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Final Report on project: Factors affecting the formation of flavour compounds and their transfer to grape berries

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INTRODUCTION

The experiments carried out were designed to detect flavour components made in grape berries which were transported into the fruit from leaves. These were sought by GC/MS of toluene extracts of the exudate from cut leaf petioles, from stems of fruit bunches, from leaf and fruit homogenates. The exudates were also dried over silica gel and ether extracts were methylated with diazomethane.

Attempts to label potential flavour terperoids by feeding [14_C] pyruvate to vine leaves gave such an extremely large number of labelled products that the method could not be used to track flavour components. At least it established that pyruvate applied to the lower surface of vine leaves can be incorporated into metabolism *in vivo*.

Abscisic acid is the antistress hormone that is synthesized when a leaf wilts so that the content increases about 40 fold. It stimulates fruit ripening and accelerates the formation of the red pigments of grape skin during ripening. ABA was abundant in the exudate of vine leaves, particularly those that had started to wilt, so it was concluded that wilting leaves could hasten ripening of grapes.

The greatest difficulty experienced with these experiments was the capacity of the excised vine leaves to absorb the water of test solutions rather than to exude their phloem contents into them. This had to be overcome by spraying the cut leaves with a mist spray and placing damp tissue paper against their lower surfaces. Hartung has found that ABA moves around the plant in the phloem.

METHODS

Collection of phloem exudate from leaf petioles

The leaf petioles were sliced near their junction with the stem by a clean cut with a razor and the cut end placed in 20 mM NaEDTA for fifteen minutes. In dry weather it was found that the leaves had a water deficit which caused them to absorb the EDTA solution so they were sprayed with a distilled water mist 10 minutes before cutting and a sheet of damp filter paper was placed against their lower surfaces.

The cut petioles were then placed in 2 mL distilled water in glass scintillation vials with up to 10 leaves sandwiched together with damp filter papers for periods of 2 to 6 hours to bleed their phloem exudates into the water. The leaves were kept damp until weighed and photocopied so that their area could be measured with a planimeter.

Exudate from grape bunch stems

The stem supporting the grape bunch is shorter than the leaf petioles and requires that the EDTA solution applied to the cut surface to prevent sealing of the phloem is held on with adhesive tape. The bunch of grapes was removed with a razor cut and the EDTA applied within 5 secs. The cut stem was then washed and a glass vial of 10 mL distilled water applied. Spraying nearby leaves with water beforehand failed to overcome the water deficits of the vines and several mL of water were absorbed by the vine in spite of attempts to saturate nearby foliage. Successful experiments were carried out on a vine in a garden which was thoroughly watered for three days beforehand and well wetted on the day of the experiment. The glass vial was held with the water level just above the cut end of the phloem.

Wilting

One of the concerns of the work was that the dry conditions of the field vines, which resulted in their taking up the dilute EDTA solutions and the water was that the drying leaf would be triggered to produce large excess of "stress" Abscisic acid (ABA) with a concurrent disruption of metabolism. This was measured by deliberately wilting leaves by placing them between filter papers soaked in 0.6 M sorbitol. This caused rapid wilting and the production of "stress" ABA. In most experiments wilting was prevented by placing wet filter paper between the 6-10 leaves exuding into one glass vial.

Measurement of ABA

The ABA in the exudate of normal wet leaves was measured by adjusting the pH to 3 with 0.5 M H_2SO_4 and extracting with diethyl ether. The ether was removed and dried under a stream of nitrogen. The acids were methylated with one or two drops of diazomethane in ether. The solution was transferred to a glass (100 mL) tapered sample tube and dried. The 100 pH toluene were added and the samples analysed by Mass Spectrometry.

Hydrolysis of flavour components

Alkali – 2 mL of the aqueous residue remaining after acidification and ether extraction was adjusted to pH 8-9 with M NaOH and warmed to 40^{o}C for 30 minutes to hydrolyse any alkali labile conjugates present in the exudate. The solution was neutralised with H_2SO_4

and extracted with ether as before. After evaporation half of the residue was taken up in 0.5 mL water and smelt and tasted for wine flavour compounds. Half was transferred to a tapered glass sample insert for automatic GC/MS analysis.

Acid – 2 mL of the aqueous residue remaining after the initial acidification was taken to pH 1.5 to 2 with 0.5M H_2SO_4 and heated to 40^{o}C for 30 min, then neutralised and extracted with ether. After evaporation half of the residue was taken up in 0.5 mL water and smelt and tasted for wine flavour compounds. Half was transferred to a tapered glass sample insert for automatic GC/MS analysis.

Extraction of Vine Leaves

The leaves (50g) were homogenised by grinding with sand in a glass pestle and mortar and then the pulp was extracted with acetone for 10-12 hours, it was filtered and evaporated. 20 mL water was added and stirred then 5 mL were taken and centrifuged. A 2 mL subsample was extracted with ether and analysed as before.

Feeding [2-^{14}C] pyruvate

[2-^{14}C] pyruvate was applied to Chardonnay vine leaves growing in a garden in Sydney by spreading it over the lower surface (twisted to be upwards). 2% Tween 80 allowed the surface to be wetted and 5% glycerol was added as a humectant. After 6 hours the leaf's petiole was cut off close to the stem and treated with EDTA as usual and allowed to exude into water for a further 3 hours. The exudate solution was evaporated in a Hetovap before tlc.

^{14}C Analysis

For analysis of ^{14}C labelled materials in the treated vine leaves, a sample of the acetone extract was evaporated as a spot near the corner of a 200 x 200 mm silica gel on glass tlc plate then chromatographed in one direction in toluene/acetic acid/ethyl acetate (50:4:30 v/v) containing 10 mg/L butylated hydroxy toluene as an antioxidant. The plate was dried then chromatographed at right angles in a different solvent (hexane/ethyl acetate (5:2 v/v) and chloroform/methanol/water (10/20/2 v/v) were used to separate less polar and more polar metabolites respectively.

After thorough drying the radioactive spots were detected by a Phosphor Imager, Molecular Dynamic, CA., USA

Collection of exudate from leaves and grape bunch stems

The area of a vine bearing healthy leaves was sprayed with a mist of water half to 1 hour before the leaves were sampled. The healthy green grape leaves were selected and cut with a sharp razor to leave the longest petiole length possible and the cut ends placed in a 20 mM solution of EDTA for 15 minutes to prevent sealing of the phloem vessels (King and Zeevaart, 1974, Plant Physiol. *53*, 96-103).

The cut ends were then held in a small volume of water in a glass vial so that any exudate would mix with the water and not be reabsorbed by the xylem. The vials were agitated at intervals to ensure good mixing.

Extraction

Exudates of excised leaves were extracted with a minimum of analar toluene (0.2 mL) on a 2 mL subsample of the water and phloem exudate.

2 mL samples in weighed, short glass tubes (25 mm high x 13 mm internal diameter) and placed in sealed jars with dry silica gel pellets. After the water had evaporated the residue was weighed and subjected to analysis.

Analysis of Exudates

2 mL of the aqueous, collected exudates were tasted to detect any grape flavour constituents. The liquid was warmed in 25 x 13 mm tubes by placing them in warm water (50^{o}C) in a beaker but separated from the observer with several layers of gladwrap. The liquid was smelt carefully as the temperature rose.

RESULTS

The main result of the experiments was that no wine flavour constituents could be detected in the phloem exudate coming out of leaves or in the exudate of grape bunches.

The major component was identified as sucrose so it is concluded that all volatile flavour components are synthesised in the grape berries. The rate of exudation was measured as 0.59 μg/cm^2leaf/hour. The only flavour compounds detected in the exudate were benzoic and jasmonic acids.

Assimilation of [^{14}C] pyruvate

One of the surprising metabolic discoveries of the last decade was that terpenoids synthesised in chloroplasts were derived from isopentenyl pyrophosphate made from glyceraldehyde phosphate and pyruvate; not from mevalonate. As many flavour compounds are terpenoid it was hoped to label them by feeding [2-^{14}C] pyruvate to vine leaves. The uptake into the cells was successful but the pyruvate was incorporated into such a host of metabolites that no useful conclusions could be drawn. Lee and Milborrow (1997) had shown that ABA in isolated chloroplasts was labelled by pyruvate but this was established by extracting and purifying the one compound.

Lee, H-S and Milborrow, B.V. (1997) Aust.J.Plant Physiol., 25, 507-512

DISCUSSION

Effect of Wilting

During the course of this work on vine leaves we found that ABA itself caused the synthesis of the 40 fold increase in a leaf's "stress" ABA. This was established by infiltrating a range of high concentrations of (+)-[$^2H_6$]ABA into normal, wet leaves. After 3 hours the amounts of endogenous, unlabelled ABA and absorbed [$^2H_6$]ABA were measured by GC/MS.AO.3 μM solution of labelled ABA caused the leaves to increase their ABA content from 0.5 to 9 μg/Kg fresh weight but higher concentrations inhibited ABA synthesis, probably by a negative feedback effect.

This observation together with Lovey's (1977) finding that most of the ABA in turgid, wet leaves is held in the chloroplasts but leaks into the cytoplasm when the leaf wilts, suggested a mechanism to account for the changes in the amounts of ABA which occur in wilting leaves.

1. When a leaf wilts the ABA leaks out of the chloroplasts.
2. The free ABA in the cytoplasm initiates the synthesis of the enzymes that make "stress" ABA.
3. The free ABA in the cytoplasm can diffuse out of the cell into the apoplast where it can move to close the stomata (within 15 minutes but without a net rise in a leaf's ABA being detectable) as found by Harris and Outlaw (1991, Plant Physiol., *95*, 171-173). The net rise in "stress" ABA occurred in vine leaves about 4 hours after wilting occurred and an increased amount of ABA was detected in the phloem exudate.

One of the effects of ABA is the acceleration of the colouring of the skin of grapes, it also hastens fruit and seed development in other plants. Thus we can expect an earlier maturation of grapes if the leaves wilt during the period of grape ripening.

The application of a solution of ABA to young grape berries was reported by Cummins to accelerate ripening. This leads to the interesting question whether the "extra" "stress" ABA formed in a wilted vine leaf, and detected in the leaf exudate, could hasten ripening of a nearby grape bunch. Time did not allow this to be done but it is a planned experiment for December 2005:-

(i) Leaves close to ripening grape bunches will be wilted by clamping filter paper soaked in hyperosmotic mannitol to their lower surfaces.
(ii) Two or three leaves near a grape bunch will have their laminae removed and their cut petioles placed in a solution of ABA. Colouring of the grape bunches will be monitored for rate of ripening.

The autonomy of the grape berries as the source of wine flavour compounds, and the main import being sucrose suggests that keeping vine leaves round the developing fruit would help good flavour development. Thus the practice of stripping leaves from around grape bunches would seem to be disadvantageous.