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ORIGINAL ARTICLE

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Melatonin: a growth-stimulating compound present in lupin tissues

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Abstract Melatonin (N-acetyl-5-methoxi-tryptamine), a well-known animal hormone synthetised by the pineal gland, plays a key role in the circadian rhythm of vertebrates. An exhaustive bibliographical revision of studies on melatonin in plants published since 1990 points to very few studies (around 20), of which only 8 have a clear plant physiological focus. The data presented in this study demonstrate that melatonin plays a physiological role in plant tissues. Melatonin is seen to be a molecule that promotes vegetative growth in etiolated *Lupinus albus* L. hypocotyls, in a similar way to IAA. The measurements of melatonin and IAA in lupin hypocotyls by high-performance liquid chromatography with electrochemical detection, and their identification by tandem mass spectrometry, point to a different distribution of these molecules in etiolated hypocotyls.

Keywords Auxin · Hormone · Indole-3-acetic acid · *Lupinus* · Melatonin · Plant growth

Abbreviations IAA: Indole-3-acetic acid · MEL: Melatonin · HPLC–EC: High-performance liquid chromatography with electrochemical detection · MS/ESI+: Tandem mass spectrometry in positive electrospray ionization mode

Introduction

Melatonin (N-acetyl-5-methoxi-tryptamine; MEL), a well known animal hormone synthetised by the pineal gland, plays a key role in the circadian rhythm, sleep regulation and seasonal photoperiodic regulation (Hardeland et al. 1993; Yu and Reiter 1993; Brzezinski 1997; Foulkes et al. 1997). Melatonin acts as a hormone, but has also been studied for its role in scavenging different types of reactive oxygen and nitrogen species (Beyer et al. 1998; Chyan et al. 1999; Reiter et al. 2001b, 2002).

Since 1995, MEL has also been detected in the roots, leaves, fruits and seeds of a considerable variety of plant species (Dubbels et al. 1995; Kolar et al. 1995). Some authors have postulated that MEL acts as a universal antioxidant because of its wide distribution in bacteria, fungi, plants and animals (Reiter et al. 2001a). Recently we characterised the antioxidant properties of MEL and of other plant indoles, such as indole-3-acetic acid (IAA), indole-3-propionic acid, indole-3-butyric acid, indole-3-methanol and tryptophan, and found that they all showed good antioxidant properties (Cano et al. 2003).

However, very little is known of the role played by MEL in plants, although inconclusive attempts have been made to seek a role for this indolic compound as a photoperiodic and circadian regulator (Kolar et al. 1997; van Tassel et al. 2001; Machackova and Krekule 2002). However, interesting results have been obtained in in vitro organogenesis studies. The biosynthetic route of MEL and serotonin from tryptophan has been characterised in cultured cells of St. John's wort (*Hypericum perforatum* L.), and it has been shown that different concentrations of MEL affect root development and play a decisive role in the auxin/cytokinin ratio in the de novo biosynthesis of roots and/or shoots (Murch et al. 2000; Murch and Saxena 2002a).

In this paper we present a study of the possible role of MEL as a growth regulator in etiolated lupin hypocotyls. The chemical structure (indolic derivate) and the biosynthetic pathway (from tryptophan) of MEL led us to believe that it might behave as an auxin in plant growth, as other authors have previously suggested (Murch and Saxena 2002a). To ensure that this molecule was present in lupin, we measured the levels of MEL and of IAA in hypocotyls by high-performance liquid

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chromatography with electrochemical detection (HPLC–EC), confirming their identification by tandem mass spectrometry in positive electrospray ionization mode (MS/ESI+).

Materials and methods

Plant material

Seeds of lupin (*Lupinus albus* L.) were sterilized in 10% hypochlorous solution for 5 min, dipped in distilled water and grown in vermiculite at 24°C in darkness for 6 days.

Reagents

The indolic compounds, indole-3-acetic acid (IAA) and melatonin (N-acetyl-5-methoxy-tryptamine; MEL) were purchased from Acros Organics (Belgium). The solvents ethyl acetate and acetonitrile, both of HPLC grade, were obtained from Baker (Holland). The different reagents and salts (analytical grade) used to prepare the incubated solutions were obtained from Merck (Spain).

Growth assays

Three different longitudinal growth assays were performed.

- Assay A: The protocol of Ludwig-Müller and Cohen (2002) was used to characterize the longitudinal growth-promoting activity of IAA and MEL. For this, 5 mm sections of lupin hypocotyl were incubated in 0.01, 0.1, 1, 10, 100 and 1,000 μM solutions for 24 h at 24°C in darkness. Any increase in length was measured using a binocular microscope with a 10-fold magnification.
- Assay B: In this assay de-rooted hypocotyls were immersed (with the cotyledons protruding) in the same range of concentrations of IAA and MEL as for assay A. The increases in hypocotyl length were estimated after 24 h (Cano et al. 1996).
- Assay C: The cotyledons of 6-day-old lupins were removed. The meristematic zone (source of auxin) between the cotyledons and the hypocotyl (5–6 mm) was also eliminated. Agar blocks (0.8%) of 5 mm diameter were placed on top of each hypocotyl and different amounts of IAA or MEL (one application of 6 μl of 0.1, 1, 10, 100, 1,000 and 10,000 μM solutions; representing a range from 0.6 pmol to 60 nmol) were applied to each agar block. The increase in length of hypocotyls grown in vermiculite was measured after 24 h. In all cases, the solutions contained 10 mM potassium phosphate (pH 6.2) with 50 mM sucrose and various salts (Ludwig-Müller and Cohen 2002).

Indole analysis

To measure the IAA and MEL contents in the lupin tissues, the extraction and chromatographic method previously developed by us for lupin (Guerrero et al. 2001) was used. The results obtained for IAA extraction were compared with those obtained with the method described by Sandberg et al. (1987), while the results obtained for MEL were compared with those obtained with the method described by Murch et al. (2000). In all cases the quantifications of IAA and MEL were very similar. HPLC (Beckman System Gold), using an RP-C18 column (ODS2) with electrochemical detection (HP model 1049A with glassy carbon working electrode and a solid state Ag/AgCl reference electrode), was used to measure IAA and MEL levels in hypocotyls because both indoles were detected at +850 mV with excellent sensitivity. Endogenous IAA and MEL were identified by tandem mass spectrometry operating in positive electrospray ionization mode (MS/ESI+) with direct-flow injection using an HPAgilent 1100 MS detector. A single ion-product ion transition was monitored for IAA: m/z 176 to m/z 130, and for MEL: m/z 233 to m/z 174. The conditions were similar to those described in Yang et al. 2002.

Statistical analysis

For the bioassay data, differences were determined from their means and standard error using the SigmaPlot graphical program. Statistical analysis of indole concentration levels was carried out by the SPSS program, applying the LSD multiple range test to establish significant differences between zones.

Results and discussion

The bioassays described are commonly used to characterize the growth-promoting activity of natural or synthetic compounds in plants (Ludwig-Müller and Cohen 2002). Figure 1a shows the longitudinal growth of tissue sections of hypocotyl when incubated in the presence of a range of IAA or MEL concentrations for 24 h (assay A). IAA produced the expected effect on the sections: an active promotion of growth at concentrations in the micromolar range but a growth-inhibitory effect at high concentrations. When MEL was assayed as a possible growth-promoting agent, a similar effect to that obtained with IAA was seen. MEL stimulated the growth of the different tissue sections in a concentration range similar to the auxin IAA. At high concentrations, MEL behaved as an inhibitor (probably because it reached toxic levels), while the optimum concentration for promoting growth was 10 μM. Taking the optimum degree of growth promotion obtained with IAA as 100, the optimum growth-promoting effect of MEL was 22% for this bioassay.

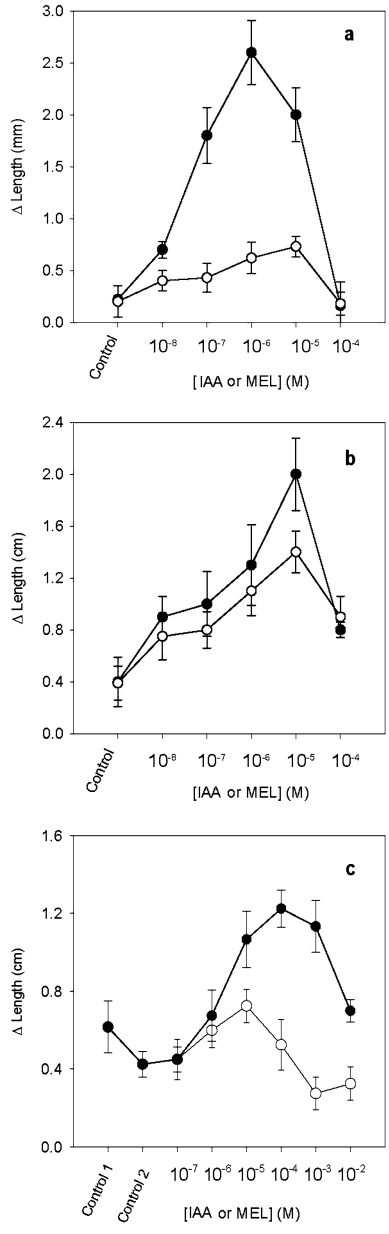

Fig. 1a–c Effect of IAA (*filled circles*) and MEL (*open circles*) on the growth of etiolated lupin (*Lupinus albus*) hypocotyls. **a** Assay A. Effect of IAA and MEL (at different concentrations) on the growth of 5-mm hypocotyl sections after 24 h of incubation (*Control* growth in buffer solution). **b** Assay B. Effect of IAA and MEL on the growth of de-rooted hypocotyls after 24 h. **c** Assay C. Effect of IAA and MEL addition in agar blocks on the growth of 6-day-old decapitated hypocotyls after 24 h. (*Control 1* intact lupin plants; *Control 2* decapitated hypocotyls with buffer solution added in agar blocks). Means ± SE ($n=4$)

In assay B, in which lupins were de-rooted and treated with IAA or MEL solutions, the promotion of growth reached similar levels to those described in assay A (Fig. 1b). In this case, both indoles promoted optimum growth at 10 μM, perhaps because a greater amount was necessary to reach the target site in the cell because the hypocotyl tissues were intact. In this case, the growth-promoting effect of MEL with respect to IAA was 63%, which must be regarded as a considerable auxinic effect on lupin hypocotyl growth.

To ascertain the possible effect of the endogenous hormone on the growth-promoting effect of IAA or MEL, we performed an assay in which the zone of hormone synthesis was eliminated (assay C). In etiolated lupin, this is the zone immediately below the cotyledons (meristematic zone), where auxin is generated and transported to the rest of the hypocotyl (Sánchez-Bravo et al. 1986). It was seen that lupins without this zone and to which only buffer solution was applied at the agar block, showed minimal growth (Control 2 in Fig. 1c). The application of IAA to the agar block restored the growth capacity of the hypocotyl in a concentration-dependent way. In this assay too, an inhibitory auxin effect was seen at high IAA concentrations (Fig. 1c). In the same way, MEL added to the agar block had the capacity to activate hypocotyl growth. In this bioassay, the growth-promoting effect of MEL with respect to IAA was 36%. Because the meristematic zone had been excised, any hypocotyl growth must have been due to cell expansion in the tissues. In this physiological aspect, MEL also would play a role.

Since IAA levels are usually high in the first stages of lupin seedling growth, we selected these stages to quantify free IAA by HPLC–EC (Guerrero et al. 2001). MEL levels in hypocotyls were also measured by the same technique. Figure 2a shows a representative HPLC–EC chromatogram of an IAA and MEL standard detected at +850 mV. Figure 2b shows a similar analysis of a lupin hypocotyl sample. IAA and MEL peaks appear with their characteristic retention times of 32.5 and 41.7 min, respectively. When known amounts of standard IAA and MEL were added to a hypocotyl sample, the peaks at 32.5 and 41.7 min increased in proportion to the quantity of standard added, strongly suggesting that these peaks were the IAA and MEL present in hypocotyls. The identification of endogenous IAA and MEL was made by MS/ESI+. Selected ion monitoring (SIM) in the multiple reaction monitoring (MRM) of the daughter ions, in the case of IAA (m/z 176 → 130) and in the case of MEL (m/z 233 → 174), in lupin samples unequivocally confirmed their identification (Harumi and Matsushima 2000; Yang et al. 2002).

Table 1 shows the IAA and MEL levels in different hypocotyl zones: apical, central and basal. According to our previous data, IAA presents a concentration gradient: apical > central > basal (Guerrero et al. 2001). For MEL, a similar distribution gradient was obtained. In all cases IAA levels were around 20 times higher than those of MEL.

Since Reiter and co-workers detected MEL in plant tissues in 1995 (Dubbels et al. 1995), there has been no concerted effort to identify its possible physiological function or role in these tissues. The few studies that exist have centred on its possible role as a regulatory molecule in circadian cycles and in aspects connected with photoperiodicity, such as flowering (Kolar et al. 1997; van Tassel et al. 2001; Machackova and Krekule

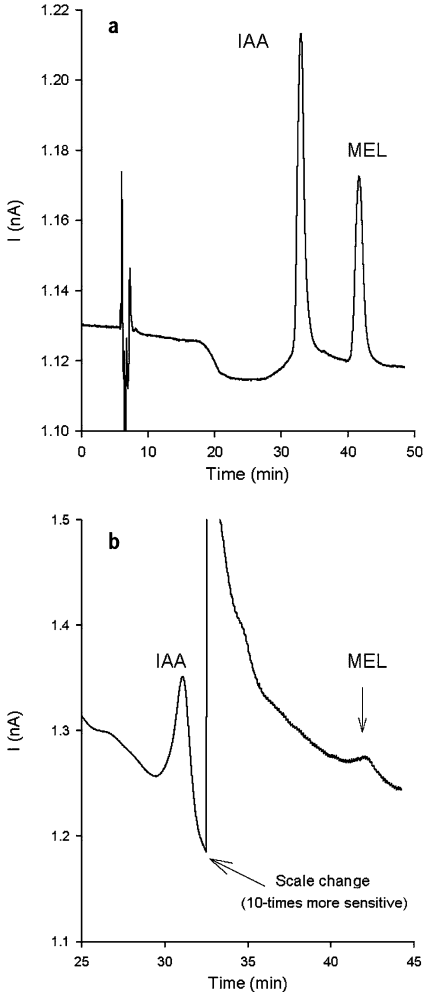

Fig. 2a,b Representative chromatograms showing HPLC–EC analysis of IAA and MEL. **a** IAA and MEL standards (0.2 nmol injected) registered at +850 mV (scale 0–500 nA) using an isocratic elution program with water–acetonitrile–acetic acid (82:16.5:1.5, by vol.) at a flow-rate of 0.7 ml min^{-1}. **b** HPLC–EC analysis of a lupin hypocotyl sample (central zone); 60 µl injected of an extraction of 2 g FW tissue in a final volume of 1 ml. After IAA elution, a scale change was made to detect MEL (scale 0–50 nA)

Table 1 Quantitation of endogenous IAA and MEL by HPLC–EC in the three zones of etiolated lupin (*Lupinus albus*) hypocotyls

Hypocotyl zone	IAA (ng g FW^{-1})	Melatonin (ng g FW^{-1})
Apical	663a*	28.1a
Central	322b	17.2b
Basal	174c	9.3c

*Within each column, values followed by a different letter are significantly different at the 0.05 confidence level ($n=5$)

2002; Murch and Saxena 2002b), although a possible auxinic role also has been discussed (Murch and Saxena 2002a). The data presented in this study show that MEL has a physiological action in plant tissues. MEL is seen to be a molecule with auxinic activity and to promote vegetative growth, in a similar way to IAA (Fig. 1). The auxin potential of this methoxyindole is between 22 and 63% of that associated with IAA, an activity that needs to be taken into account since other auxins, such as phenylacetic acid, show much lower activity than IAA (Ludwing-Müller and Cohen 2002). Thus, IAA is transported from the biosynthesis tissue to the whole hypocotyl, diminishing its concentration still further. This spatial distribution of IAA (and MEL) is in accordance with the growing potential of the different zones: the zones with highest growth rate are also the zones with highest endogenous IAA content (Sánchez-Bravo et al. 1986; Guerrero et al. 2001).

In conclusion, the detection of MEL together with IAA in the tissues studied, the number of papers which describe high levels of this indolic compound in plant tissues (Burkhardt et al. 2001; Chen et al. 2003) and the identification of its biosynthetic pathway from tryptophan (Murch et al. 2000) strongly suggest that we are dealing with a new molecule of possible hormonal status. Whatever the case, we feel that a new perspective is opening up in the field of auxinic hormones.

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