined by the activity of ACC synthase, not by EFE activity.

A variety of water-soluble oligo- and polysaccharides is likely released into the suspension culture medium during cell growth (11, 23). It is possible that ethylene biosynthesis during cell growth is induced, in part, by these released carbohydrates. Ethylene biosynthesis might also be induced by other internal or external factors during the growth phase. Whatever the induction mechanism, cells synthesizing ethylene at a maximum rate during the cell growth cycle appear to be insensitive to changes in the concentration of pectic oligomers, a pattern consistent with the model of ethylene regulation outlined above.

Oligomer Induction of Ethylene and Wound Responses

Pectic oligomers and other cell wall polysaccharides can induce responses typical of wounded tissues when added to intact plant tissues or to cultured cells (16, 19, 20, 22, 27). Ethylene is generally recognized as a component of the response to wounding in intact tissues (6–9, 22, 26), but its relation to induction of associated responses in suspension culture has been largely neglected. Detailed comparison of the regulation of ethylene biosynthesis and of similar responses in suspension culture might provide insight into underlying regulatory mechanisms and into ethylene’s role as a signal controlling cellular responses to environmental events.

LITERATURE CITED