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NEW LIQUID CHROMATOGRAPHIC APPROACHES FOR FREE AMINO ACID ANALYSIS IN PLANTS AND INSECTS

II. THIN-LAYER CHROMATOGRAPHIC ANALYSIS FOR EIGHTEEN VARIETIES OF PALM TREES

MARIA DE LOS ANGELES BARCELÓN*, RANDOLPH E. McCOY and HENRY M. DONSELMAN

University of Florida, Agricultural Research and Education Center, 3205, S.W. College Avenue, Fort Lauderdale, FL 33314 (U.S.A.)

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SUMMARY

The free amino acids in the foliage of eighteen varieties of palm and the free amino acids in the phloem exudate of three varieties of palm were studied. The fluorescent label 5-dimethylaminonaphthalene-1-sulfonyl chloride was used for amino acid analysis using two-dimensional thin-layer chromatography on polyamide sheets. A possible correlation between the presence of arginine in the palm varieties studied and susceptibility to lethal yellowing disease was observed.

INTRODUCTION

Lethal yellowing (LY) is a disease causing extensive damage to palm trees in many parts of the world[1–4]. Contrary to the initial belief that a virus caused LY, there is now evidence linking LY with mycoplasma-like organisms (MLO)[5–8]. Efforts to culture the MLO associated with diseased palms have not been successful.

The incubation period of LY in coconut palms is 6 months to 2 years and "the presumed susceptible age" is about 2 years[9,10]. There are no known methods for early detection of the disease. Once the disease has progressed to a point where the symptoms are visible, the tree can rarely be saved. While efforts are being made to unravel the mysteries of LY, a long-range solution to the ravage caused by the disease could be the replacement in infected areas of susceptible palms with resistant varieties. Screening palms for resistance to LY is done by exposure to the disease[1,2,11] in trial gardens. The method requires years and yields somewhat uncertain results but it is presently the only viable technique.

Intrigued by the many unanswered questions surrounding LY in palm trees, we decided to compare the free amino acid patterns in the foliage from seven different varieties of palms with known susceptibilities to see if there was any relationship. Our method of choice, as the initial step, was the formation of the 5-dimethyl-

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TABLE I

AMINO ACID ABBREVIATIONS

Amino acid	*Abbreviation*	*Amino acid*	*Abbreviation*
Alanine	Ala	Isoleucine	Ile
Asparagine	Asn	Leucine	Leu
Aspartic acid	Asp	Bis-Dns-lysine	Bis-Lys
Arginine	Arg	Methionine sulfoxide	Meo
α-Aminobutryic acid	AABA	Mono-Dns-diamino acids	m-DAs
γ-Aminobutryic acid	GABA	Methionine	Met
Mono-Dns-cadaverine	m-Cad	Norleucine	Nle
Carboxymethylcysteine	Cmc	Ornithine	Orth
Cysteic acid	Cya	Phenylalanine	Phe
Cystine	Cys	Pimelic acid	Pim
Dns-amide	Dns-NH_2	Proline	Pro
Diaminobutyric acid	DABA	Serine	Ser
Diaminopropionic acid	DAPA	Taurine	Tau
Glutamic acid	Glu	Threonine	Thr
Glutamine	Gln	Tryptamine	TrN
Glycine	Gly	Tryptophan	Trp
Histidine	His	bis-Tyrosine	Bis-Tyr
Hydroxyproline	HO-Pro	Valine	Val

aminonaphthalene-1 sulfonyl (Dns) derivatives coupled with two-dimensional thin-layer chromatography (TLC). The method requires no sophisticated instrumentation and is known to have a detection sensitivity in the nanomole range[12–14]. The results were encouraging, so we expanded the analysis to include foliar extracts from a total of eighteen palm varieties and the phloem sap from three palm varieties.

TABLE II

AMINO ACID ANALYSIS OF FOLIAGE

Symbols: – = not observed; + = observed; + + = bright; ± = faint; *S* = susceptible; *R* = resistant.

Species	*Susceptibility range (by exposure to LY) SI > 2 > 3 > 4 R*	*Ala/Dns-NH_2*	*Asn*	*Asp*	*Arg*	*Cya*	*Glu*
Arecastrum romanzoffianum	4	+ +	+	+ +	–	+ +	+ +
*Carpentaria acuminata**	4	+ +	±	+ +	±	±	+ +
Chrysalidorcarpus lutescens	4	+ +	+	+	–	+ +	+
Cocos nucifera (yellow Malayan dwarf)	3–4	+ +	±	+	–	+	+
Phoenix dactylifera (Halawi)	2–1	+ +	±	+ +	+	+	+ +
Cocos nucifera (Jamaica tall)	1	+ +	±	+ +	+	+ +	+ +
Veitchia merrillii	1	+ +	±	+ +	+	+	+

* Analysis by method 2 only.

EXPERIMENTAL

Specimens

Foliage were taken from the following palm varieties: (1) *Arecastrum romanzoffianum* (Queen palm), (2) *Arikuryroba schizophylla*, (3) *Carpentaria acuminata*, (4) *Caryota mitis* (Fishtail palm), (5) *Chrysalidocarpus lutescens* (Areca palm), (6) *Cocos nucifera* (Jamaica tall coconut), (7) *Cocos nucifera* (Malayan dwarf coconut), (8) *Cocos nucifera* (Maypan coconut), (9) *Phoenix dactylifera* (Deglet moor data palm), (10) *Phoenix dactylifera* (Halawi date palm), (11) *Phoenix roebelenii* (Dwarf date palm), (12) *Pritchardia thurstonii* (Thurston fan palm), (13) *Ptychosperma elegans* (Solitaire palm), (14) *Roystonia regia* (Royal palm), (15) *Sabalpa metto*, (16) *Veitchia merrillii* (Christmas palm, Manila palm), (17) *Wallichia disticha* and (18) *Washingtonia robusta*.

Phloem sap were taken from (1) diseased *Veitchia merrillii*, (2) healthy *Veitchia merrillii*, (3) diseased Jamaica tall coconut, (4) healthy Jamaica tall coconut and (5) Malayan dwarf coconut.

Extraction and preparation of free amino acids

Foliage. Leave tissue (20 g), with the hard ribs removed, was cleaned and cut into small pieces and blended for 2 min with 90 ml of methanol–water–12 *M* hydrochloric acid (90:5:5). The supernatant liquid was vacuum filtered through Celite 545 (Supelco, PA, U.S.A.). The procedure was repeated using the remaining leaf pulp for a second extraction. The combined total filtrate was *ca.* 160 ml. This leaf extract was divided into four aliquots for replicated analyses. About 40 ml of the leaf extract were diluted with 15 ml of deionized water and extracted with 15 ml of chloroform to remove most of the non-polar organic components, leaving the free amino acids in the acidic aqueous layer. The aqueous layer was passed through an ion-exchange column (7 × 1 cm; Amberlite IR-120 H, medium porosity; Mallinckrodt, KY, U.S.A.). The column was washed with deionized water until neutral to pH paper (*ca.*

Gln	*Gly*	*His*	*Ile*	*Leu/Nle*	*Bis-Lys*	*Met*	*Phe*	*Pro*	*Ser*	*Thr*	*Trp*	*Bis-Tyr*	*Val*
++	++	+	+	+	++	−	+	++	++	+	+	+	+
+	+	−	+	+	++	−	+	+	++	+	+	+	+
+	+	−	+	±	+	−	+	++	+	+	+	±	+
±	+	−	+	+	+	−	+	+	+	+	+	+	+
±	+	−	+	+	±	−	+	++	++	±	±	+	+
+	+	−	+	+	++	−	+	+	++	+	+	+	+
+	±	−	+	+	+	−	+	++	+	+	+	+	+

50 ml of water). The amino acids were eluted using 10 ml of 4 *M* ammonia solution followed by 5 ml of deionized water. The eluate was evaporated to dryness in a rotary evaporator. The resulting residue was then Dns derivatized.

Phloem sap. Phloem exudate (15–20 ml) was mixed with an equal volume of the methanol–water–hydrochloric acid mixture and treated the same way as the leaf extract.

Preparation of Dns derivatives

The residue containing the free amino acids from the foliage and/or phloem sap was dissolved in 1.5 ml of sodium hydrogen carbonate buffer (pH 10.5, 0.1 *M*), then 0.5 ml of Dns-Cl (Sigma, St. Louis, MO, U.S.A.) solution (15 mg/ml in acetone) was added, mixed thoroughly and incubated at 40°C for 2 h. The final step, *viz.*, the preparation of the Dns-amino acid mixture for TLC spotting, was carried out in three different ways.

TABLE III

AMINO ACID ANALYSIS OF PHLOEM SAP

Symbols as in Table II.

Amino acid	*Cocos nucifera (Jamaica tall)*		*Cocos nucifera (Malayan dwarf)*	*Veitchia merrillii (Christmas palm)*	
	Healthy	*Diseased*	*Healthy*	*Healthy*	*Diseased*
Ala/Dns-NH_2	++	+	+	++	++
Asn	++	++	++	−	−
Asp	+	±	+	+	±
Arg/m-DA	+	+	−	±	+
AABA/GABA	++	++	±	++	+
Cmc	+	++	++	++	++
Cya/Tau	−	−	−	−	−
Cys	−	±	−	−	−
Glu	++	+	+	++	+
Gln	+	++	++	++	++
Gly	++	+	+	++	+
His/m-DA	−	+	+	+	+
HO-Pro	+	+	−	−	−
Ile	+	+	+	++	+
Leu/Nle	+	+	+	++	+
Bis-Lys	+	±	+	+	±
Met	+	±	±	+	+
Meo	−	−	±	+	+
Phe	+	++	+	++	++
Pim	+	+	+	+	+
Pro	++	++	++	++	++
Ser	++	++	++	++	++
Thr	+	±	±	±	±
Trp	+	+	+	+	+
Bis-Tyr/TrN	±	−	+	+	+
Val	+	+	++	++	++
Susceptibility	1	1	3–4	1	1

Method 1. After incubation the reaction mixture was evaporated to dryness under reduced pressure. The residue was taken up in methanol (HPLC grade) (Fisher Scientific, NJ, U.S.A.) and filtered through a Pasteur pipet plugged with glass-wool.

Method 2. After incubation the acetone in the reaction mixture was removed by passing nitrogen gas. It was neutralized using 1 *M* hydrochloric acid, then extracted with ethyl acetate at least four times with thorough mixing using a vortex (Sybron/Thermolyne Maxi-mix). The combined ethyl acetate extract was evaporated to dryness under reduced pressure and the residue dissolved in methanol (HPLC grade).

Method 3. After removing the acetone from the reaction mixture, the pH was checked and, when necessary, adjusted to pH 8–9 (pH paper). This was extracted once with a less polar solvent such as toluene, chloroform or dichloromethane. This extract was evaporated to dryness and dissolved in methanol for a separate analysis. The pH of the remaining aqueous layer was adjusted to pH 6–7 and extracted with ethyl acetate several times. The same procedure was repeated at pH *ca.* 5 and *ca.* 3. The combined ethyl acetate extract was then evaporated to dryness and dissolved in methanol.

The aqueous layer left after the ethyl acetate extraction was also evaporated to dryness. The residue was taken up in methanol and filtered through a Pasteur pipet plugged with glass-wool.

Thin-layer chromatography

Spotting was done on 5 × 5 cm polyamide plates (Schleicher & Schüll, Keene, NH, U.S.A.) using a 10-μl Hamilton syringe. The plates were developed in a filter-paper-lined 250-ml beaker with two pieces of glass rod cut to fit diagonally at the bottom of the beaker to hold the plates upright and covered by an inverted 600-ml beaker. Solvent I (1.5% formic acid) and solvent II (benzene–acetic acid, 4.5:1) were run perpendicular to each other. The chromatograms were viewed under a UV chromatogram viewer equipped with both a longwave (366 nm) and a shortwave (254 nm) UV light source.

RESULTS AND DISCUSSION

The Dns-amino acid mixtures from the first group of palms were prepared according to both methods 1 and 2, except for *Carpentaria acuminata*, which was prepared by method 2 only. (See Table I for amino acid abbreviations). The results of the TLC analyses are given in Table II.

We grouped the amino acids into basic, acidic or neutral types, and those with sulfur (Cys, Met) or with hydroxy groups (Ser, Thr, Tyr), but could not find any meaningful correlation between the relative concentrations of these amino acid groups and the known susceptibility to LY. On the other hand, we observed that arginine, separate from the other basic amino acids, did show a rather close correlation, *i.e.*, arginine was not observed in specimens known to be resistant to LY. The fact that some MLO are known to require arginine as an essential nutrient[1,15], and the fact that MLO are associated with LY, makes this observed correlation significant.

Phloem sap from healthy and diseased *Veitchia merrillii*, healthy and diseased Jamaica tall coconut and healthy Malayan dwarf coconut were analyzed for free

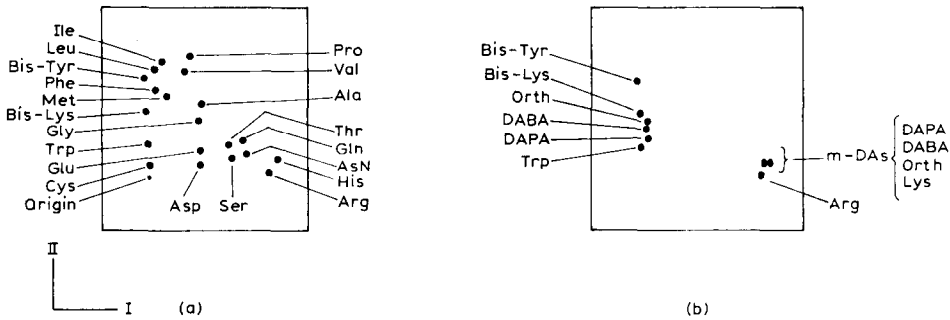

Fig. 1. (a) Chromatogram of a mixture of the twenty common amino acids. (b) Chromatogram showing the position of the mono-Dns- and bis-Dns-diamino acids relative to some standard Dns-amino acid markers.

amino acids. The results are given in Table III. A correlation between the presence of arginine and susceptibility to LY was again observed.

We then broadened the selection of palm specimens to include susceptibilities to LY, varying from very susceptible to slightly susceptible to resistant. The analysis of this larger group of speciemens required a number of adjustments in the sample

TABLE IV

AMINO ACID ANALYSIS FOLIAGE

Symbols as in Table II.

Species	*Susceptibility range (by exposure to LY) S 1 > 2 > 3 > 4 > R*	*Ala/Dns-NH$_2$*	*Asn*	*Asp*	*Arg/m-DA*	*AABA/GABA*	*m-Cad*	*Cmc*	*Cya/Tau*	*Cys*	*DAPA*
Arecastrum romanzoffianum	4	++	−	++	−	+	−	+	+	−	−
Carpentaria acuminata	4	++	+	++	−	+	+	±	±	−	−
Chrysalidocarpus lutescens	4	++	+	++	±	+	−	+	++	−	−
Phoenix roebelenii	4	++	+	++	−	++	−	−	+	+	+
Ptycosperma elegans	4	+	±	++	−	++	−	−	+	+	±
Roystonea regia	4	++	+	++	−	+	−	±	+	+	+
Sabal palmetto	4	++	±	++	−	++	−	−	±	±	−
Washingtonia robusta	4	++	+	++	−	++	+	−	++	−	+
Cocos nucifera (Malayan dwarf)	4 3	++	+	++	±	+	−	−	++	±	±
Cocos nucifera (Maypan)	4–3	++	+	++	−	++	−	−	+	+	+
Caryota mitis	3–2	+	+	++	±	++	±	−	+	±	−
Arikuryroba schizophylla	2	++	+	++	±	+	−	−	+	+	−
Phoenix dactylifera (Deglet moor)	2–1	++	+	+	±	++	−	−	+	±	−
Phoenix dactylifera (Halawi)	2–1	++	+	+	±	+	−	−	+	+	−
Cocos nucifera (Jamaica tall)	1	++	+	++	+	++	−	−	++	−	+
Pritchardia thrustonii	1	++	+	+	+	++	−	−	+	+	±
Veitchia merrillii	1	++	+	++	+	+	−	−	+	+	+
Wallichia disticha	−	++	+	++	++	++	−	−	±	+	+

handling. Numerous trial runs with control of variables in the different steps of the extraction, separation, Dns derivatization and TLC spotting had to be determined to obtain parallel and comparable analyses.

Method 3 in the preparation of the Dns mixture for TLC spotting was found to give more readable TLC plates by splitting the chromatogram into three. The organic extract at pH ≈ 9 contained most of the Dns- and/or poly-Dns-amines, while the combined ethyl acetate extract in the pH range 7–3 contained most of the Dns-amino acids. A pH range of 7–5 instead of 7–3 was found to yield incomplete extraction of the Dns derivatives of the acidic amino acids. The remaining water layer retained most of the dansylic acid and, when present in the sample, mono-Dns-pimelic acid (2,6-diaminoheptanedioic acid) and some unextracted Dns-Arg, Dns-Cya and the mono-Dns-diamino acids (m-DAs).

Experiments using known concentrations of standard Dns-amino acids, have shown that pH control was critical in the extraction of Dns-Arg in microgram amounts (the optimal pH was *ca.* 7), but was not so critical in higher concentrations (a pH above 5 was sufficient). Unextracted Dns-Arg, m-DAs and mono-Dns-pimelic acid in the aqueous residue appeared in the chromatogram as a single unresolved spot that moved with solvent system I. The spot usually has a quenched background that could be due to the presence of non-fluorescent polar substances.

Glu	*Gln*	*Gly*	*His/m-DA*	*HO-Pro*	*Ile*	*Leu/Nle*	*Bis-Lys*	*Meo*	*Orth/DABA*	*Phe*	*Pim*	*Pro*	*Ser*	*Thr*	*Trp*	*Bis-Tyr/TrN*	*Val*
+	−	+	±	−	+	+	+	−	+	+	+	++	++	+	+	±	+
+	±	+	−	+	+	+	+	−	+	+	+	++	++	++	+	±	+
+	+	+	+	±	+	+	++	±	+	+	+	+	+	+	+	+	+
+	+	+	+	−	+	+	++	±	+	+	+	++	++	+	+	+	+
++	±	+	−	±	+	+	++	±	±	+	−	++	++	+	±	+	+
+	++	+	±	−	+	+	++	±	±	+	+	++	++	+	+	±	+
+	±	++	−	−	±	±	++	−	−	+	−	++	+	+	±	+	+
++	++	+	+	−	+	+	++	±	+	+	−	++	++	+	+	+	+
+	+	+	−	±	+	+	+	±	+	+	−	++	+	+	+	+	+
++	++	+	−	±	+	+	++	+	+	+	±	++	+	+	±	+	+
++	++	+	±	+	+	+	+	±	+	+	−	++	++	+	±	+	+
++	+	++	+	±	+	+	+	±	±	+	−	+	++	+	+	±	+
+	+	+	±	−	+	+	++	−	+	+	±	++	++	+	+	+	+
+	+	+	+	−	+	+	+	−	+	+	+	++	++	+	+	+	+
++	++	+	+	+	+	+	++	+	±	++	−	++	+	+	+	+	++
+	++	+	+	−	+	+	++	±	+	++	+	++	+	+	+	+	+
++	+	+	+	−	+	+	++	+	+	+	−	++	++	+	+	+	+
++	++	+	+	±	+	++	++	+	+	+	+	++	++	+	+	+	+

At this point of the investigation, we were focusing more carefully on Dns-Arg and factors that could affect the reliability of the identification of this spot. In specimens such as *Wallicha disticha* in which the arginine seems to be present in relatively large amounts, the identification of arginine was straightforward. This, however, was not the case with most of the specimens where the Dns-Arg spot was not so intense. In our previous work on Dns-amino acids[16] we mentioned that the mono-Dns derivative of amino acids such as 2,6-diaminohexanoic acid (lysine), etc., appear in the same general area as Dns-Arg, and when present can make the identification of Dns-Arg difficult. We are of the opinion, however, that under the conditions we used for Dns derivatization, the diamino acids will be mostly in the di-Dns forms, which have very different R_F values from those of Dns-Arg.

From Fig. 1 it can be seen that the R_F values of m-DAs are slightly larger than those of Dns-Arg in both systems I and II. They also have a characteristic double spot which can be useful in the identification. This double spot is probably due to the slight difference in polarities of the structural isomers of the m-DAs. Careful spiking, the use of standard Dns-amino acid markers and the use of identification maps were all utilized in the analysis of the thin-layer chromatograms.

In Table IV we report the analyses for the eighteen different palms based on the combined results obtained from an average of nine analyses taken from foliage harvested at three different dates. *Wallichia disticha* did not have an LY susceptibility ranking, but this palm species disappeared in south Florida with the advent of LY in the area, much like the Jamaica tall coconut.

Pritchardia thurstonii and *Veitchia merrillii* are species ranked as very susceptible to LY. Foliage taken from young plants, unlike foliage taken from mature plants (2 years of age or more), did not show the Dns-Arg spot.

CONCLUSION

After studying the free amino acid pattern of eighteen varieties of palms and correlating them with the known susceptibilities of these different palms to LY, we believe that there is some correlation between the presence of free arginine in the palm and its susceptibility to LY. If proven true, this correlation can be of tremendous help in the selection of disease-resistant palms for replanting areas affected by LY. However, the TLC identification of free arginine in the palm extracts cannot be taken as exact and definitive because of the difficulties in the spot identification noted in the Results and discussion section. Therefore the significance of the presence of free arginine in susceptible palms and its absence in resistant palms will have to be confirmed by another route*.

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