Genetic Differences in Wound-Induced Ethylene Production Among Different Clones of Salix viminalis L.

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(Received 20th July 1992)

Summary

Ethylene production from wounded leaves of *Salix viminalis* L. has been followed. The study included 24 clones belonging to 8 families. The relation between wound-induced ethylene accumulation for the clones and several resistance characteristics were studied. Ethylene accumulation differed significantly both among families and among clones, for example 75 ppm to 217 ppm and 70 ppm to 203 ppm respectively, 9h after wounding. No correlations between ethylene production and resistance to gall midge, rust, leaf beetle and trysin inhibitor production were observed. Both resistance to gall midge and rust varied considerably in the clones studied, so the lack of correlation indicates that we do not have a clear relationship between ethylene production and these traits.

Key words: Salix viminalis, ethylene accumulation, wounding, genetic variation.

Introduction

Salix is of interest as a bioenergy source in Sweden. In order to increase biomass production, intensive breeding is being conducted at our department. However, it is also important to reduce losses due to external factors such as pathogens and insects. To achieve control of the parasites we follow 2 lines. On the one hand we use our present day knowledge to select plant material and management regimes that minimize the negative effects of the parasites. On the other hand we aim at more knowledge of the host-parasite relationship so that we can improve the management. An important aspect of this relationship is the induced defence of the host.

Active defence of plants is induced by various pathogens and insects, and several times it has been shown that mechanical wounding can be made to mimic an attack. The same set of metabolic changes is found in many cases of active defence reactions. Alterations include cell wall thickening resulting from the production of macromolecules, and the production of defence enzymes and proteins (Fritig et al., 1987). Defence enzymes fall into 2 classes: enzymes that catalyse the production of various metabolites participating in resistance (ethylene, phytoalexins, aromatic compounds, oxidized metabolites) and direct defence enzymes (hydrolases such as chitinases and glucanases). The defence proteins include inhibitors of proteases.

Ethylene has been shown to transcriptionally regulate a number of plant genes. However it has been found that inhibition of stress ethylene accumulation by treatment with an inhibitor of ethylene production allowed induction of various stress responses following applications of fungal elicitors, and it was concluded that ethylene was not acting as an endogenous elicitor (Paradies et al., 1980; Mauch et al., 1984). The nature of the ethylene effect is not clear, but since ethylene is produced following wounding, this might be an amplification system to superstimulate the levels of wound inducible gene expression (Kernan and Thornburg, 1989).

Induction of ethylene biosynthesis is an early reaction of plants to pathogens. In recent years much interest has been focused on the dual role of ethylene: in the development of disease symptoms in susceptible plants and the converse in resistance reactions. Although the significance of enhanced ethylene biosynthesis in plant pathogen interaction is not clear, ethylene evolution has been shown to enhance the activity of specific enzymes thought to be involved in resistance (Stahmann et al., 1966; Chalutz et al., 1968; Toppan et al., 1982; Boller et al., 1983; Boller and Vögeli, 1984; Roby et al., 1986; Broglie et al., 1986; Schlumbaum et al., 1986; Ecker and Davis, 1987; Vögeli et al., 1988; Mauch and Staehelin, 1989; Broglie et al., 1989; Weiss and Bevan, 1991; Keefe et al., 1990; Ishige et al., 1991).

The aims with this work were 1) to compare wound-induced ethylene production in different clones of *Salix viminalis* L., and 2) to correlate the rate and amount of ethylene production after wounding with other traits, e. g. wound-induced production of proteinase inhibitor (PI), resistance to a specialist leaf beetle (*Galerucella lineola* Müll.), a specialist gall midge (*Dasineura marginemtorquens* Bremi) and a rust fungus (*Melamspora* ssp.).

Material and Methods

2.1 Resistance studies

The willow clones (*Salix viminalis* L.) that have been used in these experiments have previously been tested in the field for growth characters.

For the 4 field experiments, 8 females and 8 males were crossed in a factorial crossing. Each family contained 6 to 12 offspring, and each offspring was represented by 6 to 8 vegetatively propagated individuals (Strong et al., 1993). Out of the 64 possible families 40 were included in the experiments. The experiments with gall midge (*Dasineura marginemtorquens* Bremi) and rust (*Melampsora* ssp.) were serendipitous. The gall midge unexpectedly colonized one of the field trials in 1988. The midge, a sucker, oviposits on not fully developed leaves in the shoot apex, and thereby initiates the gall. The degree of resistance was expressed as the number of leaves with galls per shoot. Clones that showed resistance in 1988 reacted similarly in 1989 (Strong et al., 1993). Dicariotic spores from the fungus *Melampsora* enter the leaf stomata and develop uridinia that grow out from the stomata and cover a circular area of the leaf. The rust was studied in the field at three different occasions during one fall (Gullberg and Ryttman, 1993). The leaves were classified according to the degree of attack. The leaf beetle (*Galerucella lineola* Müll.), a leaf-chewing insect, was tested

Table 1. — Ethylene accumulation induced by wounding from leaves from 24 different clones belonging to 8 different families. Ethylene was measured 1h or 9h after wounding. Data shown are mean values from 2 independent analyses.

Ethylene production, nl / µl							
Clone comparisons					Family comparisons		
	1h		9h			1h	9h
Clone	mean	s.d	mean	s.d	Family	mean	mean
22-08	54	10	230	10	22	63	217
22-11	53	17	170	0			
22-12	82	6	250	11			
23-08	37	6	133	1	23	26	100
23-09	11	1	70	6			
23-10	23	0	98	17			
30-06	14	3	85	1	30	13	75
30-08	12	0	65	4			
31-06	18	1	103	15	31	36	125
31-08	17	4	70	7			
31-09	74	4	203	1			
38-05	15	1	75	2	38	18	107
38-10	14	4	65	16			
38-13	21	10	161	33			
38-14	22	8	125	22			
39-09	20	2	123	20	39	12	104
39-10	13	1	85	20			
46-08	21	3	95	11	46	17	75
46-09	19	0	61	3			
46-10	15	1	130	2			
46-11	15	1	79	3			
46-13	14	3	10	7			
47-10	17	2	107	8	47	16	62
47-14	16	4	16	2			

for time of development and dry weight of female versus male cocoons for a subset of the clones in the experiment (J. FORSBERG, personal communication).

In the present study we have used 24 clones from 8 families (*Table 1*).

2.2 Plant material

Shoots from the plants of the different clones in the field experiments were collected and stored at 4° C. Five cm long cuttings were collected from the stems and placed into pots (diam, 8 cm) with planting substrate. They were grown in a greenhouse with 18 / 6 h light / dark regime, with a light intensity of 500 μE m^{-2} s^{-1}, and a constant temperature of 22° C. After 5 weeks the plants were randomly placed into two separate blocks containing eight individuals from each of the 24 clones. After 10 weeks to 12 weeks, when they were 30 cm to 40 cm in height, they were used for experimentation. Every plant was used for only one experiment.

2.3 Ethylene determination

Ethylene accumulation was determined for wounded and unwounded leaves. One leaf, with a weight of about 0.1g, was excised and crushed between the end of a small circular glass dowel and the rough surface of a flat file. The wounds were always made from the petiole and along the midrib. The wound size was 0.5 cm^2. The wounds were made at time 0 (immediately after excision). Another leaf at approximately the same physiological age was excised from the same plant and used as a control. The leaves were incubated in gastight glass tubes (40 ml Vari-Clean Vials from Pierce, No. 13510). The tubes were placed in a growth chamber at a light intensity of 30 μE m^{-2} s^{-1} and a constant temperature of 22° C. If not otherwise stated, samples were taken 1 h, 3 h, 5 h, 7 h and 9 h after wounding. Before 500 μl gas samples were taken with a Hamilton syringe, the tubes were shaken gently. Ethylene was determined by gas chromatography (Chrompack CP 9000 gas chromatograph). Ethylene was identified and calculated in comparison to the retention time and peak area of ethylene standards. Each clone was analyzed twice, in different plants. The total number of samples measured was 96.

2.4 Proteinase inhibitor assay

Trypsin inhibitor activity was induced in intact willow plants by mechanical wounding, identical to that used for ethylene. The wounds were made 24 h before harvest, and the 6 to 7 uppermost unwounded leaves were collected. As control the 6 to 7 uppermost leaves were collected from unwounded plants at the same time. The leaves were placed in aluminium foil, frozen in liquid N_2, and then stored at —70° C until use.

Protein extracts were prepared from the frozen leaf tissues. Unless otherwise indicated, all steps were performed at 4° C. Approximately 500 mg of willow leaves (fresh weight), were frozen in liquid N_2 and then ground by using a mortar and a pestle until a fine powder was obtained. Five ml of buffer I (10 mM 2-mercapto-ethanol, 4% PVP 360, 50 mM phosphate buffer, pH 7) was added to the powder and ground again. The homogenate was sonicated for 2 min, and stirred overnight. To the homogenate, 1.5 ml saturated ammonium sulphate was added and after 15 minutes the supernatants were collected and centrifuged at 20 000 g for 10 minutes. The supernatants were collected and supplied with ammonium sulphate to 90% saturation and allowed to stir overnight. The supernatants were recentrifuged at 20 000 g for 10 minutes. The pellets were resuspended in 130 μl buffer II (50 mM Tris/HCl pH 8.2, 10 mM 2-mercapto-ethanol), and desalted with Nick Spin Columns, Pharmacia, containing Sephadex G-50 at 500 g. The samples were assayed immediately for protein by the method of BRADFORD.

Trypsin inhibitor activity was assayed spectrophotometrically according to the method described by HILDER (personal communication). The rate of change of O. D. was measured at 405 nm with a model Hitachi U 2000 spectrophotometer. Leaf extracts were preincubated with 25 μg trypsin SIGMA, No. T-8253) in 1 mM HCl, and 50 mM tris buffer (pH 8.2), to give a final volume of 0.8 ml. Trypsin inhibitor activity was measured after 4 min. The assay was initiated by adding to the preincubation mixture 0.2 ml of the substrate BAPNA (15.75 mg/ml DMSO, SIGMA, No.B-4875). All steps were performed at 30° C. Each clone was analyzed twice. The total number of samples analyzed was 96.

2.5 Statistical calculations

All statistical calculations were made by using SAS, General Linear Models Procedure, on an Apple software program (JMP). The model used for analysis of variance was, ethylene=family+clone[family]+block+error.

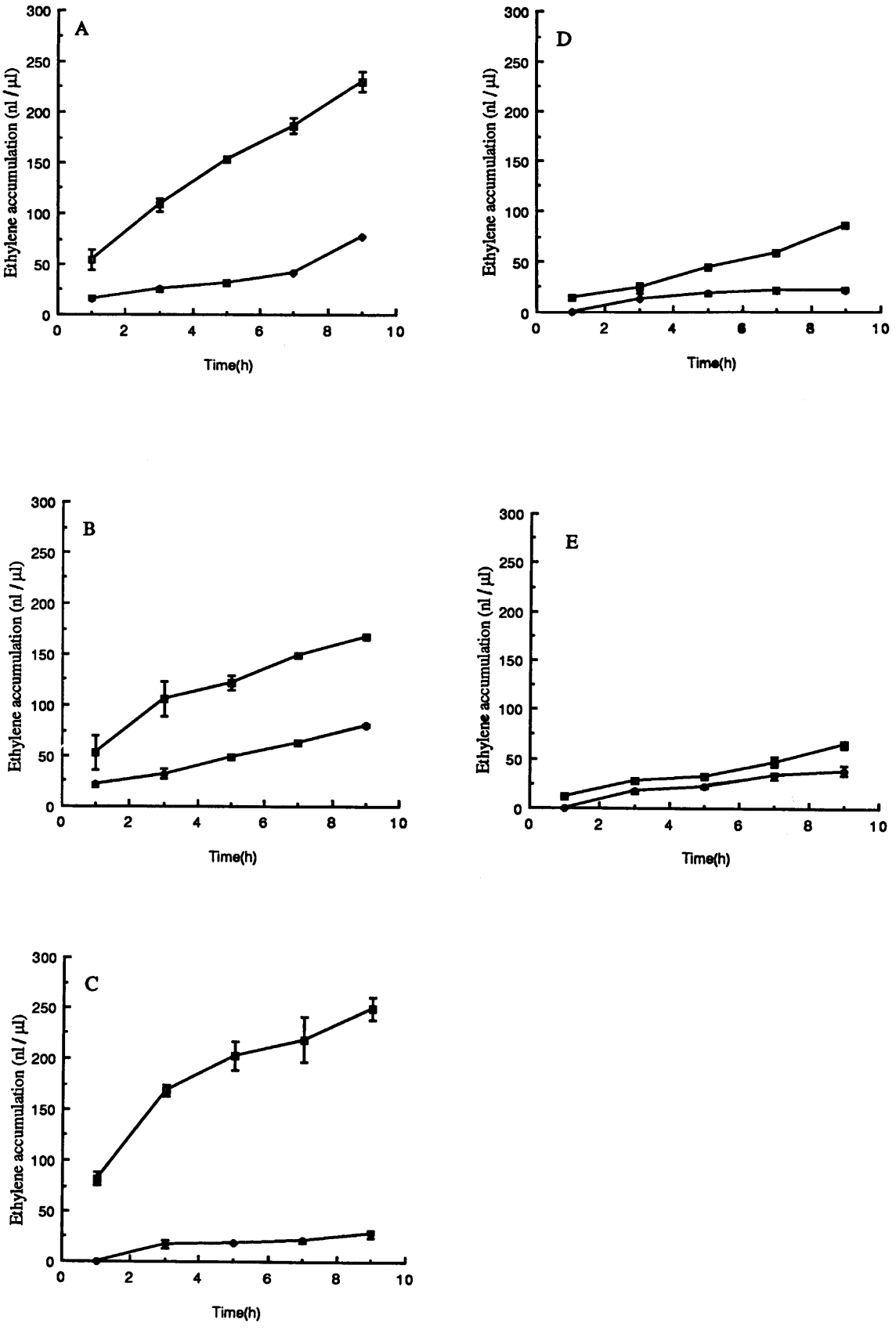

Figure 1. — Ethylene accumulation in wounded and unwounded leaves of *Salix viminalis*. A, B and C show data from three different clones (22-08, 22-11 and 22-12 respectively) belonging to family 22. D and E show data from two different clones (30-06 and 30-08) belonging to family 30. Ethylene was measured for times up to 9h. Filled squares (-■-) give data for control leaves and open squares (-□-) for wounded leaves. Data are based on 2 analyses. Bars show the SD.

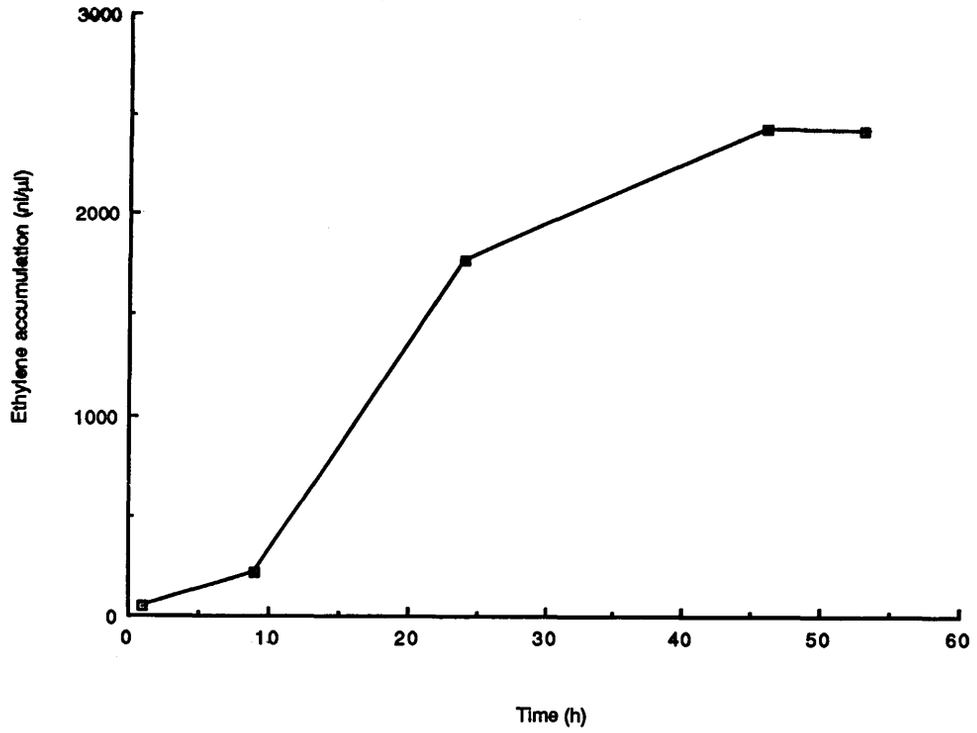

Figure 2. — Wound-induced ethylene accumulation in leaves from clone 22-08 of *Salix viminalis*. Ethylene was measured 1h, 9h, 24h, 46h, and 53h after wounding.

Results and Discussion

3.1 Time-course for ethylene production

The time-course for ethylene production in control and wounded leaves was followed in 24 clones. Data for some of the clones are presented in *figure 1*. In general the relationship in ethylene accumulation between wounded and control leaves was almost the same for most clones. A clone producing high levels of ethylene in the wounded leaves also produced relatively high levels in the control leaves compared to low producing clones. However, as shown in *figure 1*, some clones behaved differently.

Change over time in ethylene production in response to mechanical wounding in willow leaves proved to be almost linear over the first 10 hours (*Fig. 1*). When the production was analysed for a longer period of time it reached a plateau after about 40 h (*Fig. 2*).

The results show that a rapid accumulation of ethylene takes place after wounding. The time-course and amount of ethylene accumulation in response to wounding in *Salix viminalis* is similar to that reported in *Solanum tuberosum* (Weiss and Bevan, 1991). No ethylene, however, was detected in unwounded leaves of *Solanum tuberosum* while as previously mentioned many clones of *Salix viminalis* produced considerable amounts of ethylene even in control unwounded leaves. This might be due to a stress reaction caused by the excision of the *Salix* leaves.

Detached leaves exhibit under experimental conditions a rapid ethylene burst following excitation and a subsequent climacteric rise in ethylene production. The initial ethylene rise is considered as an excision-related response (Kao and Yang, 1983). The second climacteric rise, which is associated with advanced senescence, was found also in attached leaves that senesce on the plant (Aharoni et al., 1979). Leaf discs of *Spinacia oleracea*, a severely wounded and fast senescing system, were characterized by an immediate and sharp burst of ethylene which subsided after a few hours (Philosoph-Hadas et al., 1991). The severe wounding altered the magnitude of the climacteric ethylene rise as well. Consequently the degree of wounding was expressed in both magnitude and duration of the wound-ethylene. We think that ethylene accumulation during the first 10 h in *Salix* leaves is wound induced and that the later accumulation is due to senescence.

3.2 Genetic variation in ethylene production

Statistical analysis of ethylene accumulation after wounding in leaves of different clones showed that there were significant differences both among families, $p = 0.024$ and among clones within families, $p = 0.040$. No block difference was observed, $p = 0.800$.

The production of ethylene 9 h after wounding varied from 62 ppm to 217 ppm in the different families (*Tab. 1*). However, even within families there was great variation; for example in family 31 it varied between 70 ppm and 203 ppm.

One hour after wounding, members of 3 families (22, 23 and 31) responded more heavily than clones from any other family (*Tab. 1*). These clones were 22-8, 22-11, 22-12, 23-8 and 31-9. Furthermore, 9 hours after wounding clone 38-13 also proved to be a high producer of ethylene when compared to other clones.

There is great genetic variation in ethylene production after wounding in *Salix viminalis*, but our data do not allow us to state anything about the mode of inheritance. These results tell us that we cannot generalize about the ethylene production in a species from studies of a single clone.

Table 2. — Trypsin inhibition in leaves from 24 different clones of *Salix viminalis* belonging to 8 different families. Trypsin inhibition was measured 24 h after wounding. Data shown are mean values from 2 independent analyses. The asterisk (*) means that only one analysis was performed.

Family	Clone	Trypsin inhibition % inhibition / µg protein mean ± s.d
22	22-08	2,7 ± *
	22-11	1,1 0,3
	22-12	1,6 0,6
23	23-08	3,9 2,2
	23-09	1,5 0,5
	23-10	2,5 *
30	30-06	1,6 0,2
	30-08	1,5 0,3
31	31-06	1,6 0
	31-08	0,7 0,3
	31-09	1,2 0,2
38	38-05	0,5 0,4
	38-10	1,6 0,3
	38-13	0,4 0
	38-14	0,7 0
39	39-09	1,3 0,4
	39-10	0,7 0,3
46	46-08	0,6 0
	46-09	0,9 0,1
	46-10	0,5 0,1
	46-11	1,2 0,2
	46-13	0,5 0,2
47	47-10	0,6 0,1
	47-14	1,7 0

3.3 Correlation between ethylene production after wounding and other traits for different clones

Wounded leaves of *Salix viminalis* produce proteinase inhibitors (*Tab. 2*). The amount of inhibitor produced varied between different families but also within families. The content of proteinase inhibitor in wounded leaves of *Salix viminalis* (>1% of the total protein) is comparable to what has been reported for tomato and potato (Green and Ryan, 1972; Ryan, 1978). No significant correlation between ethylene production and PI content after wounding was observed (*Fig. 3A*). In this experiment we had some

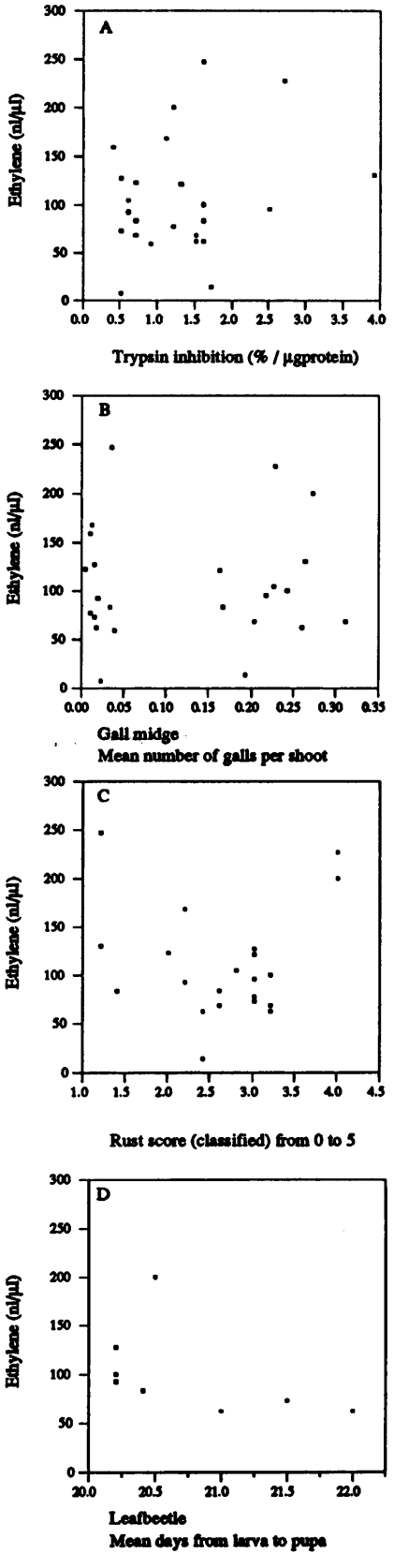

Figure 3. — Correlation between wound-induced ethylene accumulation in different clones of *Salix viminalis* to the production of trypsin inhibitor after wounding and to resistance to insects and a pathogen. Ethylene accumulation was determined in leaves 9h after wounding. Each spot represents mean values per clone.
A. Production of trypsin inhibitor after wounding. Trypsin inhibition was measured in 24 clones 24h after wounding.
B. Resistance to gall midge. The data show mean number of galls per shoot in 24 clones.
C. Resistance to rust. The data show rust score (classified from 0 to 5 per leaf) in 22 clones.
D. Resistance to leaf beetle. The data show the mean number of days for larvae to develop into pupae on leaves from 8 different clones.

insects in the greenhouse that could have influenced the induction of PI, at least for some clones where the s. d. were high (*Tab. 2*). However, for most of the clones we think that these data are representative. For wound induced PI it has previously been shown that ethylene induced the expression of PI genes (KERNAN and THORNBURG, 1989). Furthermore, systemic expression of *win2* in *Solanum tuberosum* required both a putative wound signal and ethylene (WEISS and BEVAN, 1991). In this study the poor correlation between ethylene accumulation and PI production indicates that ethylene alone is not an inducer of PI (*Fig. 3A*).

No significant correlations between ethylene production and resistance to gall midge and rust were observed (*Figs. 3B* and *C*). Both resistance to gall midge and rust varied considerably in the clones studied, so the lack of correlation indicates that we do not have a clear relationship between ethylene production and these traits. The resistance to leaf beetle was less variable among the genetic units observed which makes it impossible to exclude a relationship between ethylene production and leaf beetle resistance ($r^2=0.2$) (*Fig. 3D*). The lack of correlation between the wound-induced metabolic changes studied and resistance could perhaps be explained by insufficient ethylene and PI being formed. However, that is not very probable since the amount of ethylene produced exceeds the levels of exogenously applied ethylene that in fact have been shown to increase other defence enzymes such as for example chitinase 40 to 50 times (BROGLIE et al., 1989). Obviously more detailed studies are required for evaluating induced defence reactions in *Salix*.

Acknowledgements

This research was supported by grants from the National Board for Industrial and Technical Development and by Troëdssons Fund. The assistance of EWA WINKLER in growing the plants for this research is gratefully acknowledged.

References

AHARONI, N., LIEBERMAN, M. and SISLER, H. D.: Patterns of ethylene production in senescing leaves. Plant Physiol. **64**: 796—800 (1979). —— BOLLER, T., GEHRI, A., MAUCH, F. and VÖGELI, U.: Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta **157**: 22—31 (1983). —— BOLLER, T. and VÖGELI, U.: Vacuolar localization of ethylene-induced chitinase in bean leaves. Plant Physiol. **74**: 442—444 (1984). —— BROGLIE, K. E., BIDDLE, P., CRESSMAN, R. and BROGLIE, R.: Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. The Plant Cell **1**: 599—607 (1989). —— BROGLIE, K. E., GAYNOR, J. J. and BROGLIE, R. M.: Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proc. Natl. Acad. Sci. USA **83**: 6820—6824 (1986). —— CHALUTZ, E., DEVAY, J. E. and MAXIE, E. C.: Ethylene-induced isocoumarin formation in carrot root tissue. Plant Physiol **44**: 235—241 (1968). —— ECKER, J. R. and DAVIS, R. W.: Plant defense genes are regulated by ethylene. Proc Natl. Acad. Sci. USA **84**: 5202—5206 (1987). —— FRITIG, B., KAUFFMANN, S., DUMAS, B., GEOFFROY, P., KOPP, M. and LEGRAND, M.: Mechanism of the hypersensitivity reaction of plants. Plant resistance to viruses. Wiley, Chichester (Ciba Foundation Symposium 133) 92—108 (1987). —— GREEN, T. R. and RYAN, C. A.: Wound-induced proteinase inhibitor in plant leaves. A possible defense mechanism against insects. Science **175**: 776—777 (1972). —— GULLBERG, U. and RYTTMAN, H.: Genetics of field resistance to *Melampsora* in *Salix viminalis*. European J. Forest Pathol., in press, (1993). —— ISHIGE, F., YAMAZAKI, K., MORI, H. and IMASEKI, H.: The effects of ethylene on the coordinated synthesis of multiple proteins: accumulation of an acidic chitinase and a basic glycoprotein induced by ethylene in leaves of azuki bean, *Vigna angularis*. Plant Cell Physiol. **32**: 681—690 (1991). —— KAO, C. H. and YANG, S. F.: Role of ethylene in the senescence of detached rice leaves. Plant Physiol. **73**: 881—885 (1983). —— KEEFE, D., HINZ, U. and MEINS, F., JR.: The effect of ethylene on the cell-type-specific and intracellular localiztion of β-1,3-glucanase and chitinase in tobacco leaves. Planta **182**: 43—51 (1990). —— KERNAN, A. and THORNBURG, R. W.: Auxin levels regulate the expression of a wound proteinase inhibitor II-chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. Plant Physiol. **91**, 73—78 (1989). —— MAUCH, F., HADWIGER, L. A. and BOLLER, T.: Ethylene: symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol. **76**: 607—611 (1984). —— MAUCH, F. and STAEHELIN, A.: Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. The Plant Cell **1**: 447—457 (1989). —— PARADIES, I., KONZE, J. R., ELSTNER, E. F. and PAXTON, J.: Ethylene: indicator but not inducer of phytoalexin synthesis in soybean. Plant Physiol. **66**: 1106—1109 (1980). —— PHILOSOPH-HADAS, S., MEIR, S. and AHARONI, N.: Effect of wounding on ethylene biosynthesis and senescence of detached spinach leaves. Physiologia Plantarum **83**: 241—246 (1991). —— ROBY, D., TOPPAN, A. and ESQUERRE-TUGAYE, M. T.: Elicitors of ethylene from *Colletotrichum lagenarium* trigger chitinase activity in melon plants. Plant Physiol. **81**: 228—233 (1986). —— RYAN, C. A. Proteinase inhibitors in plant leaves: A biochemical model for pest-induced natural plant protection. Trends Biochem. Sci. **3**: 148—150 (1978). —— SCHLUMBAUM, A., MAUCH, F., VÖGELI, U. and BOLLER, T.: Plant chitinases are potent inhibitors of fungal growth. Nature **324**: 365—367 (1986). —— STAHMANN, M. A., CLARE, B. G. and WOODBURY, W.: Increased disease resistance and enzyme activity induced by ethylene production by black rot infected sweet potato tissue. Plant Physiol **41**: 1505—1512 (1966). —— STRONG, D., LARSSON, S. and GULLBERG, U.: Discontinuous and continuous genetic variation in resistance of *Salix viminalis* L. to gall-midge attack. Evolution (In press) (1993). —— TOPPAN, A., ROBY, D. and ESQUERRE-TUGAYE, M. T.: Cell surfaces in plant-microorganism interactions. Plant Physiol. **70**: 82—86 (1982). —— VÖGELI, U., MEINS, F., JR. and BOLLER, T.: Coordinated regulation of chitinase and β-1,3-glucanase in bean leaves. Planta **174**: 364—372 (1988). —— WEISS, C. and BEVAN, M.: Ethylene and a wound signal modulate local and systemic transcription of *win2* genes in transgenic potato plants. Plant Physiol. cription of *win2* genes in transgenic potato plants. Plant Physiol. **96**: 943—951 (1991).

Quantitative Genetic Parameters for Seven Characters in a Clonal Test of Salix eriocephala

II. Genetic and Environmental Correlations and Efficiency of Indirect Selection

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(Received 10th August 1992)

Summary

The relationship between seven characters that are important in biomass production was examined using 20

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