ETHYLENE BIOSYNTHESIS DURING LEAF MATURATION AND SENESCENCE IN WHITE CLOVER

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ABSTRACT

Aspects of leaf senescence in relation to ethylene biosynthesis in the plagiotropic herbaceous plant white clover (*Trifolium repens* L.) have been studied. Two stolons growing from clonally propagated plants were trained over a dry substratum that inhibits nodal root growth, and all axillary stolons and flowers were removed. White clover plants grown using this method produce leaves at all stages of development along a single stolon from initiation at the apex, through expansion, maturity, senescence and then necrosis. The study shows that modification of the stolon growth habit of white clover by the suppression of nodal roots provides a suitable system for the study of leaf development and senescence in relation to ethylene metabolism.

The pattern of leaf development (the number of leaves at each stage of development present on the stolon) and senescence (as measured by changes in leaf chlorophyll content) along the white clover stolon is consistent between plants of the same genotype growing under the same environment, but varied greatly between the different cultivars and genotypes examined. The rate of change between the different stages of leaf development and senescence within the one genotype used in this study, AgResearch Grassland genotype 10F, differed when grown under two different environments.

On mature stolons (stolons with 6 or more nodes with senesced leaves) of genotype 10F grown using the modified stolon system, the number of green leaves was maintained at a constant number as the leaf appearance rate was balanced by the senescence rate. However, the number of leaves maintained on the stolons differed between the two growing environments used in this study, from 9.85 +/- 0.23 for plants grown at Levin, to 14.57 +/- 1.99 for plants grown at Palmerston North.

The total chlorophyll concentration in the leaves from plants grown at Levin increased from leaf one (the youngest opened leaf; 740 µg/g.fw) to a maximum in leaf five (mature green leaf; 2240 µg/g.fw), declined rapidly from leaf five to leaf seven (senescing leaves; 1500 µg/g.fw), and then remained constant from leaf seven to leaf ten. A similar pattern of change in chlorophyll concentration was measured in leaves from plants grown at Palmerston North, but the maximum concentration was found in leaf 4 (1750 µg/g.fw), remained relatively constant to leaf 8, before decreasing in leaf 9 (750 µg/g.fw) and declining to a minimum in leaf 15 (250 µg/g.fw). The chlorophyll *a:b* ratio in mature green leaves from plants grown at Palmerston North (1.46:1 to 2.63:1) was lower than the ratio in leaves from plants grown at Levin (3.72:1 to 4.98:1).

Leaves of white clover produce ethylene. Ethylene evolution from attached

leaves varied from 1 nL/g.fw/h (mature green leaves) to 3 nL/g.fw/h (senescing leaves). Ethylene evolution from detached leaves was initially high (12.6 nL/g.fw/h at 15 min) but declined to 3.8 nL/g.fw/h by 45 min before increasing again.

Detached mature green leaves (leaves four to six) of white clover are sensitive (as measured by chlorophyll loss) to low concentrations (<1 ppm) of exogenous ethylene. The chlorophyll concentration in these leaves after four days of ethylene treatment (1, 10 or 100 ppm ethylene) was significantly lower than the chlorophyll concentration in freshly harvested leaves. However, the chlorophyll concentration in leaves two and three (early mature green) treated with ethylene was not significantly different from the concentration in freshly harvested leaves.

1-aminocyclopropane-1-carboxylic acid (ACC) concentration was low in leaves one to four (<1 nmoles/g.dw), increased to reach a maximum concentration of 11.4 nmoles/g.dw in leaf seven and declined to 2 nmoles/g.dw in leaf ten. 1-aminocyclopropane-1-carboxylic acid (MACC) concentration was highest in leaf one (11.3 nmoles/g.dw), declined to 6 nmoles/g.dw in leaf two, and remained constant for all other leaves.

ACC synthase activity could not be determined in protein extracts from white clover leaf tissue. ACC oxidase activity in protein extracts varied in the different leaves examined. The activity versus substrate concentration curve for leaves one, three, five and six displayed saturation kinetics with respect to the substrate, ACC, whereas the data for leaves eight and ten did not show saturation kinetics over the range of ACC concentrations used. The ACC oxidase activity varied from 0.81 nL/mg.protein/h in extracts from leaf six, to 1.64 nL/mg.protein/h for leaf five. The apparent Km varied from 61 μM for leaf six to 138 μM for leaf five, while the Vmax varied from 0.92 for leaf six to 2.06 for leaf five.

Degenerate oligonucleotide primers corresponding to conserved regions found among diverse ACC synthases were used for reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify DNA fragments from RNA extracted from white clover leaf tissue. A DNA clone, ACS7, showed 88% homology at the nucleotide level to ACC synthase from *Glycine max.* The ACS7 sequence contained the three conserved domains (including the reaction centre, and the three residues known to bind the pyridoxal phosphate coenzyme) identified in published ACC synthase sequences. The derived amino acid sequence for the conserved domains are identical with other published sequences. Southern analysis indicates ACC synthase is represented by a multigene family in white clover. Northern analysis of the expression of ACC synthase using ACS7 as a hybridisation probe was unsucessful.

Preliminary screening of a white clover leaf cDNA library produced a clone with 72.5% homology to a putative cysteine proteinase from *Pisum sativum,* and 63.4% homology to a cysteine proteinase from *Vicia sativa.*

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ATP	adenosine triphosphate
AdoMet	s-adenosylmethionine
a.i.	active ingredient
AOA	aminooxyacetic acid
AVG	aminoethoxyvinylglycine
BSA	bovine serum albumin
CAP	calf alkaline phosphatase
CTP	cytosine triphosphate
cDNA	complementary DNA
CoA	coenzyme A
DMF	N, N-dimethylformamide
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EFE	ethylene-forming enzyme
HOAc	acetic acid
IAA	indole-3-acetic acid
KOAc	potassium acetate
MACC	1-aminocyclopropane-1-carboxylic acid
PAGs	photosynthesis-associated genes
PCR	polymerase chain reaction
pfu	plaque forming units
ppm	part per million
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SAGs	senescence-associated genes
SA-PMP's	strepdavidin para-magnetic particals
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane