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APPLICATION OF POLYAMINES IN OIL PALM (*Elaeis guineensis* Jacq.) STOPS ADVANCE OF BUD ROT DISEASE

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ABSTRACT

*Bud rot complex (PC) is a disease that affects oil palm (*Elaeis guineensis *Jacq.). It is a gradual rot of the young tissue which can kill the palm if the meristem is affected. Certain predisposing factors seem involved in development of the disease. However, in Colombia, many sick palms recover naturally. Knowing the multiple effects of polyamines in cell division and morphogenesis processes, we proposed some relationships between the polyamine content and bud rot disease. The endogenous contents of polyamines (PA) putrescine, spermidine and spermine were determined, using HPLC, in meristematic and bud tissues from oil palms in different sanitary stages (healthy and sick palms). The PA content in meristems and buds gradually decreased in sick adult palms. Oil palms recovered when the PA content increased. Application of PA to the stems of oil palms suffering from the disease, sped up their recovery and maintained healthy palms in areas where the disease incidence was high. The findings reported here introduced a novel and complementary management practice against one of the most devastating diseases of oil palm in Latin America.*

Keywords: putrescine, spermidine, spermine.

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INTRODUCTION

The oil palm (*Elaeis guineensis* Jacq.) gives the highest yield per hectare of all oil crops at present (Corley and Tinker, 2003). However, in Latin America, yields are highly reduced due to bud rot disease (named PC, for its abbreviation in Spanish), caused by the fungus *Thielaviopsis paradoxa* (Nieto, 1996). PC causes the gradual rotting of buds leading to a decrease in the number of leaves and, therefore, reducing the oil quality and yield (Darus, 2000). When the rot reaches the meristematic tissues, the palm dies.

It has been suggested that extreme climatic conditions and poor soil management encourage the spread of the disease (Gómez *et al.*, 2000; Swinburne *et al.*, 1996). Frequently flooded soils are poorly oxygenated, have a variable pH, low redox potential and high electrical conductivity. These conditions make the palm prone to diseases such as PC (Acosta *et al*., 1996; Munévar *et al*., 2001).

PC develops in four clear stages. Firstly, the spear leaves develop increasing chlorosis. Second, the whole plant dries out with gradual rotting of the spear leaves. The third stage is characterized by a necrosis that generates epinasty and short young leaves. Finally, in the advanced PC stage, the palm does not produce either leaves or fruits. PC mainly affects adult palms (Gómez, 1995) although some nursery palms have also been strikened (Unipalma plantation, Cumaral, Colombia). Palms can suffer the disease for six months to three years and then recover naturally. If the rot does not reach the meristem, the palm may be able to overcome the disease and produce new spear leaves (Nieto, 1996). The recovery process requires high in the meristematic activity, including high rates of cell division, morphogenesis and organogenesis (Davies, 1987). A hormonal

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balance regulates these processes, and is responsible for controlling the plant growth and development (Davies, 1987). Plant hormones that participate in this balance include polyamines (PA), low molecular weight and nitrogen-containing molecules, that affect the cell activity and modulate the plant development (Bais and Ravishankar, 2002; Mendoza and Rocha, 2002). It is known that application of PA to abiotically stressed plants can increase their growth and yield, and minimize the damage caused by the abiotic stress (Montenegro, 1995; Norato and Romero, 1995; Mendoza *et al*., 2000; Navakoudis *et al*., 2003). In addition, pathogen attack in plants leads to a defense response that includes several metabolic changes like accumulation of PA and variation in its content (Greenland and Lewis, 1984; Legaz *et al*., 2002; Walters, 2000).

The main goals of this work were to study the relationship between PC infection and the levels of PA putrescine (Put), spermidine (Spd) and spermine (Spm) in oil palm, and to evaluate the effect of applying PA in the prevention or recovery of affected palms.

MATERIALS AND METHODS

Determination of Endogenous Content of Polyamines

Plant material. The free-PA content was determined in the meristematic and bud tissues of *tenera* palms (Unilever material) of different ages: 10-month-old nursery seedlings and 15-year-old adult palms. The palms were in the commercial plantation Unipalma, in the Eastern Zone of Colombia, at 302 m above sea level. PA analysis was also carried out on oil palms with different stages of PC (young palms with initial PC), and adult palms in the three stages: initial PC (IPC), advanced PC (APC) and in natural recovery (REC). The controls were healthy young and adult palms from the same genetic origin and ages and grown in the same plot.

Tissue collection. Selected palms were sampled using a band saw. The bud and meristematic tissues were exposed by cutting with a machete. The meristem and bud were removed and placed in a 50-ml Falcon tube containing 5% (w/v) trichloroacetic acid (TCA). The samples were kept at 4°C for three days and stored at -20°C until use.

Polyamine extraction. PA analysis was carried out as previously described (Escribano and Legaz, 1988; Smith, 1991). Three grammes of frozen tissue (either from the meristem or bud) were ground in 5% (w/v) trichloroacetic acid (TCA) and 170 µl 20 mM butylamine (ButA, Merck Darmstadt), as internal standard. The tissues were incubated overnight at 4°C and, the following day, centrifuged at 11 000 rpm for 30 min at 4ºC (Sorvall, USA). An aliquot of 2 ml of the supernatant (containing free-PA) was washed with ether and kept at 4°C until the derivation reaction. For this reaction, 0.4 ml of the extract was mixed with 100 mg sodium bicarbonate and 0.4 ml dansyl chloride (20 mg dissolved in 1 ml acetone) and incubated for 16 hr at room temperature. The reaction was stopped by incubation with 0.4 ml 60 mg ml^{-1} L-proline for 30 min at room temperature. The mixture was air-dried at 60°C under a fume hood. The dried micture was resuspended in 0.2 ml bi-distillate water, and then washed with 3 ml toluene (to extract dansyl-PA), and again air-dried. Because dansyl is light sensitive, from this point the process was always carried out in the dark. The mixture was cleansed by alkaline hydrolysis (to eliminate the traces of phenolics and tannins). The dried extract was resuspended in 1.5 ml 5 M KOH and incubated at 50°C for 45 min. After incubation, a volume of 2 ml phosphate buffer (1M KH_2PO_4 and 0.75 M NaH_2PO_4) was added. The PAs were finally extracted by washing with 3 ml toluene. The extract was air dried and kept at -20°C until HPLC analysis.

HPLC of PA. Dried extracts containing free-PA were re-dissolved in 1.5 ml 100% methanol (HPLC grade) and microfiltered through PVDF 0.45 µm membranes immediately before HPLC analysis. HPLC was performed with a LaChrom Merck Hitachi system using a LicroCart LiChrospher RP 18e 100 (5 µ) column (125 mm x 4 mm diameter; Merck®). An aliquot of 25 µl microfiltered extract was injected into the HPLC system and eluted with a gradient mobile phase of 60:40 (v/v) to 85:15 (v/v) methanol:water in 8 min, with a flux of 1 ml min^{-1}. The total running time was 23 min. The dansyl-PA was detected by using a UV detector (excitation and emission wavelengths of 365 nm and 510 nm, respectively).

To quantify the PA, calibration curves were drawn using ButA as internal standard. The standards for Put, Spm, Spd, ButA (all from Merck, Germany) were prepared in pure methanol. All the organic solvents (all from Merck, Germany) used were of HPLC grade.

EXOGENOUS APPLICATION OF PA TO THE PALM STEM

Different concentrations of Put, Spd and Spm (*Table 1*) were injected into the trunks of *tenera* palms (Unilever, sown in 1988) in four stages of the disease (healthy, IPC, APC and REC). The palms were at commercial plantation, Unipalma, in the eastern zone of Colombia. Three applications (once a month) were done from October 2002.

TABLE 1. LEVELS OF PA APPLIED TO OIL PALMS WITH DIFFERENT DEGREES OF PC

Treatment	PA	Dose
T 1		500 µM
T 2	**Put**	1 000 µM
T 3		2 000 µM
T 4		50 µM
T 5	**Spd**	100 µM
T 6		200 µM
T 7		50 µM
T 8	**Spm**	75 µM
T 9		100 µM
T 10	**Control**	-

TABLE 2. SCALE FOR EVALUATING PHYTOSANITARY STAGE OF PALMS AFFECTED BY PC

Stage	Disease index	Description
IPC	1	Slight chlorosis and necrosis of leaves and spear leaves
APC	2	Deep damage in bud
Instable	3	Deformed spear leaves, short fronds with rotting at their bases
Beginning of REC	4	Fronds of 2 to 4.5 m with rotting in their apices
Good REC	5	Healthy fronds until frond number 9
High REC	6	Minimum 12 healthy fronds
Healthy	7	Minimum 17 healthy fronds and bunch production

To apply the PA solutions, frond 33 was pruned and on its attached petiole base a hole of 2 cm diameter and 15 cm deep was drilled, and 20 ml of one of the PA solutions (prepared with distilled water as solvent) injected using a syringe (without needle) (Mendoza, 2003; Mendoza and Rocha, 2003). A volume of 20 ml distilled water (PA-free) was injected into the control palms.

Evaluated Parameters

To assess the effect of the exogenous PA, the palms were evaluated monthly for their disease severity on the scale in *Table 2*. The percentages of healthy palms and palms in recovery were determined for each treatment.

Statistical Analysis

Statistical analysis was performed using SAS.

RESULTS

Endogenous Content of PA in Oil Palm

In all the oil palms (young and adult) the Put, Spd and Spm contents were determined in the meristematic and bud tissues by HPLC (*Table 3*).

The highest concentrations of total free-PA were detected in healthy adult palms and naturally recovered palms (REC) at 130 to 183 nmol g^{-1} FW. The PA content in adult and young palms in IPC was 82 to 100 nmol g^{-1} FW. The lowest free-PA amount was registered for young healthy palms and adult palms at APC (60 to 74 nmol g^{-1} FW) (*Table 3*).

For both tissues, the levels of Put (41 to 163 nmol g^{-1} FW) were higher than those of Spd (12 to 34 nmol g^{-1} FW) and Spm (1.8 to 15 nmol g^{-1} FW) (*Table 3*). Interestingly, the Spd and Spm levels were, respectively, 12 and 26 times lower than that of Put in the meristems of healthy adult palms (*Table 4*). In buds, the Spd and Spm amounts were lower than that of Put (*Table 4*). Spd presented a concentration 2

TABLE 3. AVERAGE LEVELS OF ENDOGENOUS PUT, SPD, SPM AND TOTAL FREE-PA (Nmol G^{-1} FW) IN MERISTEMS AND BUDS OF OIL PALMS IN DIFFERENT PHYTOSANITARY STAGES

Age	Stage	Meristem				Bud			
		PUT	SPD	SPM	Total PA	PUT	SPD	SPM	Total PA
Young (10-month-old)	Healthy	41.6 d	16.8 d	1.8 e	60.2 e	45.2 d	24.8 b	4.0 e	74.1 c
	IPC	56.6 d	21.3 c	4.3 d	82.3 d	67.2 b	26.1 a	6.8 c	100.2 b
Adults (15-year-old)	Healthy	163.1 a	13.8 e	6.2 c	183.2 a	95.6 b	35.8 a	12.2 a	143.6 a
	IPC	63.2 c	22.0 b	7.4 b	92.7 c	71.0 c	20.6 c	7.2 c	98.9 b
	APC	44.8 b	11.3 e	4.1 d	60.2 e	43.6 d	14.9 d	5.3 d	63.8 d
	REC	81.1 d	34.0 a	15.1 a	130.3 b	108.1 a	34.8 a	9.3 b	152.3 a
Variance coefficient		4.5	5.8	1.2	3.8	4.5	5.8	1.2	3.8
F-value		447	157	91	416	447	157	91	416

Note: Means with different letters in the same column are significantly different according to Tukey´s test ($p<0.05$).

TABLE 4. RATIOS OF FREE PUT, SPD AND SPM, IN MERISTEMS AND BUDS IN OIL PALMS IN DIFFERENT PHYTOSANITARY STAGES

Age	Stage	Meristem			Bud		
		Put/Spd	Put/Spm	Spd/Spm	Put/Spd	Put/Spm	Spd/Spm
Nursery	Healthy	2.5	23.5	9.5	1.8	11.4	6.3
(10-month-old)	IPC	2.6	13	4.9	2.5	9.8	3.8
Adults	Healthy	11.8	26.1	2.2	2.6	7.8	2.9
(15-year-old)	IPC	2.9	8.5	2.9	3.4	9.8	2.9
	APC	3.9	11	2.7	2.9	8.2	2.8
	REC	2.4	5.3	2.2	3.1	11.5	3.7

to 9.5 times more than that of Spm. In all the analysed tissues or samples, Spm was the PA in lowest concentration.

In the young palms, there were no fluctuations in the PA content, either between the tissues or phytosanitary stages. Only a slight increase in the Spm concentration was observed in the buds and meristems from palms with IPC. Healthy palms at nursery stage displayed lower concentrations (up to a third) of PA than healthy adult palms (*Tables 3* and *4*).

Healthy adult palms had the highest accumulation of Put (163 nmol g^{-1} FW) in their meristematic tissues, while Spd and Spm had higher accumulations in buds. Adult palms with IPC had higher Spd levels in meristem than the healthy adult palms (22 and 13 nmol g^{-1} FW, respectively). Palms with APC had low concentrations of PA in their meristems and buds. The PA concentration in naturally recovering palms was higher than those in the palms with IPC and APC.

Effect of Exogenous Application of PA

PA application had positive effects on the infected oil palms. The effects included preventing the disease and faster recovery of the infected palms with disease symptoms. The effects were measured using different quantitative and qualitative parameters (*Table 2*).

In the palms treated with Put 1000 μM, no symptoms of PC were observed (*Table 5*), but in the control palms without PA treatment (but applied with water) PC symptoms appeared from the fourth month after water application.

In the palms with IPC symptoms, the recovery was accelerated by the application of Spd 100 μM (*Table 5*). This treatment was protective even after more than 18 months. For palms with advanced PC, faster recovery was observed with applications of Spd 50 μM and Spm 75 μM (*Table 5*). Infection by PC did not recur in any of the treated palms.

In addition, PA application did not affect the yield. We did not observe any changes in the bunch production (values between 0 to 6.5 kg) and sex ratio of the inflorescences. Palms are currently in a female cycle (sex ratio > 0.5) (*Table 6*).

TABLE 5. EFFECTS OF PA APPLICATION ON PREVENTING PC AND ASSISTING IN RECOVERY OF PALMS FROM PC

Month	% of healthy palms			% of recovered IPC palms			% of recovered APC palms		
PA dose (μM)	1	6	12	1	6	12	1	6	12
Put 500	100	80	80	0	0	60	0	100	20
Put 1000	100	100	100	0	40	80	0	60	60
Put 2000	100	80	40	0	100	80	0	100	80
Spd 50	100	100	80	0	20	60	0	100	100
Spd 100	100	80	40	0	100	100	0	40	40
Spd 200	100	60	60	0	20	80	0	100	60
Spm 50	100	80	40	0	40	80	0	100	40
Spm 75	100	100	60	0	80	60	0	80	100
Spm 100	100	80	40	0	60	40	0	80	80
Control	100	60	40	0	40	80	0	0	40

TABLE 6. EFFECT OF PA APPLICATION ON SEXUAL EXPRESSION

PA dose (µM)	Healthy palms							IPC palms							APC palms						
	FI	MI	TI	FI/Total	BN	TBW	SBW	FI	MI	TI	FI/Total	BN	TBW	SBW	FI	MI	TI	FI/Total	BN	TBW	SBW
Put 500	2.3	3.0	3.8	0.4	2.4	17.5	6.5	2.5	1.0	2.8	0.9	1.2	3.8	3.3	2.5	3.0	3.7	0.5	2.0	9.2	5.1
Put 1000	2.4	1.3	3.2	0.8	2.0	11.9	5.0	2.4	1.0	2.6	0.9	0.6	2.8	5.1	1.7	4.0	2.3	0.8	1.4	5.0	3.7
Put 2000	2.5	1.5	2.6	0.8	1.4	6.3	5.0	3.0	4.0	4.0	0.7	2.0	7.5	4.1	2.7	2.5	3.3	0.6	1.2	4.9	5.3
Spd 50	2.2	2.0	2.2	0.8	1.2	6.1	5.1	2.3	4.0	2.6	0.8	2.0	7.3	3.6	3.0	1.0	3.3	0.9	2.0	8.0	4.3
Spd 100	2.2	1.7	3.2	0.7	1.6	10.4	6.2	2.8	0.0	2.6	1.0	1.2	5.4	4.7	2.5	4.0	3.0	0.7	2.2	8.1	3.7
Spd 200	3.5	2.7	4.4	0.6	1.4	10.6	5.0	3.0	0.0	3.0	1.0	2.4	11.3	4.3	1.5	1.0	1.8	0.8	0.8	2.4	3.3
Spm 50	2.3	1.3	2.6	0.7	0.6	2.5	4.2	3.5	0.0	2.8	1.0	1.8	8.7	5.0	1.7	3.0	2.0	0.8	0.0	0.0	0.0
Spm 75	2.8	2.0	3.2	0.9	1.6	10.3	4.6	3.3	1.0	3.7	0.9	0.8	4.9	6.1	2.3	2.5	3.8	0.5	1.4	5.5	4.0
Spm 100	2.5	2.7	2.8	0.3	2.0	9.4	5.3	2.4	2.0	2.7	0.8	0.6	1.9	2.5	4.7	2.5	3.8	0.6	1.0	3.8	4.0
Control	3.4	1.0	3.0	0.8	1.0	3.8	3.6	2.4	2.0	2.8	0.9	0.8	3.3	4.5	3.0	0.0	3.0	1.0	1.6	4.5	2.9

Notes: FI: female inflorescences, MI: male inflorescences, TI: total inflorescences, BN= mean bunch number per palm, TBW = total bunch weight, SBW= single bunch weight in kg. Average values obtained after 15 months of exogenous application of PA.

DISCUSSION

PA have been implicated in a vast number of plant growth, developmental and defense processes (Walters, 2000; Galston and Kaur-Shawney, 1990; Evans and Malmberg, 1989; Davies, 1987). However, there are few reports on the PA levels in oil palm and their role in this plant (Mendoza and Rocha, 2002; Norato and Romero, 1995). Experiments were thus carried out to determine the endogenous content of PA in oil palm of different ages and phytosanitary stages.

Endogenous Content of PA in Oil Palm

In the meristem, the levels of Spm in young sick palms and Spd in adult sick palms compared with those in healthy palms support the suggestion that pathogen attack can alter the PA metabolism and accumulation in plants (Walters, 2000; Evans and Malmberg, 1989).

The higher accumulation of Put in the meristem and Spd and Spm in the buds in healthy adult palms suggests that Put was mainly acting on cell division in the meristem, while Spd and Spm were acting on organogenesis and cell elongation in the buds. Similar results have been reported in *Saccharomcyes cerevisiae* in which Put was necessary to maintain cell division, while Spd and Spm were directly involved in cell differentiation (Tabor and Tabor, 1984). Apparently, in all the cases, PA acted by stabilizing the different cell components.

The lowest contents of Put, Spd, and Spm were in the meristem and bud from palms with APC, and could be due to the fact that the damage to the tissues drastically reduced cell division and morphogenesis, with a consequent difference in the rates of production and accumulation of PA. However, the PA in the palms with APC may also be due to PA from microorganisms in the affected tissues than from the tissue *per se*. It is likely that the amount of PA from the proper plant was lower than the value registered.

The Put, Spd, and Spm levels in palms in REC were higher than those in the palms with IPC and APC. These differences could be due to production of the spear leaves because they were the result of active cell division in the meristem and cell differentiation and elongation in the bud, the processes in which PA are required (Egea and Mizrahi, 1991; Bais and Ravishankar, 2002).

The results suggest that PA have specific functions in each stage of the disease. For example, in healthy palms, PA contributes to maintaining the vital processes associated with the reproduction (Bais and Ravishankar, 2002). In palms affected by PC disease (IPC and APC), PA could be important in conferring additional protection against the pathogen (Walters, 2000). In palms in REC, PA seems involved in the induction of morphogenetic processes that result in the production of more spear leaves even though they may be abnormal. The production of new spear leaves helps the palm remove its affected tissue and, subsequently, recover its normal production of fronds and bunches (Nieto, 1996).

As a conclusion, bud rot alters contents of free-polyamines in oil palm. The palm responds by initially increasing the levels of PA when it is attacked. Then, with development of the disease, the levels fall. Finally, the palm again increases its levels of PA, in its recovery from disease.

Effect of Exogenous Application of PA in Oil Palm

The correlation between the endogenous PA content and the severity of PC was the base for using PA to prevent or help palms recover from the disease (Mendoza and Rocha, 2002). Several studies on PA application have been carried out on *in vitro* cell lines of different species (Bais and Ravishankar, 2002). In addition, *in vivo* applications have been performed on important crops and tropical trees (Norato and Romero, 1995a; Romero, 1999; Mendoza, 2000). Therefore, the objective of this experiment was to evaluate the effects of application of different PA on the growth and development of oil palm and to minimize the damage caused by the disease stress.

It is known that at least in the eastern zone of Colombia, a considerable number of palms with advanced PC recover naturally, in contrast to PC disease in other regions such as Tumaco (South West of Colombia) and plantations in Ecuador and Brazil (Swinburne *et al.,* 1996). However, this recovery is very slow with up to two years before the palm shows any signs of rejuvenation (Nieto, 1996).

In the experiments reported here, palms with APC and only water as treatment recovered very slowly. However, the afflicted palms injected with PA, in particular, Spd 50, 100 µM and Spm 75 µM, recovered much faster (*Table 5*) in only about six months compared to the almost three years of natural recovery. This difference in time implies that the oil palm grower can recover production in about 12 months from the initial detection of PC in contrast to the three or four years of the natural process.

In palms with APC, the bud is highly damaged and it is likely that the positive effect of PA is due to the stimulation of cell division in the meristem (Galston and Kaur-Shawney, 1990) or activation of growth, as this has been shown with applications of Put and Spm to dormant tubers of *Helianthus tuberosus* L. (Serafini, 1991) and seedlings of native trees (Norato and Romero, 1995; Mendoza *et al*., 2002). So, the high activity of the meristem will result in production of new spear leaves, which although aberrant, help expel the damaged tissues from the palm (Nieto, 1996).

In healthy palms, that is, palms without PC symptoms, the solution containing Put 1000 μM prevented appearance of the disease, in contrast to the control palms treated only with water. It seemed that Put was somehow conferring resistance against the pathogen, possibly a result of the cell stability function associated with the presence of these molecules. This has been suggested for *Phaseolus* sp. and grass, in which PA application reverted the damage caused by low temperature (Guye *et al*., 1986; Marquínez *et al*., 2001). In addition, it was also possible that PA prevented appearance of the disease by the production of secondary metabolites derived from polyamines (Walters, 2000). It is clear that PA confer some protection against the pathogen, as reported for sugar cane affected by *Ustilago* sp. (Legaz *et al*., 1998). In the case of oil palm, application of PA prevented the typical rot caused by PC on the meristem.

After 18 months´ evaluation, it was clear that PA application to the oil palms did not affect their sexual expression (*Table 6*). This is very important because PA play so many roles in the plant development (Egea and Mizrahi, 1991; Evans and Malmberg, 1989) that it was likely that the imbalances caused by the application could have affected the flowering and sex ratio. However, no differences were observed compared to the control.

CONCLUSION

In conclusion, Put 1000 μM acted on healthy palms to prevent PC. In sick palms (IPC and APC), Spd 100 μM and Spd 50 μM sped up their recovery process (from three years to six months). In palms naturally recovered (REC), PA application had no effect compared to the control. In addition, PA application of PA did not affect the sexual expression of the palms. Therefore, PA application can be considered a novel and complementary management practice against the most important disease of oil palm in Colombia and Latin America.

Finally, it is not clear if the changes in PA levels are the cause or consequence of bud rot. However, the fact that PA could significantly reduce the disease incidence and accelerate the recovery of stricken palms is an indication that they play a major role in the defense mechanism against PC disease in oil palm.

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