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Production of *Curcuminoids* in Different *in vitro* Organs of *Curcuma longa*

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Curcuma longa L. (turmeric) is one of the most important spice and safe food additives. Its main constituents, curcuminoids, showed anti-inflammatory, antitumor and antioxidant effects. In the present work, an *in vitro* propagation method was developed to achieve selected plant organs with quantified curcuminoid content. I*n vitro* plants were obtained from sprouting buds as primary explants. The major curcuminoid constituents, such as curcumin (CUR), demethoxycurcumin (DEM), and *bis*-demethoxycurcumin (bis-DEM) were examined in different organs by LC-DAD-ESI-MS. A significant production of curcumin (more than 260 µg g^{-1} fresh weight) was obtained from *in vitro* microrhizomes, especially grown in a Murashige and Skoog medium (MS) supplemented with kinetin (0.1 mg L^{-1}), α-naphthaleneacetic acid (NAA, 1 mg L^{-1}), sucrose (6%), agar (5%) and activated charcoal (0.1%). The analyzed microrhizomes showed reduced amounts of DEM and bis-DEM in comparison with CUR levels. In addition a shoot culture line was suitable to biosynthesize curcuminoids, in a ratio very similar to that identified in the fresh rhizomes of parent plants. This study represents the first direct quantification of curcuminoids in turmeric *in vitro* shoots and microrhizomes to be used in dietary supplements.

Keywords: Curcumin, Demethoxycurcumin, *bis*-Demethoxycurcumin, Turmeric*, In vitro* shoots, Microrhizomes, LC-DAD-ESI-MS.

Turmeric (*Curcuma longa* L., Zingiberaceae) is an important spice crop widely used also by pharmaceutical and cosmetic industries [1, 2]. The drug, a rhizome, contains volatile and non-volatile components [3]. The medicinal use of turmeric rhizomes is well documented in Indian and Chinese health systems [4-5]. The biological effects have been attributed especially to curcumin that has been widely studied for its anti-inflammatory, antioxidant, and anti-cancer effects [6-10]. In fact, a large number of clinical studies on curcumin have been completed in the last years suggesting that the consumption of turmeric may reduce the risk of different diseases [11-13].

Moreover, turmeric uses have been justified as a food additive (e.g. dye agent), but also for its antimicrobial activity in the preservation of certain foods [14]. *C. longa* rhizomes are the spice-yielding part of the plant, and contain about 1% of an aromatic volatile oil, called turmeric oil. High levels of curcuminoids (>3% dry weight), which represent the non-volatile portion, responsible for the yellow color, are specifically accumulated in the cortex and central cylinder of the rhizome [15]. Curcuminoids are characterized by a diarylheptanoic nucleus with varying degrees of oxidation and unsaturation (Figure 1), of which curcumin (CUR), demethoxycurcumin (DEM) and *bis*-demethoxycurcumin (bis-DEM) represent the main constituents of turmeric rhizomes [16].

Turmeric is a monocotyledonous species, rarely flowering, classified as a sterile triploid plant (2n = 3x = 63) [17], generally reproduced by vegetative propagation of the rhizomes. During the growing season (8-10 months) each rhizome can produce 10-25 lateral buds, but only 4-6 of them actively develop plantlets in the wild or under field conditions. Special care should be taken to avoid damage of the rhizomes during harvest management because these are subjected to accumulation and transmittance of pathogens [15,17]. Curcuminoids are slowly accumulated in rhizomes throughout the growing season [18]. Variability among genotypes, and field and processing conditions trigger differences in the chemical composition of commercial turmeric rhizomes. Improvement of this crop through conventional breeding is, therefore, difficult.

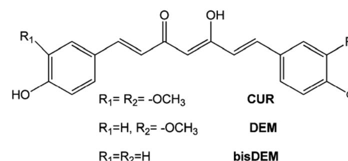

Figure 1: chemical structure of curcuminoids based on the oxidation and unsaturation of the common diarylheptanoic nucleus: curcumin (CUR), demethoxycurcumin (DEM) and *bis*-demethoxycurcumin (bis-DEM).

In vitro propagation has been generally regarded as a method to escape all these problems. The *in vitro* cultures of *C. longa* are known to give different responses depending on the selected organs and the media employed [19-21]. These studies generally described procedures to establish *in vitro* cultures, but in most of them the curcuminoid content was not investigated; in some cases the presence of the acid yellow pigment curcumin was only checked by its property to turn to magenta at alkaline pH [22]. Another phytochemical investigation was carried out in regenerated plantlets from other *Curcuma* species (*C. zedoaria)* [23], but these authors defined only the essential oil composition. Recently, CUR and DEM contents were evaluated in turmeric during a study to establish the biosynthetic pathway of curcuminoids [24]. The determination was performed by feeding ^{13}C-labeled precursors of curcuminoids to very young plantlets from an *in vitro* culture system. The

accumulation of the labeled curcuminoids was correlated with the *in vitro* rhizome growth. Furthermore, the authors suggested that the formation of the curcuminoid skeleton took place before the hydroxylation and *O*-methylation of the final compounds [24], giving more insights about their synthesis, previously correlated with the phenylpropanoid pathway [25].

This research is a part of an EU-funded project called "NUTRA-SNACKS", with the aim to select *in vitro* plant cultures as potential source of natural antioxidants in the development of nutraceutical products.

In the present study, different tissues of *in vitro C. longa* were produced with the aim of obtaining healthy *in vitro* cultures for a further massive production of plant material useful for the accumulation of curcuminoids. Three major curcuminoids of *C. longa*, CUR, DEM and bis-DEM, were monitored in the different established *in vitro* organs (shoots, roots and rhizomes) by LC-DAD-ESI-MS.

Young vegetative buds from sprouting rhizomes were used as explants for *in vitro* culture initiation. Special sterilization care was necessary to eliminate the endogenous contamination of fungi and bacteria, usually high in this species. After the sterilization procedure, 30% of healthy explants were successfully obtained (data not shown) and the sterilized buds, each originated from different rhizomes, were then proliferated in different media.

Table 1: Effect of different culture media on shoot proliferation in *C. longa*. Data recorded after 4 weeks of culture. Mean values were obtained from three independent replicates ± SD. Numbers followed by different letters in the same column are statistically different at $p \leq 0.05$. (MS= Murashige and Skoog medium; phytoregulator concentration is given in mg L^{-1}).

Medium	Proliferation rate (n°shoot/explant)	Shoot height (cm)	Root formation
A = MS + BAP 1	1.50 ± 0.50 a	5.45 ± 0.83 b	abundant
B = MS + BAP 2	1.33 ± 0.57 a	5.60 ± 0.72 b	abundant
C = MS + BAP 3	3.00 ± 1.00 ab	4.25 ± 0.35 a	scarce
D = MS + BAP 2,5 + NAA 0.5	4.67 ± 2.08 bc	3.50 ± 0.50 a	scarce
E = MS + TDZ 0.1 + BAP 0.5 + AA 100	6.19 ± 2.06 c	3.85 ± 1.39 a	scarce

The data regarding the effect of different culture media on shoot height and shoot proliferation are summarized in Table 1. Media A and B, containing different amounts of benzylaminopurine (BAP) alone, induced low proliferation of shoots (1.5 or 1.33 shoots per explant, respectively). The axillary shoots were well developed in length (5.45 cm and 5.60 cm, respectively) and abundant root systems were present (Table 1, Figure 3A). Medium C, containing the highest concentration of BAP (3 mg L^{-1}), induced a sufficient multiplication rate (3 shoots/explant), although the obtained shoots were short and with a poor root system. The strong action of BAP is well documented on shoot proliferation of ginger [26,27], turmeric and *C. zedoaria* [20,28].

It is well known that the addition of very high amount of cytokinins (1–30 mg L^{-1}) is effective for shoot proliferation in several *Curcuma* species [23, 29]. In this work high amounts of BAP were avoided since it was known that high amount of cytokinins showed a potential inhibitory effect on further microrhizome production [22]. For this purpose medium E was tested, which included a low amount of thidiazuron (N-phenyl-N'-(1,2,3-thidiazol-yl)urea, TDZ), BAP and ascorbic acid (AA). This medium produced the highest multiplication rate (6.19 shoot/explants) with short shoots and a scarce root formation. (Table 1, Figure 2b). It is well known that TDZ is a compound with a strong cytokinin-like activity especially

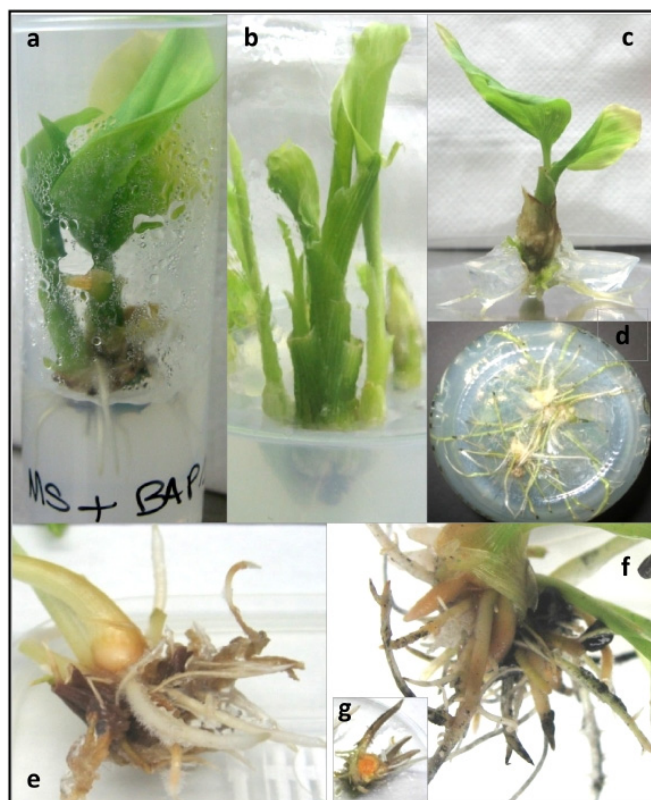

Figure 2: Micropropagation phases of turmeric plants. Phase I: Multiple shoot formation: shoots grown on A medium (a), on E medium (b) and on D medium (c). Phase II root formation: roots produced after 4 weeks in root F medium (d). Phase III: rhizome formation: *in vitro* 12-week turmeric rhizomes produced on H medium (e) and produced on I medium (f, addition of charcoal). g: rhizomes produced in I medium.

for woody plants [30]. It has been shown to stimulate turmeric shoot proliferation, when used for a short period in liquid media at high concentration [31].

However high shoot multiplication rates could be obtained in *Curcuma* species with a combination of cytokinins and auxins [32]. Therefore, medium D was prepared with the addition of a mixture of different phytoregulators, such as BAP (2.5 mg L^{-1}) and α-naphthaleneacetic acid (NAA) (0.5 mg L^{-1}). The concentration of these phytoregulators did not stimulate callus formation; on the other hand, this medium resulted in successful proliferation since multiple shoot formation was obtained, although the roots were scarcely developed (Table 1, Figure 2c).

This work confirmed that the addition of TDZ improved the multiplication efficiency [15]; therefore, medium E was chosen for the further scale up of *in vitro* turmeric cultures.

The micropropagation of *Zingiberaceae*, and in particular of turmeric, was usually carried out to induce simultaneous shoot multiplication and rooting, in order to obtain plantlets ready to be transplanted to the soil. In this study we preferred to induce the rooting phase subsequently to the shoot proliferation phase, in order to enhance the secondary metabolite yield produced by hypogeal organs. The shoots were transferred to the rooting medium F, containing low amounts of Kinetin (Kin) (0.1 mg L^{-1}) and NAA (1 mg L^{-1}). In 4 weeks complete plants were produced. The newly formed roots appeared thin and white (Figure 2d), and subsequent subcultures in the same medium did not promote the formation of microrhizomes (data not shown).

Table 2: The extract yields and curcuminoid contents obtained from the commercial rhizome (adult plant) and selected *in vitro* organs of *Curcuma longa*.

sample	Plant organ	medium	extract yield (%w/w)	bisDEM	curcuminoid content DEM	CUR
	in vivo adult plant				(mg g^{-1} FW)	
A	Commercial rhizomes		5.02	2.6± 0.16	2.8± 0.09	4.0± 0.61
	in vitro biomass *				(μg g^{-1} FW)	
1-TUR	C1 shoots	Medium E	3.51	26.1 ± 2.8	29.9 ± 2.2	33.7± 4.1
2-TUR	C2 shoots	Medium E	3.19	n.d.	n.d	59.8± 3.5
3-TUR	C1, C2 roots	Medium F	3.17	n.d.	n.d.	n.d.
4-TUR	C1 rhizomes	Medium H	3.96	n.d.	n.d.	n.d.
5-TUR	C1 rhizomes	Medium I	4.11	20.6 ± 0.5	147.2 ±11.8	266.5±20.5
6-TUR	C2 rhizomes	Medium H	1.82	11.4 ± 0.4	75.8 ±6.2	n.d
7-TUR	C2 rhizomes	Medium I	4.62	24.7 ± 2.0	40.9± 1.3	275.7±10.6

n.d. = not detected. * C1 and C2 represented two different explant lines.

In the literature turmeric microrhizomes were obtained in MS medium with a high sucrose concentration and a short photoperiod [33-34]. In this work the induction of microrhizomes was achieved by using the proposed short photoperiod to facilitate the storage phase of roots, and introducing the use of two different media, with a reduced agar percentage (0.5%) to facilitate microrhizome expansion. Medium H was characterized by a high amount of sucrose (6%). The second medium used, medium I, was characterized by sucrose (6%) and the addition of active charcoal (0.5%) to mimic the natural darkness of the *in vivo* soil environment. Microrhizome formation was observed after 10-12 weeks (Figure 2e, f, g). Medium H produced microrhizomes with very slight orange colour, while those grown in medium I were thick and orange coloured, very similar to young rhizomes of turmeric adult *in vivo* plants (Figure 2e, f). Furthermore, the olfactive perception of the peculiar turmeric flavour was perceived after rhizome cutting. These results confirmed that an increase in sucrose concentration (6%) and a short-day photoperiod were necessary conditions for a good development of *in vitro* turmeric rhizomes [22,34]. Moreover the addition of charcoal represents a good tool for the establishment of the characteristic colour of these organs.

Curcuminoids represent a class of antioxident compounds characteristic of turmeric plants conferring the typical yellow color to their rhizomes [7]. Curcumin (CUR), demethoxycurcumin (DEM), and *bis*-demethoxycurcumin (bis-DEM) were utilized as marker compounds for the phytochemical analyses. *In vitro* samples coming from different turmeric organs (shoots, roots and rhizomes) were analyzed in two different lines C1 and C2, chosen to compare the variability in curcuminoid content between different explants (1-7 TUR, Table 2). Fresh commercial *C. longa* rhizomes (A) were chosen as reference source of curcuminoids in comparison with the *in vitro* organs. Each sample was extracted by an ultrasonic method to avoid the instability and degradation of these metabolites when submitted to extraction with organic solvents at elevated temperatures. In fact, curcuminoids have a very high reactivity due to an extended aromatic portion and many functional groups suitable for the formation of hydrogen bonds [35]. Therefore, particular attention was taken during this experimental work, handling with care both the extracts and the standard solutions. The samples were prepared and analyzed on the same day, keeping them in protected vials to avoid photochemical degradation. An ethanolic aqueous mixture was selected as a safe solvent for the extraction, according to the Pharmacopoeial rules, in order to exclude any toxic residues into the final extracts that are potentially usable as food ingredients. In addition, the use of ethanol has already been reported to increase curcuminoid amounts, because it promoted the extraction of the more polar compounds present in the turmeric rhizomes [36].

In the present work, the extract yields of the selected *in vitro* samples ranged from 2 to 5%, very similar to those obtained from the commercial fresh rhizomes (Table 2).

LC-DAD-ESI-MS was used to screen the extracts obtained from the several established *in vitro* lines. The total UV scan detection in the range 220-600 nm was not very useful because the absorption spectra and chromatographic behavior of the three marker compounds were very similar (Table 3). Therefore, quali-quantitative analysis was performed by LC-ESI-MS in full scan (80-500 *m/z*) and MS/MS mode, which guaranteed peak identification of CUR, DEM, and bis-DEM in the extract samples (Table 3).

Internal standard calibration was performed by linear regression curves by using six different concentrations of CUR, DEM, and bis-DEM (0.25-100 μg mL^{-1}), along with a constant amount of 4-fluoro-4'-hydroxybenzophenone. These curves were represented by the following equations and correlation coefficients: $y = 911744x + 5237$ ($r^2 = 0.99886$) for CUR, $y = 877305x + 7404$ ($r^2 = 0.99744$) for DEM, and $y = 901245x + 1293$ ($r^2 = 0.99886$) for bis-DEM, where x is the concentration expressed as μg mL^{-1} and y the correspondent peak area.

The analysis of fresh commercial rhizomes showed the production of the three marker curcuminoids in significant quantity (A, Table 2). *In vitro* shoots and microrhizomes showed the ability to biosynthesize the three target curcuminoids, although not all simultaneously, and in very reduced amount in comparison with the levels determined in the fresh commercial rhizomes. The aerial part of turmeric *in vitro* cultures exhibited low amount of curcuminoids: in C1 line (1-TUR) the different curcuminoids were detected with similar amount, whereas in the C2 line (2-TUR) only curcumin was quantified, even if in higher amount than the other line. Roots did not represent good samples for the production of curcuminoids, since no level was detectable by the analytical method used in both lines.

The most significant production of the curcuminoids was observed in microrhizomes. *Bis*-DEM, DEM and CUR were detected in the rhizomes grown in medium I; in particular, curcumin was quantified in 5-TUR and 7-TUR (266 μg g^{-1} FW and 276 μg g^{-1} FW, respectively), together with DEM (147.2 μg g^{-1} FW in 5-TUR and 41 μg g^{-1} FW in 7-TUR) and *bis*-DEM (20.6 μg g^{-1} FW in 5-TUR; 7-TUR, 24.7 μg g^{-1} FW). On the other hand rhizomes from both lines grown in medium H showed variable amounts of *bis*-DEM and DEM, and the absence of the yellow curcumin. The darkness of solid medium represented the optimum condition to enhance the production of curcuminoids, especially for curcumin, in 5-TUR and 7-TUR. On the other hand the presence of light (medium H) limited greatly the production of curcuminoids, since only *bis*-DEM (11 μg g^{-1} FW) and DEM (76 μg g^{-1} FW) were produced in the C2

line (6-TUR), as evidenced by the absence of pigmentation in rhizomes (Figure 2 f, g).

It has already been reported that curcumin has a poor light stability and decomposes into several degradation products such as vanillin, vanillic acid, ferulic acid, and ferulic aldehyde when exposed to sunlight [37,38]. Kita *et al.* [24] studied directly the biosynthesis pathway of curcuminoids in *in vitro* microrhizomes of *C. longa* and suggested that the phenylpropanoid precursors (cinnamic acid or *p*-coumaric acid) were incorporated into both the two aromatic rings, and the hydroxy- and methoxy-functional groups were introduced after the formation of the curcuminoid skeleton. Our results are in agreement with the feeding experiments carried out in darkness by Kita *et al.* [24].

The absence of curcuminoids in *in vitro* roots is simply correlated to their predominant synthesis in rhizomes, the characteristic turmeric storage organ that can be developed under particular conditions, such as high level of sucrose and short photoperiod length [33-34].

Besides the significant levels of the curcuminoids detected in the rhizomes, a poor production of these compounds occurred also in the young shoots. Between the two shoots lines tested, the best results were obtained in 1-TUR samples. CUR, DEM and *bis*-DEM were simultaneously present (Table 2), whereas only curcumin was detected in 2-TUR (59.8 µg g^{-1} FW).

Curcuminoids are accumulated in the rhizomes, but also shoots can be generally involved in their biosynthesis depending on the activity of specific enzymes. Ramirez-Ahumad *et al*. [25] studied the enzymes of the phenylpropanoid pathway in leaf, shoot and rhizome extracts from turmeric adult plants. All the extracts showed both COMT (caffeic acid *O*-methyltransferase) and CCOMT (caffeoyl-CoA *O*-methyltransferase) activities, key enzymes for the biosynthesis of curcumin precursors [25], supporting the evidence that such a pathway is present in different plant organs. Our results confirmed the ability of *in vitro* shoots to produce curcuminoids, although in low levels. These organs could represent a good raw material considering the significant reduction in time and costs to produce this *in vitro* biomass, if compared with the production of rhizomes.

Experimental

Plant material and culture initiation: Fresh rhizomes of turmeric (*Curcuma longa* L.) were purchased from Raziel srl Company, Padova, Italy. Sprouting rhizomes were thoroughly washed under running tap water for 1 h to remove adhered soil particles. Sprouting buds (2-3 cm) derived from different rhizomes were used as explants, and immersed in a water solution with a few drops of Tween 80, then sterilized by soaking them in 70% ethanol for 30 seconds. After rinsing 3 times with distilled water, the buds were treated with 10% sodium hypochlorite (NaClO) for 15 min, then washed several times with sterile distilled water and dissected with a sharp scalpel to remove the outer leafy scale.

C. longa micropropagation: Sterilised sprouting buds were the primary explants to initiate *in vitro* culture in different media, and a single bud represents an individual line. Media A, B, and C were composed of Murashige and Skoog (MS) [39] salts and vitamins added with 6-benzylaminopurine (BAP) at the amount of 1, 2, and 3 mg L^{-1}, respectively; medium D was composed of MS salts and vitamins, 2.5 mg L^{-1} of BAP and 0.5 mg L^{-1} of α-naphthaleneacetic acid (NAA). Medium E was composed of MS salts and vitamins, 0.5 mg L^{-1} BAP, 0.1 mg L^{-1} of thidiazuron (*N*-phenyl-*N*'-(1,2,3-thidiazol-yl)urea, TDZ) and 100 mg L^{-1} ascorbic acid (AA). Each medium was supplemented with 30 g L^{-1} of sucrose, and 0.05% Plant Preservative Mixture (PPM Plant Cell Technology Inc., USA). To all media, 8 g L^{-1} of Bacto –agar was added and adjusted to pH 5.8 before autoclaving at 121°C at 1 atm for 20 min. The explants were cultured in Magenta-like vessels and incubated in a growth chamber at constant temperature of 22 ± 1°C, at an irradiance of 80 µmol m^{-2} s^{-1} for a photoperiod of 16/8 h. After 4 weeks, all induced shoots were transferred to E medium for proliferation under the same environmental conditions. Subcultures were prepared at 4-week intervals.

Root and microrhizome formation: The shoots obtained after 2 subcultures in medium E were transferred to rooting medium (F) composed of MS salts and vitamins, to which were added 1 mg L^{-1} of NAA, 0.1 mg L^{-1} kinetin (Kin), supplemented with 30 g L^{-1} of sucrose and 8 g L^{-1} of Bacto –agar and subcultured at 25°C ± 2°C using a 16/8 h photoperiod and at an irradiance of 80 µmol m^{-2} s^{-1}. The shoots were subcultured in this medium at 4-week intervals at least for 3 subcultures and then transferred to medium G for the formation and development of microrhizomes. Medium G had the same composition as medium F except for the increased amount of sucrose (60 g L^{-1}). The explants were subcultured 3 times at 4-week intervals. The plantlets were then transferred onto media H and I: medium H was composed of MS salts and vitamins, to which were added 1 mg L^{-1} of NAA, 0.1 mg L^{-1} of Kin and supplemented with 60 g L^{-1} of sucrose and solidified with 5 g L^{-1} Bacto –agar, while medium I had the same composition as medium H except for the addition of 5 g L^{-1} of active charcoal. Two subcultures were made at 4-week intervals. To promote the induction of microrhizomes the plantlets were subcultured at a constant temperature of 28°C ± 1°C, held in a short photoperiod of 8/16 h at an irradiance of 80 µmol m^{-2} s^{-1}.

Extraction of plant material: Samples (0.5-18 g fresh weight, FW) of *in vitro* organs (shoots 1-TUR and 2-TUR, roots 3-TUR and rhizomes 4-7TUR) of *C. longa,* maintained in a freezer at -80°C after harvest, were extracted using an ultrasonic apparatus and ethanol/water (EtOH:W, 7:3) for 15 min. Fresh commercial rhizomes (4-5 g FW, A) were extracted by the same method and used as reference material. Each extract was filtered, evaporated under vacuum in a rotavapor at 40°C and diluted (2-5 mg/mL) in a suitable volume of MeOH-water and filtered through a luer-lock syringe filter (polytetrafluoroethylene, PTFE, 2.5 mm, 0.45 mm) before LC injections.

LC-DAD-ESI-MS analysis: The LC system consisted of a Surveyor ThermoFinnigan liquid chromatograph pump equipped with an analytical Lichrosorb RP-18 column (150 x 4.6 mm i.d., 5µm, Synergy Fusion), a Thermofinnigan Photodiode Array Detector and a LCQ Advantage mass detector. The analyses were carried out with a linear gradient using water with 0.1% HCOOH (solvent A), CH_3CN (solvent B) at a flow rate of 0.3 mL min^{-1} in the following conditions: from 40:60, v/v (B-A) (0-10 min) to 80:20 (B-A) (10 min) for 22 min, and then conditioning to the initial condition (40:60 v/v B-A) for 10 min. Chromatograms were detected at 262 nm for all analyzed compounds. Both full scan and MS/MS mode chromatograms (mass range *m/z* 100 to 500 amu, negative ion mode) were registered (Table 3).

ESI-MS were obtained using the following parameters: sheath gas flow-rate 62 arb units, auxiliary gas flow 6 arb units, capillary voltage -4 V and capillary temperature 280°C, collision energy: 28 %. An aliquot (20 µL) of each sample was analyzed in triplicate and the quantitative analysis was performed according to the method of

Table 3: Experimental data of the target curcuminoids used for the quali-quantitative analyses of the *C. longa* extracts obtained from *in vivo* and *in vitro* plant material.

Target compound	UV (λ, nm)	Molecular weight	Base peak [M-H]$^-$ *m/z*	Collision energy (%)	Fragment ions MS^n (*m/z*)
curcumin	257, 420	368	367.4	28	216.9 (28)*, 173.3, 149.1
demethoxycurcumin	258, 419	338	337.2	27	216.9 (28), 173.4, 145.1
bis-demethoxycurcumin	249, 417	308	307.2	28	187.2 (28), 143.1, 119.1

*collision energy used for MS^n experiment

Hiserodt *et al.* [40] by an internal standard (4-fluoro-4'-hydroxy-benzophenone) co-injected with the solutions of extract samples under the same conditions used for the calibration curves of the 3 target curcuminoids (CUR, DEM, bis- DEM, 0.25-100 µg mL^{-1}).

Statistical analysis: Statistical analysis was undertaken using specialized software (Statgraphics 5.1, Statistical Graphics Corp., USA). Data were analyzed by one-way ANOVA and mean comparison was performed by Student Neumann Keuls" (SNK) test at $p<0.05$.

Reagents and chemicals: Reagents used for *in vitro* experiments were purchased from Sigma-Aldrich (St Louis, Mo, USA). The phytoregulators (BAP, NAA and TDZ) were obtained from Micropoli (Duchefa). Organic solvents were purchased from J.T. Baker (Mullinckrodt, Buker, Inc, Phillipsburg, NI 088659. Curcumin (CUR), demethoxycurcumin (DEM), and *bis*-demethoxycurcumin (bis-DEM) were bought from Sigma Aldrich.

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