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MONITORING PHOSPHORUS COMPOUNDS IN AVOCADO TISSUES

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OPSOMMING

Die toepassing van bioluminessensie in die bepaling van A TP en ander adeniennukleotiede en gaschromatografie in die bepaling van fosfiet en etielfosfonaat word bespreek.

SUMMARY

The application of bioluminescence in measuring ATP and other adenine nucleotides and gas chromatography in estimating phosphite and ethylphosphonate are discussed.

INTRODUCTION

Phosphorous is a structural and functional component of cells of all living organisms. Its presence in cell constituents such as nucleic acids, nucleotides and phospholipids and use in certain phosphorus fungicides such as fosetyl-Al warrants a study into the role of phosphorus compounds in avocados.

Most analysis for phosphorus in plants provides information about the total phosphorus. Even if such figures are only required to ascertain the nutritional state of the plant, Chisholm, Blair & Bowden (1981) pointed out the benefit of analyzing for certain phosphorus fractions instead of total phosphorus. Two further examples can be provided to demonstrate the need of analyzing for specific phosphorus compounds, the one example is concerned with adenosinetr¡phosphate (ATP) and the other one is related to the use of fosetyl-Al.

The P compound, ATP, is a universal energy currency in the metabolism of all organisms. The ratio between ATP and the other two adenine nucleotides (AMP and ADP) is, except for certain physiological stress conditions, remarkably constant (Pradet & Raymond, 1983). By determining ATP, ADP and AMP a value for this ratio (adenilate energy charge) may be calculated to indicate the presence of stress conditions.

Phosphorus compounds naturally present in plants, such as ATP, phospholipids and others, are in the fully oxidized state, exemplified by PO_4^{3-}. But when treating plants with fosetyl-AI less oxidized P compounds (for example PO_3^{3-}) may be found in the plant.

In this report the results in estimating ATP, related adenine nucleotides, fosetyl-Al and its decomposition product PO_3^{3-} are discussed.

MATERIALS AND METHODS

1. Adenine nucleotides

The hot water extraction technique (Lumac, 1981) was used in conjunction with the Luseferine-Luciferase reaction to estimate ATP. The two other adenine nucleotides (ADP and AMP) were enzymatically converted to ATP and estimated. Standard methods for converting these two nucleotides were used (Lumac, 1981). Using known amounts of ADP and AMP the conversion efficiencies of these two compounds to ATP were respectively 89% and 83%. In estimating ATP in unknown samples these conversion factors were taken into account.

The adenilate energy charge (AEC) of the sample was calculated from the formula: AEC = (ATP + 1/2 ADP) / (ATP + ADP + AMP)

2. Cooling as a possible stress factor in the ripening of fruit

Freshly picked Fuerte fruit were divided into four samples and incubated for 17 days at two temperatures as indicated in Table 1. At days 7, 10 and 17 three fruit of each sample were collected and three 1 cm^3 portions tissue of the mesocarp removed. The portions were homogenized in an "Ultra-Turrax" blender in 100 ml Tris buffer (pH 7,2; 0,02 N) at 90ºC. An aliquot of this extract was analyzed for ATP, ADP and AMP, and the AEC was calculated.

3. Determination of PO_3^{3-} and ethylphosphonate

The basic principle of obtaining derivatives of P compounds by diazomethane or bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) as described by Blay & King (1980) was used.

A 20 g sample was homogenized in 200 ml water, centrifuged at 5000 g for five minutes and 2 ml of the upper water layer collected and freeze dried. The residue obtained by freeze drying was either methylated by diazomethane or Dilated by BSTFA.

Diazomethane was prepared by the usual method from N-nitrososulfonamide (Blay & King, 1980). After dissolving the freeze dried residue in 90% methoxyethanol the generated diazomethane was bubbled through the sample. When the sample started to turn yellow the production of diazomethane was diverted to another sample. The excess diazomethane in the samples were removed with a stream of nitrogen gas. Methylated samples were analyzed by means of gas chromatography. Details for the gaschromagraphic conditions are presented in Table 2.

Silating of the freeze dried samples was done with BSTFA by dissolving the sample in 2 ml water. This solution was passed through a Cation-exchange column in the NH_4+ form (Amberlite 120 (H), 4 cm x 0,3 cm). The elute was freeze dried after which 0,1 ml BSTFA was added to the sample, sealed and incubated for 24 h at room temperature. This mixture was analyzed on the gas chromatograph with operating conditions as presented in Table 2.

4. Plant samples analyzed for PO_3^{3-} and ethylphosphonate

Lupine seeds were planted in sand-perlite mixtures containing up to 0,1 % a.i. fosetyl-Al. The plants were watered with Hoagland's solution and after two weeks analyzed for $P0_3^{3-}$ and ethylphosphonate.

Two year old avocado seedlings were treated with fosetyl-Al at a rate of 2 g a.i. m^{-2} canopy. Two months later the leaves and roots were collected and analyzed.

Avocado leaves and roots from commercial orchards treated with fosetyl-Al (2ga.i. m^{-2} canopy) were analyzed 5,8,10 and 12 weeks after being treated by the fungicide for PO_3^{3-} and ethylphosphonate. Leaves and roots from trees never treated with any form of ethylphosphonate, collected at the University of Pretoria research farm, served as controls.

RESULTS

1. Effect of cooling on the adenilate energy charge of avocado fruit

Only in one case was a difference in the AEC values of the mesocarp detected between the cooling treatments (Table 3). Treatment 2, in which the fruit were incubated for 10 days at room temperature and then cooled, showed a significant drop in the AEC compared to the other treatments. Except for treatment 2 a general increase in the AEC with incubation was found.

2. Determination of PO_3^{3-} and ethylphosphonate

Using pure substances, a good separation between ethylphosphonate, $P0,^{3-}$ and PO_4^{3-} was obtained. The minimum practical detection limit was 0,01 ppm for these substances. With the BSTFA-technique a slight oxidation from $P0_3^{3-}$ and PO_4^{3-} occurred during silation. This problem was diminished by analyzing all the samples and standard reference solutions after a constant (24 h) silation reaction time.

The results for the analysis of lupine treated with fosetyl-Al showed a good correlation between the concentration of ethylphosphonate added and the amount of ethylphosphonate and PO_3^{3-} in the sample (Table 4). The correlation between the results of the two methods of obtaining derivatives, BSTFA and diazomethane, was highly significant.

Both methods for derivatising were applied to avocado tissues (Table 5). No PO_3^{3-} nor ethylphosphonate could be detected in avocado plants nottreated with fosetyl-Al. In material obtained from a commercial orchard the time between treatment date and analysis time showed no correlation with either the PO_3^{3-} or ethylphosphonate content of tissues Leaves usually contain less PO_3^{3-} and ethylphosphonate than roots.

DISCUSSION

Bioluminescence was successfully applied in the measurement of adenine nucleotides in avocado fruit. The results indicated that the adenilate energy charge of fruit may be

influenced by cooling. The possibility exists thus that the effects of cooling and other postharvest practices such as controlled atmosphere on fruit might be studied by using the adenilate energy charge as an index of the physiological state of the fruit. In view of the simplicity of the technique and the rapid way results in which can be obtained with bioluminescence this technique should be considered for further application in avocado research.

In contrast with the determination of adenine nucleotides, gas chromatographic estimation of $P0_3^{3-}$ and ethylphosphonate in avocado tissues is not a simple task. Strictly controlled conditions especially with the formation of derivatives, and experience in handling the NSPD detector are required. Another drawback is the length of time required for the analysis. About one hour is required for preparation of the sample and completion of each analysis.

Lupine treated with different concentrations of fosetyl-Al showed that a correlation exists between the added amount of ethylphosphonate and the PO_3^{3-} and ethylphosphonate measured. The results given by the two methods of obtaining derivatives, by the BSTFA and diazomethane techniques, agreed well but the BSTFA technique is preferred due to its better peak resolution and toxicity of diazomethane.

The results for PO_3^{3-} and ethylphosphonate indicate that these compounds may be present in avocado tissues at least 12 weeks after the application of fosetyl-Al. In general lower values for $P0_3^{3-}$ and ethylphosphonate were obtained from leaves than from roots. Preliminary results indicated that these compounds can also be detected in fruit from treated trees.

Experiments are now in progress where the transformation rates of ethylphosphonate and $P0_3^{3-}$ in relation to *Phytophthora* root rot are studied. The results of these experiments will be presented at a later stage.

TABLE 1: The incubation periods at two temperature regimes for four Fuerte avocado fruit samples.

Sample	Room Temperature	Refrigeration (4-8 °C)
1	day 1 to 17	---
2	day 1 to 10	day 11 to 17
3	day 8 to 17	day 1 to 7
4	---	day 1 to 17

TABLE 2: Details for gaschromatographic conditions for analysing methylated PO_3^{3-} and ethylphosphonate obtained by diazomethane or BSTFA.

Parameter	Conditions for	
	BSTFA	Diazomethane
Column type	10% SE 30 ON Chromosorb 80/100	20% Carbowax ON Chromosorb 80/100
Column length	2m	2m
Column temperature: Initial (°C/Min)	75/2	75/2
Rate (°C/Min)	10	10
Final	130	160
Injector temperature (°C)	135	170
Detector type	NPSD (P mode)	NPSD (P Mode)
Detector temperature (°C)	145	180
Flow rate (ml/min)		
Nitrogen (carrier)	20	40
Air	230	230
Hydrogen	30	30
Sample size (1)	2	2

TABLE 3: The adenilate energy charge in the mesocarp of Fuerte avocados incubated at various temperatures (mean value of three fruit).

Treatment	Day 1-7 Temperature	AEC at day 7	Day 8-10 Temperature	AEC at day 10	Day 11-17 Temperature	AEC at day 17
1	Room	0,85	Room	0,87	Room	0,94
2	Room	0,86	Room	0,89	4-8°C	0,76★
3	0-8°C	0,84	Room	0,90	Room	0,93
4	4.8°C	0,82	4-8°C	0,85	4-8°C	0,89

★ Significantly different ($p \geq 0,95$) from other values in the column according to Duncan's new multiple range test.

TABLE 4: The concentration of ethylphosphonate and PO_3^{3-} estimated according to the BSTFA and diazomethane technique in lupine treated with fosetyl-Aℓ.

% Aℓ-ethylphosphonate added	PO_3^{3-} (ppm)		Ethyl phosphonate (ppm)	
	BSTFA	Diazomethane	BSTFA	Diazomethane
0,000	0	0	0	0
0,005	1,2	0	0	0
0,010	13,6	2,8	11,4	22,7
0,015	13,4	13,0	21,7	43,0
0,050	37,9	26,4	62,9	141,3
0,100	217,3	319,2	178,6	234,7

TABLE 5: The PO_3^{3-} (ppm) and ethylphosphonate (ppm) content of avocado fruit tissue in various samples estimated by the BSTFA (B) or diazomethane (D) technique. Where fosetyl-Al was added it was at a rate of 2 g a.i. m^{-2} canopy.

Sample description	Tissue	Fosetyl-Al added	PO_3^{-3}		Ethylphosphonate	
			B	D	B	D
Seedling 2 years old	root	yes	43	28	32	51
	leaf	yes	27	16	11	49
Untreated trees	root	no	0	0	0	0
Commercial orchard	leaf	yes	3-16	1-21	4-7	2-9
	root	yes	5-17	3-14	3-11	2-12

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