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SHORT COMMUNICATION

Endogenous profiles of indoleamines: serotonin and melatonin in different tissues of *Coffea canephora* P ex Fr. as analyzed by HPLC and LC-MS-ESI

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Abstract Endogenous indoleamine profiles in various ex vitro and in vitro tissues of commercially important *Coffea canephora* were analyzed by using a high performance liquid chromatography and further confirmed with electrospray ionization mass spectrometry. High content of serotonin (SER) (98.54 ± 5 μg/g) and melatonin (MEL) (115.25 ± 6 μg/g) were found in freshly harvested seeds of *C. canephora* followed by zygotic embryo (65.25 ± 4 and 96.54 ± 5 μg/g fresh weight) and endosperm (34.08 ± 2 and 51.08 ± 4 μg/g fresh weight) of ripened fruits. Similarly endogenous pools of SER and MEL were moderate in in vitro tissues of *C. canephora*, i.e. callus (25.85 ± 2 and 75.74 ± 4), somatic embryos (31.88 ± 2 and 19.30 ± 2 μg/g fresh weight) and in vitro regenerated plant stalk (15.78 ± 1 and 38.25 ± 3 μg/g fresh weight), respectively. In view of significant levels of both SER and MEL in various tissues and beans of *Coffea*, further investigations on their physiological role needs to be investigated.

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Abbreviations

LC-MS-ESI	Liquid chromatography mass spectrometry electrospray ionization
MS	Murashige and Skoog
MEL	Melatonin
SER	Serotonin

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine; MEL) and serotonin (5-hydroxytryptamine; SER) are indole group containing organic compounds found in animals (Reiter 1991; Veenstra-VanderWeele et al. 2000) fungi (Hardeland and Fuhrberg 1996) and plants (Bardia 2002). SER has been reported in more than 42 plant species (Roshchina 2001). Fruits and seeds are the major tissues in which SER occurs abundantly. In plants, SER was implicated in several physiological roles such as flowering, morphogenesis and adaptation to environmental changes [Kang et al. 2007; reviewed by Ramakrishna et al. (2011a)]. MEL was first identified in edible plants by Hattori et al. (1995) then reported in different parts, viz. roots, stem, leaves, fruits and seeds of many plants (Murch et al. 2000; Manchester et al. 2000; Ramakrishna et al. 2009; Posmyk and Janas 2009). As both the MEL and SER are structurally related to the plant hormone, indole-3-acetic acid (IAA), possibly they influence physiology and development in plants (Hernandez-Ruiz et al. 2005). Consumption of edible plant tissues that contain the SER and MEL would be useful as their antioxidant activity is linked to their medicinal value for treatment of human diseases (Reiter et al. 2005). Its

antioxidant activity protects different plant tissues particularly reproductive tissues, fruits and germ tissues of the seed, from oxidative stress due to abiotic and environmental stresses (Van Tassel and O'Neill 2001). In view of this, now there is a growing interest to screen various plant species for the presence of SER and MEL. Free biogenic amine levels in some arabica and robusta coffee samples were used as chemical markers for the two coffee species (Sridevi et al. 2009). The presence of SER in coffee beans is interesting since it has physiological action in humans as a neurological mediator (Casal et al. 2002). To our knowledge, there are no reports on the profiles of MEL in *Coffea canephora*, but SER was detected in green and roasted beans (Casal et al. 2002) and coffee wax (Kele and Ohmacht 1996). In view of various health benefits attributed to coffee, investigations on endogenous pools of indoleamines in *Coffea*, and their physiological role is warranted. The objective of the study was to analyse the endogenous profiles of SER and MEL in both ex vitro and in vitro tissues of *C. canephora* which would be of physiological relevance to plant.

Materials and methods

Plant source and establishment of in vitro cultures

The zygotic embryos and endosperm of 7 months old green immature fruits and 9 months old ripened fruits along with freshly harvested green beans of *C. canephora* P. ex. Fr., CxR variety plants grown at Central Coffee Research Institute, Balehonnur, Karnataka, India were used for endogenous profiling of SER and MEL and also for establishment of in vitro cultures. Freshly harvested seeds were used for raising in vitro seedlings, and then for establishment of callus, somatic embryos and in vitro plantlets as described earlier (Giridhar et al. 2004).

Analysis of melatonin and serotonin by LC-MS-ESI

The HPLC fractions (20 µL) are directly infused on mass spectrometer for mass ion analysis, the ESI-MS of the compounds were obtained with an alliance waters 2695 mass spectrometer (QTOF Ultima Waters Corporation, Micromass Ltd, UK, Manchester) operating at ESI positive mode. The mass spectral data was analysed by a Mass Lynux 4.0 SP4 data acquisition system. The following ion optics was used. capillary voltage: 3.5 kV; cone temperature: 100°C, source temperature: 120°C, desolvation temperature: 150°C, cone gas flow 50 L/h, desolvation gas: 500 L/h, collision energy 10, TOF (kV): 9.10. The scan range *m/z* 50–1,000, scan speed 1,000 amu/s. The occurrence of the compounds using the molecular ions in the spectra are identified based on molecular mass of the compounds in the mass spectra obtained.

Estimation of MEL and SER by HPLC with fluorimetric detection

Extraction of MEL and SER was done according to Murch et al. (2000). One gram fresh weight tissue samples were ground in 1 mL of Tris buffer (1 M Tris–HCl, pH 8.4) and homogenized in 500 µL of buffer [0.4 M perchloric acid, 0.05% sodium meta bisulfate, 0.1% ethylenediaminetetraacetic acid (EDTA)]. Particulate matter was removed by centrifugation at 12,000 rpm for 15 min. The resulting supernatant was dried with nitrogen gas, in total darkness, and resuspended in 500 µL methanol for injection. Known quantity of authentic samples of MEL and SER (Sigma-Aldrich, St. Louis, MO, USA) were prepared by dissolving in pure water (milli-Q quality). For quantification, 20 µL of sample was injected for HPLC analyses. MEL and SER were separated using C-18 Column (Waters, Atlantis dC18, 3.9 × 150 mm, particle size 3 µm, Ultra Lab products, Bangalore, India). Isocratic buffer consist of 0.1 M sodium acetate, 0.1 M citric acid, 0.5 mM sodium octanylsulfonate, 0.15 M EDTA, adjusted to pH 3.7 and 5% methanol was used as a mobile phase at a flow rate of 1 mL/min. The peak identities and λ_{max} values of these compounds (MEL 255, SER 260) 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.

Statistical analysis

Analyses of three different samples and their average values (mean ± SD) were depicted in Table 2.

Results and discussion

Molecular mass of each peak in LC-MS-ESI was observed by monitoring the parent molecular ion 233 and 176 peak of MEL and SER, respectively coupled with H^+, $2H^+$. MEL and SER were detected and mass fragments occur as molecular ions (*m/z*) are either $(M + H)^+$, $(M + 2H)^+$. The molecular ion (*m/z*) 234 $(M + 2H)^+$ in in vitro leaves, (*m/z*) 233 $(M + H)^+$ in callus confirm the occurrence of MEL (Table 1). Similarly, the molecular ion (*m/z*) 177 $(M + H)^+$ in in vitro leaves, and (*m/z*) 177 $(M + H)^+$ in callus cultures of *C. canephora* confirm the occurrence of SER (Table 1).

Analysis of indoleamine titers through HPLC in *C. canephora* fruit tissues, i.e. zygotic embryos and endosperm tissues, seedling parts and in vitro established

Table 1 Identification of melatonin and serotonin in *Coffea canephora* by LC-MS-ESI

Sample	MS (*m/z*)	Identification
Melatonin standard	233 $(M + H)^+$	Melatonin
Serotonin standard	177 $(M + H)^+$	Serotonin
In vitro leaves	234 $(M + 2H)^+$	Melatonin
	177 $(M + H)^+$	Serotonin
Callus cultures	233 $(M + H)^+$	Melatonin
	177 $(M + H)^+$	Serotonin

cultures showed significant differences in their levels (Table 2). In early stages of fruit development (green immature fruit) the indoleamines level in seeds was low and subsequently elevated with the fruit development (ripened fruit). The levels of two major indoleamines SER and MEL were abundant in ex vitro samples than in vitro tissues, which was evident with zygotic embryos and somatic embryos. Considerable levels of SER and MEL were found in both zygotic embryos (65.25 ± 4 and 96.54 ± 5 μg/g fresh weight) and endosperm (34.08 ± 2 and 51.08 ± 4 μg/g fresh weight) of ripened fruits compared to immature green fruits. The difference in SER and MEL profiles in 2 weeks old ex vitro (8.25 ± 1 and 6.04 ± 1) and in vitro (9.10 ± 1 and 4.07 ± 1 μg/g fresh weight) regenerated plant leaves was meager. Among the in vitro cultures MEL content of 4 weeks old callus (75.74 ± 4 μg/g fresh weight) and 6 weeks old regenerated plant stalk (38.25 ± 3) was high followed by in vitro roots (16.08 ± 2 μg/g fresh weight).

It is interesting to find a clear demarcation in both SER and MEL content among ex vitro grown seedling parts, wherein, high levels of MEL content was found in cotyledons (46.23 ± 4) which was slightly more than that of hypocotyls (44.25 ± 4 μg/g fresh weight). In roots of seedlings, MEL content was more (15.04 ± 1 μg/g fresh weight) than that of shoot tip (6.25 ± 2 μg/g fresh weight) and first leaves (4.00 ± 1). It may be noted that there was a drastic fall in MEL content in first leaves compared that of cotyledonary leaves (46.23 ± 4 μg/g fresh weight). However, in case of SER the levels were more or less same in both cotyledonary and first leaves in seedlings. The levels of SER are four folds more than that of MEL in these first leaves of seedlings. High content of MEL (115.25 ± 6 μg/g dry weight) and SER (98.5 μg/g dry weight) were found in freshly harvested seeds of *C. canephora*.

In the present study, we have initially relied on HPLC with fluorescence detector, which provided sensitive methodology to identify MEL and SER in different tissues of *Coffea*. Moreover, in *Chenopodium rubrum* (Kolar et al. 1997), grape (Iriti et al. 2006) and *Dunaliella bardawil* (Ramakrishna et al. 2011b). Their detection was done by HPLC with further confirmation by LC-MS-ESI. The presence of SER during the differentiation stage of embryos in *Coffea* was further supported by its existence in other tissues, i.e. seeds and leaf wax (Kele and Ohmacht 1996) and also in polished coffee (Strance 1997) called

Table 2 Endogenous levels of melatonin and serotonin in different tissues of *Coffea canephora* determined by HPLC

Plant tissue	Age of tissue	Melatonin (μg/g fresh weight)	Serotonin (μg/g fresh weight)
In vitro cultures			
Callus	4 weeks	75.74 ± 4	25.85 ± 2
Somatic embryos	6 weeks	19.30 ± 2	31.88 ± 2
Regenerated plant stalk	6 weeks	38.25 ± 3	15.78 ± 1
In vitro leaves	2 weeks	04.07 ± 1	09.10 ± 1
Roots	3 weeks	16.08 ± 2	12.06 ± 1
Plant tissues (ex vitro)			
Zygotic embryos (green fruits)	12 weeks	28.45 ± 2	45.68 ± 3
Zygotic embryos (ripened fruits)	16 weeks	96.54 ± 5	65.25 ± 4
Endosperm (green fruits)	12 weeks	35.50 ± 3	31.06 ± 3
Endosperm (ripened fruits)	16 weeks	51.08 ± 4	34.08 ± 2
Ex vitro leaves	2 weeks	06.04 ± 1	08.25 ± 1
Seedling			
Cotyledons	2 months	46.23 ± 4	18.20 ± 2
Hypocotyls		44.25 ± 4	15.25 ± 1
Leaves		4.00 ± 1	19.40 ± 1
Shoot		6.25 ± 2	10.35 ± 1
Roots		15.04 ± 1	05.08 ± 1
Harvested beans[a]	9 months	115.25 ± 6[a]	98.54 ± 5[a]

Values are mean of three determinants

[a] Values are on the dry weight basis

Astra, which contained 20–40 mg SER/100 g. Its quantities in plants vary greatly among species and tissues, ranging from 0.007 μg/g fresh leaves to 2,000 μg/g in seeds of *Griffonia simplicifolia* (Fellows and Bell 1970). Calcium is reported to significantly influence somatic embryogenesis in *C. canephora* (Ramakrishna et al. 2011c). Similarly; SER/MEL endogenous pools in somatic embryos might influence their subsequent development in to plantlets. Recent studies have shown that sweet cherries, which contain substantial amounts of MEL and SER, may have a several health benefits and would be of relevance in a healthy diet (Gonzalez-Gomez et al. 2009). High MEL content in seeds, compared to other tissues, could be a necessity for the plant, to protect the delicate lipid rich tissues of the embryo from oxidative stress (Manchester et al. 2000). Presence of MEL in plant products and its consumption is reported to elevate MEL profiles of blood leading to improved antioxidant activity (Hattori et al. 1995) and amphipathic potential (Borah and Mohanakumar 2009).

Indoleamines levels varied in different parts of ex vitro seedling and also in in vitro cultures of *C. canephora.* High content of SER and MEL in callus and somatic embryos of *C. canephora* was evident in our study, but in subsequent stages of growth their levels declined. As the content of SER and MEL in seeds is high compared to all other parts of coffee plant, investigations on in planta physiological role is required. Further, studies on consumption of SER and MEL through coffee beverage on human physiology would be interesting to investigate.

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