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Indoleamines and calcium channels influence morphogenesis in in vitro cultures of *Mimosa pudica* L.

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Keywords: melatonin, serotonin, calcium channels, *Mimosa pudica* L, shoot multiplication, root induction

Abbreviations: SER, serotonin; MEL, melatonin; p-CPA, p-chlorophenylalanine; EGTA, [ethylene glycol-bis (β-amino ethylether]-N, N, N', N'-tetra acetic acid)

The present article reports the interplay of indoleamine neurohormones viz. serotonin, melatonin and calcium channels on shoot organogenesis in *Mimosa pudica* L. In vitro grown nodal segments were cultured on MS medium with B5 vitamins containing Serotonin (SER) and Melatonin (MEL) at 100 μM and indoleamine inhibitors viz. serotonin to melatonin conversion inhibitor p-chlorophenylalanine (p-CPA) at 40 μM, serotonin reuptake inhibitor (Prozac) 20 μM. In another set of experiment, calcium at 5 mM, calcium ionophore (A23187) 100 μM, and calcium channel blocker varapamil hydrochloride (1 mM) a calcium chelator EGTA (100 μM) were administered to the culture medium. The percentage of shoot multiplication, endogenous MEL and SER were monitored during shoot organogenesis. At 100 μM SER and MEL treatment 60% and 70% explants responded for shoot multiplication respectively. Medium supplemented with either SER or MEL along with calcium (5 mM) 75%–80% explants responded for organogenesis. SER or MEL along with calcium ionophore (A23187) at 100 μM 70% explants responded for shoot multiplication. p-CPA, prozac, verapamil and EGTA, shoot multiplication was reduced and endogenous pools of SER, MEL decreased by 40–70%. The results clearly demonstrated that indoleamines and calcium channels positively influenced shoot organogenesis in *M. pudica* L.

Introduction

Mimosa pudica L. (Mimosaceae) is a herbaceous species native to Brazil and largely naturalized through out the world.[1] It is very popular ornamental due to its ability to exhibit tactile movements of leaflets. This plant is known to contain indoleamines and its alkaloid, mimosine, (3-hydroxy-4-pyridine-1-yl L-alanine) present in leaves and stem. Melatonin (N-acetyl-5-methoxy tryptamine; MEL) and Serotonin (5-hydroxy tryptamine; SER) are two important indoleamines, which participate in neuronal transmission in animals. Interestingly they are also found to occur in several organisms including, unicellular algae and also higher plants.[2] The precise function of SER/MEL in plant system is not clearly known. MEL has been found in many plants and in the different parts of plants[3-5] and seeds.[6] And also been reported in extremely high levels in several herbs including St. John's wort (*Hypericum perforatum*).[7] SER has been reported in different edible portions of plants such as banana, tomato and walnuts etc.,[8] and also in coffee wax.[9]

Tryptophan acts as a precursor for MEL and SER biosynthesis[10] (**Fig. 1**). As both the MEL and SER are structurally related to the plant hormone, Indole-3-acetic acid (IAA), their possible role in various developmental aspects of plant morphogenesis is worth investigating.[11] Interestingly, high concentrations of SER is found in organs of movements, the pulvini and tendrils of *Albizzia julibrissin*, *Pisum sativum*, *Mimosa pudica* and *Passiflora quadrangularis* compared with other vegetative parts.[12] SER is implicated in adaptation to environmental changes, flowering, morphogenesis and ion permeability.[13]

Calcium is crucial for plant growth and development as it exerts profound influence on various biological processes.[14] The Ca^{2+} permeable channels exist in a variety of cell types as demonstrated in suspension cultures of Carrot[15] and Capsicum[16] and such channels can be activated by calcium ionophore. Calcium channel inhibitors bind to plant membranes block Ca^{2+} entry into protoplast.[17] There is no direct link established so far between the calcium channels and SER/MEL mediated morphogenesis. Hence, it was of interest to understand the correlation of calcium and calcium channels on MEL and SER mediated morphogenetic response, which has not been reported earlier in any plant species.

In this communication we are reporting the influence of SER, MEL and the interplay of calcium channels on morphogenesis in in vitro cultures of *M. pudica* L.

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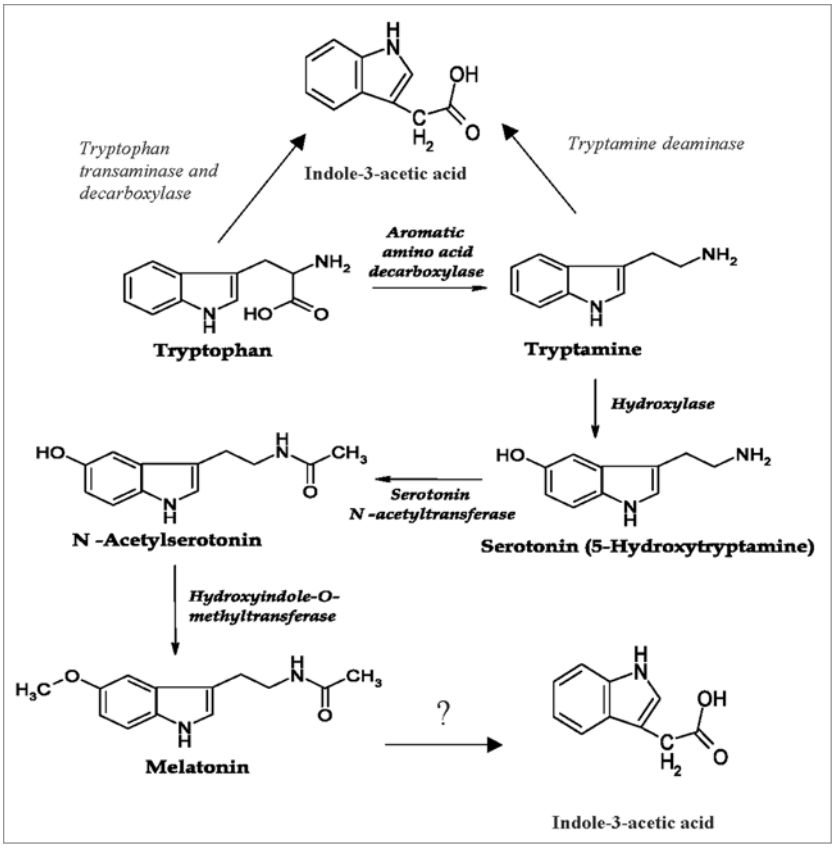

Figure 1. Biosynthetic pathway of serotonin and melatonin in plants. Serotonin, melatonin and IAA synthesized from Tryptophan. SER is synthesized from tryptophan by two enzymes, *tryptophan decarboxylase* (TDC) and *tryptamine 5-hydroxylase* (T5H).[20] Serotonin is converted in to melatonin by two enzymes, *serotonin N-acetyl transferase* and *Hydroxy-o-methyl transferase*.[10] Recent report suggests that exogenously applied melatonin raises Indole-3-acetic acid levels in roots of etiolated seedlings of *Brassica juncea*.[30]

Results and Discussion

Role of indoleamines on multiple shoot induction. Nodal segments cultured on MS medium supplemented SER or MEL at 100 µM induced multiple shoots in *M. pudica* (**Table 1 and Fig. 2**). Various concentrations (50, 100, 250 µM) of MEL or SER were used separately in preliminary experiments and 100 µM of MEL or SER were found to be optimum for induction of multiple shoots in *M. pudica.* MEL treatment induced 60% explants to respond for shoot multiplication, wherein formation of 15 ± 5.0 shoots from each nodal segment was noticed. Endogenous MEL content increased by 10-fold in MEL fed medium compared to control (**Table 1**). Similarly, SER incorporation into the medium induced 22 ± 2.0 shoots with increase in endogenous SER levels (**Table 2**). Whereas control cultures on MS basal medium failed to elicit morphogenesis. This study indicates that exogenous feeding of MEL or SER could influence organogenesis.

Influence of indole inhibitors on multiple shoot induction in *Mimosa pudica*. In order to investigate the influence of MEL on morphogenesis MS medium was supplemented with 100 µM MEL with p-CPA 40 µM (SER to MEL converting inhibitor) this resulted in reduction in response to multiple shoot formation only from 10% explants, in contrast to 60% response in MEL treatment alone (**Table 1**). Moreover, only 2–3 shoots were produced from nodal segment in presence of p-CPA 40 µM, showing that the SER to MEL conversion appear crucial for morphogenesis. Similarly in the medium supplemented with SER (100 µM) + p-CPA only 10% explants responded for multiple shoot induction and only 3–4 shoots were produced from each nodal segment (**Table 2**). Similar results were obtained in the medium containing SER (100 µM) + 20 µM Prozac (serotonin uptake inhibitor) with the production of 4 shoots per explant. These studies imply that the endogenous MEL, SER is crucial in influencing morphogenesis. In control, only 5% explants responded with only one shoot slowly proliferating on MS medium. Prozac treatment showed low endogenous levels of MEL and SER (**Tables 1 and 2**). These results imply that, the exogenously supplied SER and MEL influenced shoot organogenesis whereas the inhibitors of indoleamines suppressed the organogenesis response.

Influence of indoleamines and calcium channels on in vitro cultures of *M. pudica*. It was interesting to understand the interplay of calcium/calcium ionophore A23187 and the interrelationship with SER/MEL mediated morphogenesis. MS basal medium supplemented with MEL (100 µM) + 5 mM $CaCl_2$ induced 80% explants responding for shoot organogenesis wherein, 22 ± 1.44 shoots were produced from each nodal segment, whereas control without growth regulators and with normal levels of calcium (2.2 mM) as in MS medium resulted in only 5% of the cultures showing the response. The endogenous MEL concentration was high by 10–15-fold in the MEL (100 µM) alone and MEL (100 µM) + 5 mM $CaCl_2$ supplemented medium and in MEL and calcium ionophore containing media compared to control (MS medium) respectively. The combination of MEL (100 µM) + Calcium ionophore A23187 (100 µM)) significantly induced shoot organogenesis wherein, 70% explants produced multiple shoots (18 ± 1.05 shoots) from each nodal segment (**Table 1**).

In MS medium with 5 mM $CaCl_2$ + calcium channel blocker verapamil hydrochloride at 1 mM, only 5% explants responded for shoot multiplication. Endogenous levels of MEL was found to be low (4.60 µg/g FW). $CaCl_2$ (5 mM) + EGTA at 100 µM treatment resulted in stunted growth of shoots with low response to shoot induction (10%), there was reduction in MEL and SER levels also (**Tables 1 and 2**). In general the trend in morphogenetic response to SER and calcium treatment was similar to that noticed for MEL + calcium treatment (**Table 1**). Furthermore SER + calcium ionophore treatment enhanced shoot formation.

Effect of indoleamines, calcium and calcium ionophore A23187 on root induction in in vitro cultures of *M. pudica*.

Table 1. Effect of melatonin, calcium and calcium ionophore (A23187) on shoot multiplication in in vitro cultures of *M. pudica*

Treatment*	% Response	Total number of shoots	Shoot length cm	Melatonin (μg/g fresh weight)
MEL (100 μM)	60	15 ± 5.0[a]	08 ± 0.68[a]	21.28 ± 2.56[a]
MEL + p-CPA (40 μM)	10	2 ± 0.4[c]	02 ± 0.24[c]	4.72 ± 0.95[cd]
MEL + Prozac (20 μM)	05	1 ± 0.05[cd]	01 ± 0.45[cd]	2.04 ± 0.84[cd]
MEL + Calcium (5 mM)	80	22 ± 1.44[a]	06 ± 0.57[a]	34.44 ± 2.98[a]
MEL + Calcium ionophore (A23187) (100 μM)	70	18 ± 1.05[c]	05 ± 0.89[a]	9.64 ± 1.54[c]
Calcium ionophore + Verapamil hydrochloride (1 mM)	05	02 ± 0.2[c]	02 ± 0.05[c]	4.60 ± 0.08[c]
Calcium (5 mM) + EGTA (100 μM)	10	03 ± 0.8[c]	03 ± 0.08[c]	6.14 ± 0.54[c]
MS basal medium with 3% sucrose	5	01 ± 0.02[cd]	01 ± 0.04[cd]	2.97 ± 0.02[cd]

Data recorded during shoot multiplication stage after 2 months of culture. Values are mean of three determinants. *All media contained MS basal salts with 3% sucrose.

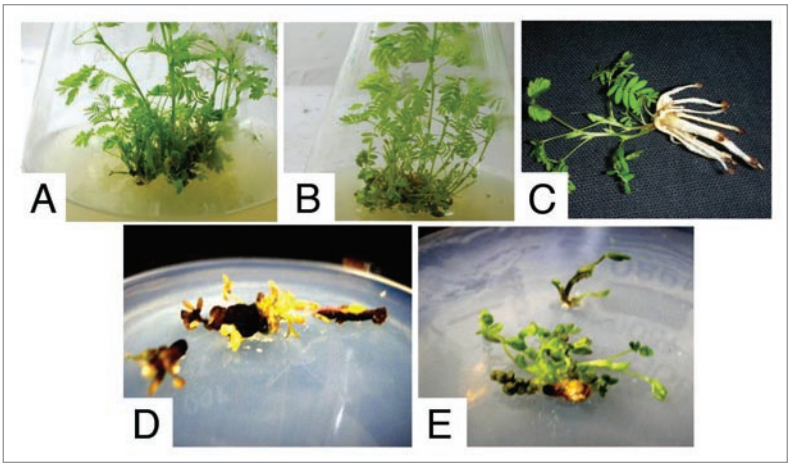

Figure 2. Effect of indoleamines and their inhibitors on shoot multiplication in in vitro cultures of *M. pudica*. (A) Elongation of shoots in *M. pudica* under the influence of Melatonin (100 μM). (B) Shoot induction by Serotonin (100 μM). (C) Root induction by Serotonin/Melatonin (100 μM). (D) Suppression of shoot elongation by p-CPA at 40 μM. (E) Suppression of shoot elongation by Prozac at 20 μM.

MS medium supplemented with MEL and SER produced 11 ± 0.5 and 8 ± 1.5 roots from each nodal segment respectively (**Table 3**). MS medium with calcium (5 mM) and calcium ionophore (A23187) produced 10 ± 2.7 and 13 ± 1.0 produced respectively. Indoleamine inhibitors and calcium channel inhibitors reduced root induction in *M. pudica* (**Table 3**). These results show similarity to shoot proliferation response to MEL & SER (**Table 2**). This rooting phenomenon was also influenced by the treatment of explants with these indoleamines (**Table 3**).

Endogenous melatonin and serotonin in different tissues of *M. pudica*. Studies on the endogenous levels in leaves and seeds of *M. pudica* revealed high quantity of SER/MEL in seeds compared to leaves. This may be of significance in seed germination and its viability (**Table 4**).

In the present study, role of indoleamines and calcium channels on shoot organogenesis in *M. pudica* was studied. Nodal segments or shoot tip explants cultured on MS medium supplemented with MEL or SER at 100 μM, after 2 months, enhanced shoot multiplication and root induction was noticed. MEL or SER incorporation into the medium resulted in the formation of 15 ± 5.0, 22 ± 2.0 shoots from each nodal segment respectively (**Tables 1 and 2**). MEL/SER fed cultures showed high endogenous indoleamines levels. This indicates that the changes in the endogenous levels of MEL and SER influence induction of shoot multiplication in *M. pudica*. Incorporation of either calcium or calcium ionophore (A23187) also showed elevation in endogenous pools of MEL, this increase was highly evident for calcium addition (**Table 1**). Similarly, the incorporation of calcium channel blockers alone into MS medium substantially reduced the endogenous pools of MEL. Even the addition of verapamil and EGTA with calcium drastically brought down the endogenous levels of

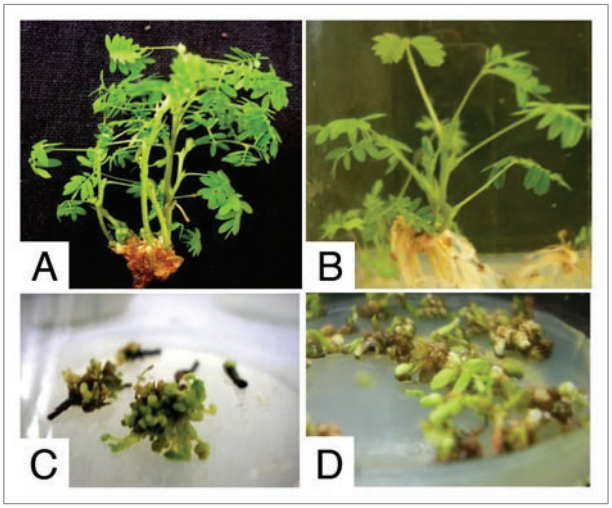

Figure 3. Effect of calcium and calcium channel inhibitors on shoot multiplication in in vitro cultures of *M. pudica*. (A) Multiple shoot induction in *M. pudica* under the influence of calcium (5 mM). (B) Root induction by calcium ionophore A23187 at 100 μM. (C) Suppression of shoot elongation by Verapamil hydrochloride at 1 mM. (D) Suppression of shoot elongation by EGTA at 100 μM.

Table 2. Effect of serotonin, calcium and calcium ionophore (A23187) on shoot multiplication in in vitro cultures of *M. pudica*

Treatment*	% Response	Total number of shoots	Shoot length (cm)	Serotonin (μg/g fresh weight)
SER (100 μM)	70	22 ± 2.0[a]	08 ± 0.84[a]	14.96 ± 1.25[a]
SER + p-CPA (40 μM)	10	4 ± 0.5[c]	04 ± 0.64[cd]	1.60 ± 0.05[cd]
SER + Prozac (20 μM)	10	4 ± 0.4[c]	06 ± 0.48[c]	2.45 ± 0.45[cd]
SER + Calcium (5 mM)	75	29 ± 2.3[a]	4 ± 0.25[cd]	8.54 ± 0.90[c]
SER + Calcium ionophore (100 μM)	70	28 ± 1.6[a]	4 ± 0.56[cd]	8.00 ± 0.85[c]
Calcium ionophore (100 μM) + Verapamil hydrochloride (1 mM)	05	02 ± 0.2[cd]	02 ± 0.05[cd]	2.94 ± 0.04[cd]
Calcium (5 mM) + EGTA (100 μM)	10	03 ± 0.8[cd]	03 ± 0.08[cd]	1.33 ± 0.01[cd]
MS medium	5	01 ± 0.02[cd]	01 ± 0.04[cd]	8.36 ± 0.85[c]

Data recorded during shoot multiplication stage after 2 months of culture. Results are mean of 3 replicates. *All media contained MS basal salts with 3% sucrose.

Table 3. Effect of indoleamines, calcium and calcium ionophore A23187 on root induction in in vitro cultures of *M. pudica*

Treatment*	% Response	No. of roots
MS	0	NIL
MS + MEL (100 μM)	70	11 ± 0.5[c]
MS + SER (100 μM)	60	8 ± 1.5[c]
MS + MEL + p-CPA (40 μM)	15	4 ± 1.09[cd]
MS + SER + p-CPA (40 μM)	15	3 ± 0.5[cd]
MS + p-CPA (40 μM)	0	Nil
MS + Calcium (5 mM)	75	10.0 ± 2.7[c]
MS + Calcium ionophore (100 μM)	80	13 ± 1.0[a]
MS + Calcium ionophore (100 μM) + Verapamil hydrochloride (100 μM)	20	4 ± 0.8[cd]
MS + Calcium (5 mM) + EGTA (100 μM)	10	2 ± 0.9[cd]
MS + Verapami hydrochloride (1 mM)	0	Nil
MS + EGTA (100 μM)	0	Nil

Data recorded during shoot multiplication stage after 2 months of culture. Results are mean of 3 replicates. *All media contained MS basal salts with 3% sucrose.

Table 4. Endogenous profiles of serotonin and melatonin in different tissues of *M. pudica*

Tissue	Age of the tissue	Melatonin (μg/g fresh weight)	Serotonin (μg/g fresh weight)
In vitro leaves	4 weeks	2.7 ± 0.6[cd]	8.3 ± 1.4[cd]
Ex vitro leaves	4 weeks	3.7 ± 0.5[cd]	17.3 ± 2.0[c]
Seeds	3 months	68.3 ± 3.5[a]	80.4 ± 4.7[a]

Results are mean ± SD of 3 replicates.

MEL to 4.60 μg/g FW and 6.12 μg/g FW respectively compare to that of MEL or calcium alone fed medium (**Table 1**). A similar trend was noticed with that of SER (**Table 2**). This indicates that both the indoleamines SER/MEL exhibited a similar influence on plant morphogenesis in *M. pudica* shoot multiplication.

SER is reported to exhibit several physiological roles in plants such as flowering, morphogenesis and adaptation to environmental changes.[20] High concentrations of SER was found in organs of movements, the pulvini and tendrils of *Albizzia julibrissin*, *P. sativum*, *Mimosa pudica* and *Passiflora quadrangularis* compared with other vegetative parts. The growth promotive effect of exogenous MEL and SER on shoot morphogenesis in in vitro cultures of *M. pudica* was evident in our study. Variation in the accumulation of tryptophan, *N, N-Dimethyltryptamine* (DMT), SER in micropropagated trees and in in vitro cultures of *Mimosa tenuiflora* was reported.[21]

Our results are in accordance with other reports[22] on the potential role of MEL as a plant growth regulator. It is further reported that, auxin-induced root and cytokinin-induced shoot organogenesis were altered by the exogenous supply of MEL, and inhibitors of the transport of SER and MEL.[22] However, in our study, indole inhibitors viz. p-CPA and prozac significantly reduced shoot organogenesis (**Tables 1 and 2**) and root induction in *Mimosa pudica* (**Table 3**). This may be due to p-CPA inhibiting the conversion of SER to MEL and altered balance of these indoleamines resulted in eliciting morphogenesis response.

Recent report suggests that MEL stimulates the expansion of etiolated lupin cotyledons.[23] On the other hand the MEL promotes vegetative growth in etiolated lupin (*Lupinus albus* L.) hypocotyls in a similar manner to Indole-3-acetic acid (IAA). The significance of these indoleamines in plant physiology may be related to auxin metabolism.[11] Similarly, SER is reported stimulates the germination of radish seeds,[24] and the pollen of *Hippeastrum hybridum*.[25] Therefore our finding of high levels of SER/MEL in seeds (**Table 4**) may be of significance in this context. A substantial stimulation of growth in cultures of tobacco (*Nicotiana tabacum*) thin cell layers and *Acmella oppositifolia* hairy root was achieved by micromolar concentrations of biogenic amines.[26]

Synergistic effect of calcium/calcium ionophore on morphogenetic response in *M. pudica* was observed in *M. pudica*. Calcium is crucial for plant growth and development as it exerts profound influence on various biological processes.[14] The Ca^{2+} permeable channels exist in a variety of cell types as demonstrated in Carrot[15] and Capsicum[16] suspension cultures

and such channels can be activated by calcium ionophore (A23187). Which leads to somatic embryogenesis in carrot cells.[27] Nevertheless, there is no direct link established so far between the calcium channels and SER and MEL mediated organogenesis in plant systems. Calcium ionophore A23187 stimulates mitosis leading to bud formation in *Funaria hygrometrica* by increasing the intracellular calcium levels.[28] Increased intra-cellular calcium levels caused by the divalent ionophore A23187.[29]

Thus we have been able to show that exogenous supply of SER and MEL to *Mimosa pudica* nodal explants exhibited shoot multiplication and proliferation and it was further enhanced by calcium and calcium ionophore treatments there by demonstrating the synergistic influence of SER/MEL and calcium treatment eliciting morphogenesis. This was substantiated by calcium channel inhibitor application also.

Materials and Methods

Chemicals. The indole compounds MEL and SER, IAA and p-CPA, Calcium ionophore A23187, and EGTA (ethylene glycol-bis (β-amino ethylether)-N, N, N', N'-tetra acetic acid) were purchased from Sigma-Aldrich, India. Prozac (Fluoxitine hydrochloride), Verapamil hydrochloride purchased from local drug stores. All these were filter-sterilized using 0.22 μ filter (Sartorius) before addition to the autoclaved medium. All the growth regulators were obtained from Sigma-Aldrich. $CaCl_2 \cdot 2H_2O$ obtained from Himedia Mumbai, India. The solvents methanol of HPLC grade obtained from Merck, India. Di methyl sulphoxide (DMSO) procured from SRL, India.

Plant material. The fresh pods of the plant *Mimosa pudica* L., were collected from the surroundings of Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore. Pods were surface sterilized with 1% (w/v) sodium hypochlorite (Qualigens, Mumbai) for 10 min., followed by rinses with sterile distilled water thrice. Later treated with 0.2% (w/v) mercuric chloride followed by rinses with sterile distilled water. Resultant aseptic seedlings were used for further experiments. The surface sterilized seeds were inoculated in MS basal medium[18] for 4 weeks for germination. One cm long nodal explants were excised from the seedlings and so also the shoot tip explants.

Multiple shoot initiation. For initiation of multiple shoots, the nodal segments were cultured on MS basal medium.[18] Mimosa nodal segments were used as inoculum to study influence of indoleamines MEL, SER. MS salts supplemented with B5 vitamins, and 3% sucrose (w/v), indoleamines MEL and SER and indolamine inhibitors p-CPA (40 μM), Prozac (20 μM) depending on the experiment. Controls received MS basal medium with 2.2 mM calcium.

In another set of experiment calcium chloride at 5 mM, calcium ionophore (A23187) at 100 μM, calcium channel blocker, verapamil hydrochloride at 1 mM, EGTA at 100 μM were added in separate treatments into MS medium. Control cultures received all MS salts containing 2.2 mM calcium.

Media and culture conditions. The pH of the medium was adjusted to 5.6 prior to autoclaving at 121°C, 1.2 kg cm^2 pressure for 20 min. The media were gelled with 0.8% (w/v) tissue culture grade agar in 200 ml glass jars, containing 40 ml of the medium. The jars were closed with polypropylene caps. The cultures were incubated at 25 ± 2°C under light (16:8 hours photoperiod). Periodic observations were made and the percentage of shoot induction, root induction was recorded.

Quantification of endogenous levels of melatonin and serotonin. *Extraction of sample.* Extraction of MEL and SER was carried out in various tissues as reported earlier.[10] One gram fresh samples were ground in 1 ml of Tris buffer (1 M Tris-HCl, pH 8.4) and homogenized in 500 μl of extraction buffer (0.4 M perchloric acid, 0.05% sodium meta bisulphate, 0.1% EDTA). Particulate matter was removed by centrifugation at 12,000 rpm for 10 min. The resulting supernatant was dried with nitrogen gas and resuspended in 500 μL methanol for further analyses by HPLC.

HPLC conditions. MEL and SER were separated using C-18 Column (3.9 x 150 mm, particle size 3 μm) (Ultralab products, Bangalore, India). Isocratic buffer consisted of 0.1 M sodium acetate, 0.1 M citric acid, 0.5 mM sodium octane sulfonate, 0.15 mM EDTA, adjusted to pH 3.7 and 5% methanol was used as a mobile phase for the separation of indolamines. Samples were injected (20 μl) for HPLC analysis and an isocratic condition was maintained at a flow rate of 1 ml/min. Indolamines were monitered using fluorescence detector (FD) at, λ Em 348/Ex280, (Shimadzu, Japan). The peak identities of these compounds were confirmed by their retention times and characteristic spectra of standard chromatograms, recorded with a Shimadzu model LC-10Avp series equipped with SPD-10AVP detector. MEL and SER were quantified from their peak areas in relation to the respective reference standards. Spiking was followed to authentically confirm the separation under the HPLC conditions.

All the experiments were repeated thrice. Ten replicates were maintained in each experiment. The data were analyzed by one way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corporation, Washington), and mean separations were performed by Duncan's Multiple Range Test at $p \leq 0.05$ by using the statistical program SPASS7.0.[19]

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