

Indole-3-acetic acid metabolism in normal and dwarf micropropagated banana plants (*Musa* spp. AAA)

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Nanism is one of the most frequent type of mutant in micropropagated banana plants from the Cavendish subgroup. The present study aimed at studying some of the hormone factors involved in this type of mutation. Rhizomes from normal and dwarf plants from the cultivar Grand Naine were incubated for 5 d in the presence of [³H]-L-tryptophan, [³H]-indole-3-acetic acid and gibberellin, to quantify the endogenous levels of indole-3-acetic acid-ester, indole-3-acetic acid-amide, free indole-3-acetic acid, and cytokinins. The endogenous levels of indole-3-acetic acid and its ester- and amide-conjugated forms were measured in normal and dwarf plants incubated for 30 d with gibberellin, indole-3-acetic acid, and L- and D-tryptophan. In normal plants, the use of [³H]-L-tryptophan resulted in higher levels of radioactivity in the retention times corresponding to indole-3-acetic acid, indole-3-acetic acid-aspartate, indole-3-acetic acid-glycine and indole-3-acetic acid-alanine. These values were higher than those observed in dwarf plants. Higher quantities of radioactive indole-3-acetic acid and of amide-forms in dwarf plants occurred in rhizomes treated with [³H]-L-tryptophan and gibberellin simultaneously. The endogenous levels of total cytokinins were the same in both materials, while the levels of indole-3-acetic acid in normal plants were 1.5 times higher than in dwarf plants. Moreover, in these ones application of tryptophan and gibberellin coincided in an increase in the levels of free indole-3-acetic acid in dwarf plants and to a decrease in the levels of indole-3-acetic acid-ester and indole-3-acetic acid-amide.

Key words: indole-3-acetic acid metabolism, growth regulators, somaclonal variation.

Metabolismo do ácido 3-indolil acético em plantas anãs e normais de bananeira micropropagada (*Musa* spp. AAA):

O nanismo em plantas de bananeira micropropagada representa uma das formas mais comuns de alteração genética no subgrupo Cavendish. Utilizaram-se rizomas de plantas normais e anãs de bananeira cv. Grande Naine, incubados durante 5 dias na presença de [³H]-L-triptofano (Trp), [³H]-ácido 3-indolil acético e giberelina (GA₃), para o estudo da biossíntese de ácido 3-indolil acético e ácido 3-indolil acético amida e dos níveis endógenos de citocininas totais (Cks) e ácido 3-indolil acético livre. Os teores endógenos de ácido 3-indolil acético e de seus conjugados éster e amida foram obtidos de plantas anãs e normais incubadas durante 30 dias na presença de GA₃, ácido 3-indolil acético e L- e D-triptofano. A aplicação de [³H]-L-triptofano resultou na obtenção de quantidades mais elevadas de radioatividade nas plantas normais, no tempo de retenção coincidente com o de ácido 3-indolil acético, ácido 3-indolil acético-aspartato, ácido 3-indolil acético-glicina e ácido 3-indolil acético-alanina, valores esses superiores aos observados nas plantas anãs. Verificou-se a ocorrência de maior quantidade de ácido 3-indolil acético radioativo e de forma amida nas plantas anãs nos rizomas tratados com [³H]-L-triptofano e GA₃ simultaneamente. Os níveis endógenos de Cks totais foram praticamente iguais em ambos os materiais, sendo o teor de ácido 3-indolil acético nas plantas normais cerca de 1,5 vez superior ao das plantas anãs. Os tratamentos com triptofano e GA₃ favoreceram incremento nos níveis de ácido 3-indolil acético livre nas plantas anãs, paralelamente a uma diminuição nos teores de ácido 3-indolil acético éster e amida.

Palavras-chave: metabolismo do ácido 3-indolil acético, reguladores de crescimento, variação somaclonal.

INTRODUCTION

The occurrence of mutants during the micropropagation of banana plants poses a serious limitation to the widespread use of this technique. Nanism is the most frequent type of mutant, occurring in up to 90% of micropropagated plants from the Cavendish subgroup (Stover, 1987; Smith, 1988; Israeli *et al.*, 1991).

Some studies related to nanism in banana plants point to the involvement of gibberellins (Sandoval *et al.*, 1995; Damasco *et al.*, 1996); in other crops such as *Hordeum vulgare* nanism is related to low endogenous levels of indole-3-acetic acid (Inouhe *et al.*, 1982). Most indole-3-acetic acid present in plant tissues is covalently linked to other compounds, the most frequent form in legumes being the aminoacid-conjugated indole-3-acetic acid, whereas in monocots the most frequent form is represented by sugar-conjugated indole-3-acetic acid (Bandurski and Schulze, 1977).

With respect to the metabolism of indole-3-acetic acid in plants, it has been shown that free indole-3-acetic acid is easily converted into esterified indole-3-acetic acid with sugar or amide-linked indole-3-acetic acid and such conjugated forms are the forms in which indole-3-acetic acid is stored in plants.

The physiological importance of indole-3-acetic acid conjugation seems to be the control of free indole-3-acetic acid in cells (Cohen and Bandurski, 1982). Previous studies with 'Grand Naine' banana plants showed that nanism was related to low sensitivity to GA and to the effect of this growth regulator in the metabolism of indole-3-acetic acid (Zaffari *et al.*, 1998).

The present study aimed at studying the metabolism of endogenous auxin in dwarf banana plants through the quantification of the levels of free, amide-conjugated and ester-conjugated indole-3-acetic acid, as well as the measurement of the biosynthesis of indole-3-acetic acid and its amide-conjugated form.

MATERIAL AND METHODS

Plant material: The dwarf and normal plants of *Musa* spp. (AAA) cv. Grand Naine used in this study were obtained from *in vitro* culture of rhizomes in liquid MS medium (Murashige and Skoog, 1962) added with 2.5 mg.L⁻¹ benzylaminopurine (BAP).

Determination of endogenous free indole-3-acetic acid and cytokinins in rhizomes: The endogenous levels of indole-3-acetic acid and the cytokinins (Cks) zeatin (Z), zeatin riboside ([9R]Z), isopentenyladenine (iP), and isopentenyladenosine ([9R]iP), were measured using 1 g of fresh dwarf and normal banana rhizome tissue, which had been incubated for 5 d in MS liquid medium. The immunoenzymatic method was performed according to Maldiney *et al.* (1986) and Sotta *et al.* (1987) with some modifications (Peres *et al.*, 1997). This method allowed for the determination of three hormonal classes in the same extract. Freeze-dried powdered tissues were stirred up and extracted with chilled 80 % methanol containing butylhydroxytoluene (0.18 mM) as an antioxidant for 60 h at 4 °C in the dark. Tritiated radiolabelled standards (IAA and ABA, specific activity 999 GBq.mmol⁻¹ and 2.37 TBq.mmol⁻¹, respectively; Amersham, UK), were added to the samples for recovery estimation after purification. Tritiated IAA and ABA have been shown to perform as internal standards for Z, ZR and iP and iPA, respectively (Maldiney *et al.*, 1986; Sotta *et al.*, 1987). The methanolic extracts were filtered and then passed through SepPak C18 cartridges, and the eluates reduced to dryness in a vacuum with a rotary evaporator. The residues were redissolved in 500 µL acid water, pH 3.0 and the hormones separated for 80 min by HPLC using a reverse-phase semi-preparative µBondapak 19 mm x 300 mm column, at a flow rate of 5 mL.min⁻¹ with a 5-50 % methanol gradient in formic acid buffered to pH 3.0. Fractions were collected at 1-min intervals and reduced to dryness in a Speed-Vac concentrator. They were then methylated with ethereal diazomethane for IAA analysis prior to being submitted to ELISA with anti-IAA antibodies. Cytokinin measurements were carried out by ELISA with anti-[9R]Z and anti-[9R]iP antibodies. The hormone levels in each sample were measured four times and the standard deviation was calculated.

Determination of endogenous free indole-3-acetic acid, indole-3-acetic acid-amide and indole-3-acetic acid-ester in leaf sheaths: Both dwarf and normal genotypes were submitted for 30 d to the following treatments to determine the endogenous levels of free and bounded indole-3-acetic acid: MS (control), MS + 75 µM GA₃, MS + 50 µM IAA, MS + 75 µM GA₃ + 50 µM IAA, MS + 500 µM L-tryptophan, MS + 500 µM L-tryptophan + 75 µM GA₃,

MS + 500 μ M D-tryptophan, MS + 500 μ M D-tryptophan + 75 μ M GA₃. The experiment was carried out in a completely randomized design with 10 replications and one rhizome per plot. Freeze-dried powdered tissues (1 g) of the leaf sheaths were stirred up and extracted with chilled 80 % methanol containing butylhydroxytoluene (0.18 mM) as an antioxidant for 60 h at 4 °C in darkness. Tritiated radiolabelled indole-3-acetic acid (specific activity 999 GBq.mmol⁻¹; Amersham, UK), was added to the samples for recovery estimation after purification. The methanolic extracts were then centrifuged at 10,000 g_n for 10 min at 4 °C, and the supernatant filtered through SepPak C18 cartridges. After filtration, each eluate was divided into three equal amounts and reduced to dryness in a Speed-Vac concentrator. For determination of ester and amide auxins, an alkaline hydrolysis was performed with two of the three samples. The alkaline hydrolysis was conducted with 1 M NaOH at room temperature for 1 h (ester bound conjugates) or with 7 M NaOH at 100 °C under N₂ for 3 h (amide bound conjugates). After adjusting the pH to 2.5 with HCl, the indole-3-acetic acid of all samples was extracted with ether three times. The fractions with ether were reduced to dryness in a Speed-Vac concentrator and then methylated with ethereal diazomethane. The measurements of the resulting free indole-3-acetic acid of the three amounts were carried out by ELISA with anti- indole-3-acetic acid antibodies as previously described (Peres et al., 1997). The hormone level in each sample was measured four times and the values were corrected for recovery.

Biosynthesis of free indole-3-acetic acid and indole-3-acetic acid-amide in rhizomes: Indole-3-acetic acid biosynthesis in dwarf and normal banana plants was analyzed in rhizomes incubated for 5 d in liquid MS medium with the following treatments: MS + 5 μ Ci.mL⁻¹ [³H]-L-tryptophan; MS + 5 μ Ci.mL⁻¹ [³H]-L-tryptophan + 75 μ M GA₃ and MS + 5 μ Ci.mL⁻¹ [³H]-IAA. The cultures were kept in a growth chamber under 100 rpm agitation and 16 h of light (10 W.m⁻²) at 28 \pm 2 °C. The experiment was carried out in a completely randomized design with 3 replications, and one rhizome per plot. Before the analysis, the rhizomes were washed 5 times with milliQ water and frozen in liquid nitrogen. For the recovery estimation after purification, 1.85 Mbq of ABA was added to the samples. After the separation in HPLC, each fraction was subjected to radioactivity counting and the incorporation picks of radioactivity were compared with the retention times for

tryptophan, indole-3-acetic acid, indole-3-acetic acid-aspartate (IAA-Asp), indole-3-acetic acid-glycine (IAA-Gly) e indole-3-acetic acid-alanine (IAA-Ala).

RESULTS AND DISCUSSION

Endogenous levels of indole-3-acetic acid and total cytokinins in dwarf and normal plants: The Cks levels were similar in both normal and mutant plants but the amounts of indole-3-acetic acid in normal plants were ca. 1.5 times higher than those found in mutants (table 1), which suggests that nanism is associated with indole-3-acetic acid metabolism. In recent studies a relationship was shown between variegated mutants and the Cks metabolism in micropropagated banana plants and in these plants the levels of chlorophylls in variegated leaves were positively correlated with the Cks levels.

Table 1. Endogenous levels of total cytokinins (Z, [9R]Z, iP, [9R]iP), indole-3-acetic acid and indole-3-acetic acid/cytokinins ratio in rhizomes from 'Grand Naine' banana genotypes after 5 d of incubation in Murashige and Skoog (1962) liquid culture medium.

Genotype	IAA ^a (pmol.g ⁻¹ FW)	Cytokinins (pmol.g ⁻¹ FW)	IAA/Cytokinins ratio
Normal	118.1 \pm 28.0	692.2 \pm 32.6	0,17
Dwarf	78.7 \pm 30.8	651.3 \pm 96.2	0,12

^a Values are mean \pm SD (n = 3).

Endogenous levels of free and conjugated indole-3-acetic acid in normal and dwarf plants: The presence of GA₃ alone in the medium decreased the level of free indole-3-acetic acid in normal plants, but did not have any effect on dwarf plants. On the contrary, indole-3-acetic acid alone in the medium depressed the amount of free indole-3-acetic acid in dwarf plants. When GA₃ was added with indole-3-acetic acid an increase was observed in the amount of free indole-3-acetic acid in dwarf plants, but no effect could be detected in normal plants (figure 1A).

L-tryptophan in the culture medium, with or without GA₃, caused an increase in the levels of endogenous free indole-3-acetic acid in normal and dwarf plants, whereas D-tryptophan caused a decrease in those levels (figure 1A). These results corroborate the suggestion given by Baldi et al. (1991) that indole-3-acetic acid is synthesized from L-tryptophan and not from D-tryptophan, as proposed by Law

(1987). It is also important to note that the amount of free indole-3-acetic acid present in normal plants incubated in the presence of L-tryptophan was higher than the amount found in dwarf plants under the same conditions, but incubation with L-tryptophan and GA₃ at the same time caused this amount to be significantly higher in dwarf plants, compared to normal plants under the same conditions.

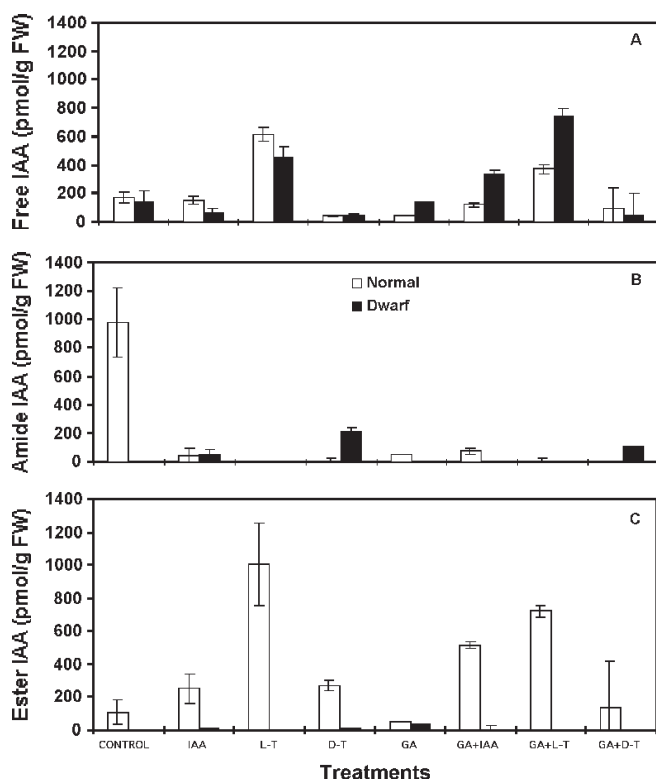


Figure 1. Endogenous levels of free indole-3-acetic acid (A), indole-3-acetic acid-amide (B) and indole-3-acetic acid-ester (C) in pseudostems of dwarf and normal 'Grand Naine' banana plants after 30 d of incubation in Murashige and Skoog (1962) culture medium (MS; control), 50 μ M IAA + MS, 500 μ M L-tryptophan + MS, 500 μ M D-tryptophan + MS, 75 μ M GA₃ + MS, 75 μ M GA₃ + 50 μ M IAA + MS, 75 μ M GA₃ + 500 μ M L-tryptophan + MS, 75 μ M GA₃ + 500 μ M D-tryptophan + MS. Values are mean \pm SD (n = 10).

In media without growth regulators or tryptophan, only normal plants accumulated indole-3-acetic acid-amide (figure 1B). The addition of these compounds, alone or in combination, caused a severe reduction in the accumulation of indole-3-acetic acid-amide in normal plants, but indole-3-acetic acid, GA₃, or D-tryptophan, also caused a moderate increase in the accumulation of indole-3-acetic acid-amide in dwarf plants. Growing evidence suggests

that different conjugates perform different functions in the plant. Certain conjugates, such as indole-3-acetic acid-amide, can be intermediates in indole-3-acetic acid destruction (Tuominen *et al.*, 1994). Often indole-3-acetic acid conjugates may serve as reservoirs of inactive indole-3-acetic acid that can be hydrolysed to provide the plant with active hormone. In maize, indole-3-acetic acid-inositol can be transported from the kernel to the shoot, and conjugate hydrolysis, rather than de novo synthesis, supplies the developing seedling with indole-3-acetic acid (Epstein *et al.*, 1980).

As observed with indole-3-acetic acid-amide, only normal plants accumulated indole-3-acetic acid-ester in medium without growth regulators or tryptophan. The addition of GA₃ or indole-3-acetic acid alone to the medium had no significant effect, but the combination of both significantly increased the amount of indole-3-acetic acid-ester (figure 1C). The most marked effect was observed when L-tryptophan was added alone or in combination with GA₃, whereas D-tryptophan caused only a marginal increase in the amount of indole-3-acetic acid-ester when applied alone. The broad distribution of indole-3-acetic acid-ester and indole-3-acetic acid-amide in plant tissues (Bandurski and Schulze, 1977; Sztein *et al.*, 1995), together with the higher concentrations of these growth regulators compared with the concentration of free indole-3-acetic acid, suggest that the synthesis and the hydrolysis of these conjugated forms represent an important mechanism in the control of the auxin levels in plants (Cohen and Bandurski, 1982). The analysis of tryptophan-auxotrophic mutants with a defect in the activity of tryptophan synthase β in maize and *Arabidopsis* revealed the high accumulation levels of indole-3-acetic acid in intact cells. Although L-tryptophan has been proposed to be a precursor of indole-3-acetic acid, these observations suggest the presence of a tryptophan-independent pathway, where indole or indoleglycerol might be a precursor of indole-3-acetic acid (Wright *et al.*, 1991; Normanly *et al.*, 1993). Recently, D-tryptophan, rather than the much more abundant L isomer, has been proposed to be a direct biosynthetic precursor of indole-3-acetic acid based on evidence of gibberellin enhanced racemization of L-tryptophan to D-tryptophan, which could then be transaminated to indolepyruvic acid (Law 1987; McQueen-Mason and Hamilton, 1989). Lantican and Muir (1969) have reported that GA₃ does not decrease indole-3-acetic acid-aspartate synthesis in pea

stems. In the long-standing argument concerning a possible role for auxin in the mediation of GA-induced elongation, it is often noted that exogenous auxin fails to increase growth to an extent comparable to that obtained with exogenous GA. However, such a proposed GA-induced increase in indole-3-acetic acid biosynthesis coupled with reduced conjugation might be relevant, especially given that exogenous auxins induce conjugation (inactivation) of indole-3-acetic acid (Venis, 1972).

However, it was observed in a previous study that the enzymatic degradation of free indole-3-acetic acid in dwarf banana plants was ca. 1.5 times higher than that observed both in rhizomes and leaves in normal plants (Zaffari et al., 2000). This observation suggests that the control of the endogenous level of indole-3-acetic acid through enzymatic degradation may be a mechanism to compensate for the low conjugation capacity found in dwarf mutants (figures 1B and 1C).

Biosynthesis of auxin in normal and dwarf plants: The retention times (RT) for the studied compounds were: tryptophan = 32.19 min; indole-3-acetic acid-asp = 40.50 min; indole-3-acetic acid-gly = 41.45 min; indole-3-acetic acid-ala = 45.92 min; and indole-3-acetic acid = 51.20 min (figure 2). The patterns of indole-3-acetic acid-amide retention times are relatively low when compared to the patterns for free indole-3-acetic acid. This small interaction with the stationary apolar phase is probably due to the higher polarity of aminoacids. The treatment with [³H]-L-tryptophan led to the incorporation of radioactivity in the RT corresponding to indole-3-acetic acid-asp, indole-3-acetic acid-gly, indole-3-acetic acid-ala and indole-3-acetic acid in the rhizomes of normal and dwarf plants, the incorporation in the RT for indole-3-acetic acid-gly being the most expressive in both genotypes (figure 3A). In general the incorporation of radioactivity in normal plants was markedly superior to that observed in mutant plants (figure 3A), which suggests that the genotypes studied presented differences in their capacity for indole-3-acetic acid biosynthesis. In addition, this biosynthesis seems to be at least partially dependent upon tryptophan, with an expressive fraction of the [³H]- indole-3-acetic acid formed being incorporated in substances with RT equivalent to those found in some aminoacid-conjugated forms. An inverse effect in indole-3-acetic acid biosynthesis and conjugation was observed between the two genotypes when incubated with [³H]-L-tryptophan + GA₃, since there was more incorporation in the dwarf mutant (figure 3B), which

is in agreement with the data from figure 1. Incubation with [³H]- indole-3-acetic acid led to an accumulation of radioactivity in the RT equivalent to that of free indole-3-acetic acid, particularly in normal plants (figure 3C).

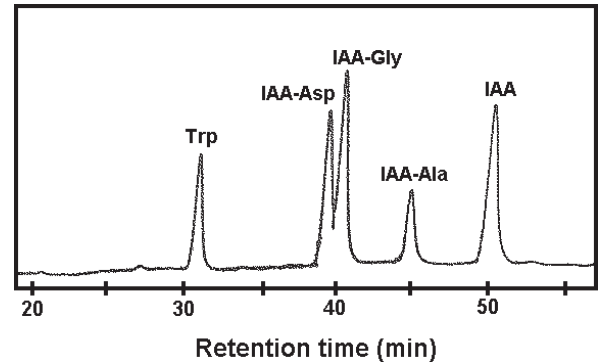


Figure 2. Radioactive patterns of tryptophan, indole-3-acetic acid-asp, indole-3-acetic acid-gly, indole-3-acetic acid-ala and indole-3-acetic acid in HPLC. CPM = counts per min.

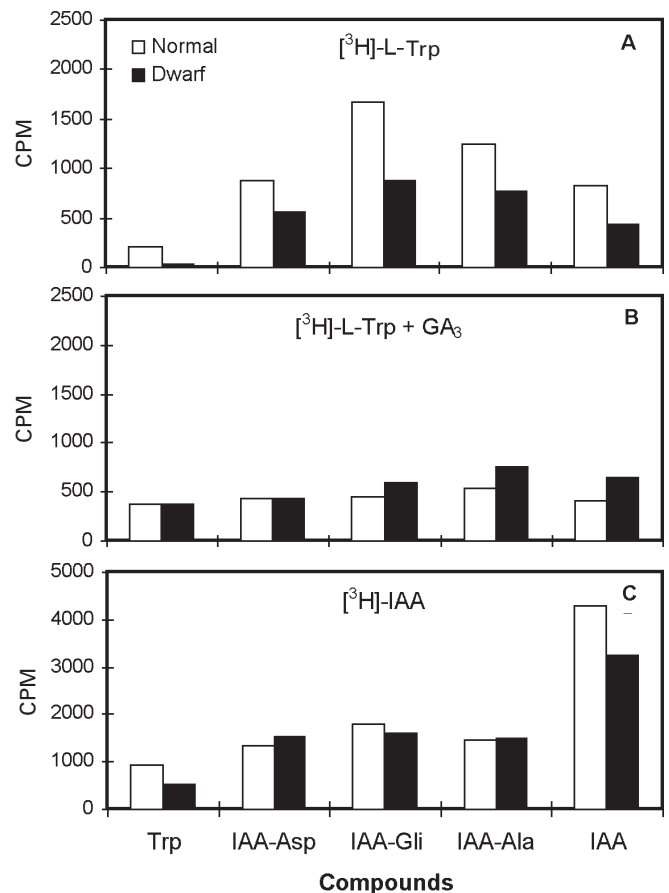


Figure 3. Radioactivity distribution in HPLC methanolic extracts, from rhizomes of 'Grand Naine' banana plants after 5 d of incubation with (A) MS + 5 µCi mL⁻¹ [³H]-L-tryptophan, (B) MS + 5 µCi mL⁻¹ [³H]-L-tryptophan + 75 µM GA₃ and (C) MS + 5 µCi mL⁻¹ [³H]-indole-3-acetic acid. CPM = counts per min.

Pseudostems from both genotypes presented low endogenous levels of indole-3-acetic acid-amide 30 d after rhizome incubation unlike rhizomes incubated for 5 d in the presence of L-tryptophan, L-tryptophan + GA₃ and indole-3-acetic acid (figures 3A, 3B and 3C, respectively). These results indicate that the amide conjugates in rhizomes could accumulate in pseudostems as free and ester forms of indole-3-acetic acid in normal plants, and as free form in dwarf banana plants (figures 1A and 1C). These results show the interaction between exogenous GA₃ and indole-3-acetic acid metabolism in both banana genotypes. Although the occurrence of interactions between the metabolism of endogenous gibberellins and indole-3-acetic acid has already been demonstrated (Tsurusaki *et al.*, 1990; Zaffari *et al.*, 2000), there is little information on the regulatory mechanisms involved.

This study suggested that dwarfism in banana plants is related not only with low endogenous levels of gibberellins, as proposed by Sandoval *et al.* (1995) and Damasco *et al.* (1996), but also with a decrease in the endogenous levels of indole-3-acetic acid. A putative interaction between the metabolism of gibberellin and indole-3-acetic acid may occur in this mutant type. Although little is known about the interaction between the metabolism of these two growth regulators, the present results corroborate previous reports in the literature pointing to the involvement of these growth regulators on the control of cell division and growth in leaves, sheaths and stems of normal and dwarf banana plants.

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