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Changes in Endogenous Cytokinins and *in vitro* Photoperiodic Flowering Induction in *Cichorium intybus* L.

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Abstract: Cytokinins were extracted from the explants of *Cichorium intybus* L. roots grown under flowering inductive (long-day and red light), or non inductive conditions (short-day). Flowering was expressed as percent after 8 weeks of their development. Overall, cytokinins levels exhibited biphasic trends with initial increasing concentrations followed by a decrease over time. The highest content of cytokinins was observed in explants exposed to either red light or long-day periods. Measured maximum levels of up to 525 ng.g^{-1} dry weight were obtained under red light exposure conditions. Cytokinins concentrations measured in materials cultured under long-day conditions were slightly lower by quite similar to those obtained under red-light growth conditions. In contrast, much lower peak values (not exceeding 155 ng.g^{-1} dry weight) were obtained under short-day experimental conditions. The *in vitro* development of *C. intybus* under both long-day periods and exposure to red light resulted in percent flowering of 55.75% and 64.28%, respectively. No flowering was observed under short-day experimental conditions. Overall, our results show that both endogenous content of cytokinins and flowering of *C. intybus* grown *in vitro* are dependent on both light quality and length of the photoperiod.

Key words: *Cichorium intybus* L., cytokinins, flowering, *in vitro* culture, photoperiodicity

INTRODUCTION

The use of *in vitro* cultures in agriculture, arboriculture, forestry and horticulture plays a significant role in meeting the continuously growing needs of human populations (Dubos, 2001; El Kbiach *et al*., 2002). Despite current efforts for the development and improvement of *in vitro* techniques by both academic and industrial institutions, knowledge on the physiology of plant materials cultivated *in vitro* remains rather limited (El Kbiach *et al*., 2002). This is because the neo-formation of plant materials such as floral buds cultured *in vitro* depends on several and complex physicochemical parameters that includes photoperiods and climatic variations and which promote different types of biochemical modifications. For instance, these parameters have been linked to changes in cellular differentiation, which in turn impacts floral organogenesis (Attibayéba and Paulet, 2004). For of *Cichorium intybus* L., the inductive process of *in vitro* flowering is a key factor for reproductive development, which is associated with important biochemical changes such as variations in enzymatic activities (Attibayéba, 1992), accumulation of chlorogenic acid (Badila and Paulet, 1986) and changes in endogenous contents of ß-indolylacetic acid (Gaspar *et al*., 1982). Therefore, understanding the different physiological mechanisms involved in the flowering process would help close current knowledge gaps.

In this paper, we investigated the potential relations between cytokinins levels and flowering induction under *in vitro* culture conditions. The study emphasizes the effect of changes in endogenous cytokinins levels on floral metabolism of explants of *Cichorium intybus* L.

MATERIALS AND METHODS

Plant materials: Roots of *Cichorium intybus* L. C.V. Witloof were used in all experiments. Under natural conditions, *C. intybus* exhibits a port in bow in a vegetative state and flowering under such conditions requires exposure to cold temperatures and diurnal cycles with long photoperiods. After collection of the plant's roots from the field and hand cleaning, they were placed horizontally in tubs filled with slightly humidified vermiculite and preserved at 4°C for about three months.

***In vitro* cultures:** At the time of their use, roots, freed of their snares, were divided into three zones from the snare. These zones are representative of the hormonal distribution gradient (Vardjan and Nitsch, 1961). Explants were taken in the median third of the root. After sowing on culture medium of Margara and Rancillac (1961a), the cultures of tissues were arranged in premises air-conditioned, maintained at 24°C during daytime and 22°C at night. In the air-conditioned premises, white light with an intensity of 56 µmoles m^{-2}s^{-1} (Mazda Fluor TFR 20 L 5L) was used for 16 h (L-D:

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long-day) or 8 h (S-D: short-days) for every 24 h. Additionally, for cultures under short-day conditions, the 16 h of darkness was followed by an illuminated by red light or R (3 µmoles $m^{-2}s^{-1}$; Mazda Fluor TF 40W/15) from the 8th day of incubation to the 18th day. Red plastic filters (Rodhoïd 227 P2F 0.3 mm, COFRAMAP, France) were used in combination with the above described fluorescent red light.
For every treatment, explants were removed from growth media starting on the 5th day of the experiment up to the 30th day. Removed explants were freeze-dried immediately until analysis for cytokinins. After 8 weeks of *in vitro* development and for the number N of replicates corresponding to a given treatment type, the number of explants with visually identifiable neoformation of floral organs or n was determined. The ratio n/N was then used to report the results on a 100% scale.

Extraction of cytokinins: A method adapted from Palni and Horgan (1983) was used. First, 10 g of freeze-dried root explants were finely crushed at room temperature using a Dangoumau grinder for 30 sec. The obtained powdered material was extracted overnight with a 100 ml of an 80% methanol solution at 4°C and without shaking to solubilize the nucleotides. The supernatant was then filtered using a Büchner the following day and the above described methanol extraction repeated twice on the same powdered material. For each sample, the three methanol extracts were combined and evapo-concentrated using a rotary evaporator at 35°C to about 20 ml. The concentrated sample was centrifuged at 9000g at 5°C for 15 min. The supernatant was recovered and its pH adjusted to 3.5. The surfactant Polyvinylpyrrolindone (PVP) was added to the sample (80 mg/mL of extract), mixed and then filtered using a Büchner to remove the excess PVP. At this stage, the obtained filtrate contains the cytokinins. The pH of the extract is next adjusted to 8.5 and a volume of water-saturated n-butanol, with a water content equivalent to that of the extract, is added to it. The mixture is shaken for 45 min (Tay and Palni, 1987) and decanted by sedimentation. The aqueous phase is then extracted 3 times with the n-butanol solution as described above to transfer the nucleotidic cytokinins to the organic phase. The butanolic supernatants are combined and evaporated to dryness under controlled pressure at 40°C. Two mL of a 80% methanol solution in water was used to resuspend the active components extracted from the samples. The pH of this obtained solution was adjusted to 8.2 and brought to 10 ml. Next, the 4 mg of alkaline phosphatase was added to the sample (Van Staden and Dimalla, 1980), then centrifuged at 40,000g at 3°C for 20 min. The recovered supernatant was adjusted to a known final volume, split in n- butanol (Palni and Horgan, 1983), before being purified using PVP following the procedure described above.

Purification of the extracts of cytokinins: The free cytokinins stemming from the butanolic phase and the bound cytokinins were separated by chromatography on whatman paper 3 mm according to the method of Van Staden *et al.* (1983), after sample treatment with ß-glucosidase. They are purified on Sephadex column LH 20 (21 x 2.5 cm) and then on Thin Layer Chromatography (TLC) before identification by Gas Chromatography (GC).

Gas Chromatography (GC): Prior to GC identification, the purified solutions of cytokinins were evaporated and dried on P_2O_5. The residues were dissolved in 10 ml of pyridine. The derivatives TMS (trimethylsylilyed) were prepared by addition of 20 ml of BSTFA (twice trimethyltrifluoroacetamid). The sylilation was conducted in conical tubes for 30 min at 90°C. Residues transformed into TMS by BSTFA were analyzed by GC (Delsi DI-700, equipped with a FID detector and a 2.5 m long Pyrex column with a 2 mm ID). The column packing material was chromosorb WHP with 3% silicon. Helium was used as carrier gas at a flow rate of 40 ml/min. The detector and the injector temperatures were maintained at 300°C. The cytokinins were analyzed using different temperature conditions. The riboylzeatine was analyzed under isotherm conditions with a column temperature of 280°C. In contrast, temperature gradient was used for the separation of zeatine (first 240°C for 5 min and increase to 260°C at a rate of 30°C per minute) and isopentenyladenosine (245°C for 5 min and up to 260°C at a rate of 30°C per minute).
For the identification of the cytokinins, 10 ng of Benzylaminopurine (BAP) were used as internal standard and co-injected with the extract (v/v). The co-chromatography is obtained by mixing cytokinins from commercial sources with those extracted from samples, volume per volume.

Analysis of the cytokinins: From a mother solution of cytokinins with a concentration of 1 mg/mL, standards were prepared. Cytokinins concentrations in samples are expressed in ng/g dry weight.

RESULTS AND DISCUSSION

Our results show that the levels of cytokinins in analyzed plant extracts increase from the 5th day during *in vitro* development, to reach their maximum on the 15th day. This initial growth phase is then followed by a decrease up to the 30th day of incubation. Figure 1 shows that explants exposed to Red Light (R) have Ribosylzeatine

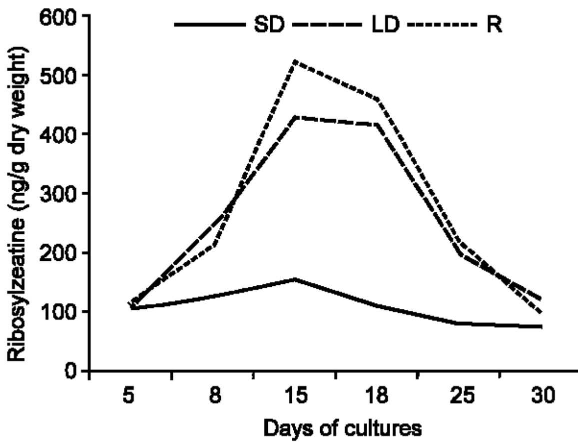

Fig. 1: Effect of photoperiodic variations on ribosylzeatine levels over time (SD = Short Day, LD = Long-Day and R = Exposure to Red Light)

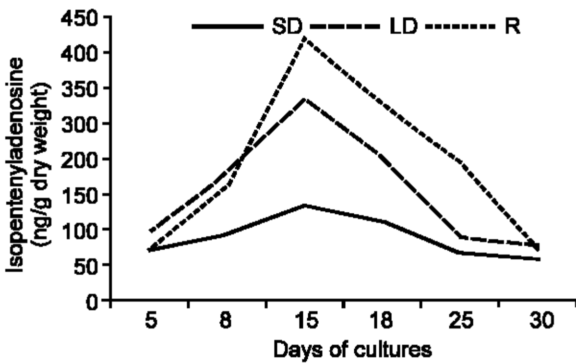

Fig. 2: Effect of photoperiodic variations on isopentenyladenosine levels (SD = Short Day, LD = Long-Day and R = Exposure to Red Light)

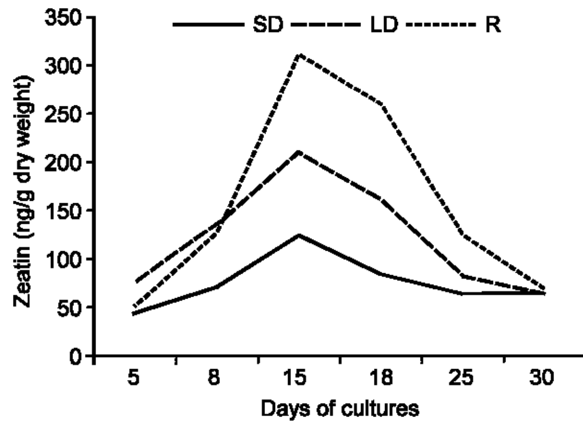

Fig. 3: Effect of photoperiodic variations on zeatine concentrations (SD = Short Day, LD = Long-Day and R = Exposure to Red Light)

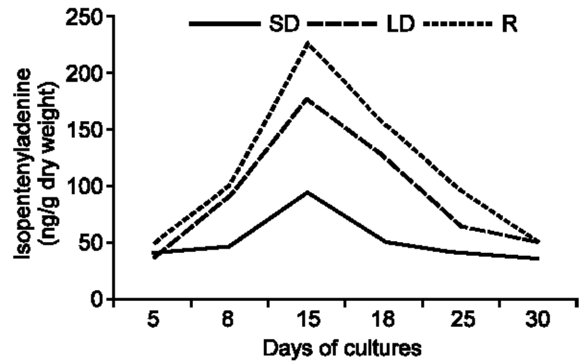

Fig. 4: Effect of photoperiodic variations on concentrations of isopentenyladenine (SD = Short Day, LD = Long-Day and R = Exposure to Red Light)

(RZ) contents that vary from 109, 525 and 100 ng.g^{-1} dry weight to 100, 430 and 120 ng.g^{-1} dry weight in L-D experiments and to 100, 155 and 75 ng.g^{-1} dry weight in S-D experiments. The contents of Isopentenyladenosine (IPA) varied from 80, 415 and 85 ng.g^{-1} dry weight in the R; to 110, 320 and 90) ng.g^{-1} dry weight in L-D incubations; to 80, 150 and 65 ng.g^{-1} dry weight in the S-D tests (Fig. 2). Also, the curves of endogenous zeatine (Z) varied from 52, 312 and 70 ng.g^{-1} dry weight in the R; to 75, 210 and 64 ng.g^{-1} dry weight in the L-D and to 44, 124 and 64 ng.g^{-1} dry weight in the S-D (Fig. 3). The Fig. 4 shows that, the curves of endogenous Isopentenyladenine (IP) changes to 48, 225 and 50 ng.g^{-1} dry weight in explants exposed to the red light; to 40, 176 and 50 ng.g^{-1} dry weight in those developing in the L-D and to 36, 94 and 36 ng.g^{-1} dry weight in the S-D. In the same way, in the R, the contents of Ribosylzeatine Glucoside (RZG) changes to 32, 112 and 34 ng.g^{-1} dry weight; to 42, 90 and 38 ng.g^{-1} dry weight in the L-D and to 30, 46 and 26 ng.g^{-1} dry weight in the S-D (Fig. 5). Finally, the Fig. 6 shows that, the contents of Zeatine Glucoside (ZG) changes to 36, 138 and 36 ng.g^{-1} dry weight in the R; to 45, 112 and 36 ng.g^{-1} dry weight in the L-D and to 36, 60 and 25 ng.g^{-1} dry weight in the S-D.

Generally, we note that, the content in RZ and IPA is more important with regard to those in Z and IP or RG and RZG. In the R and in the L-D the contents of endogenous cytokinins are higher than in S-D explants. What seems to justify itself because, in the L-D and the R, explants differentiates respectively 55.75% and 64.28% of flowers; in the S-D, no culture differentiates *in vitro* flowers (Table 1). Light conditions of the study led to an increase of cytokinins and the capacity to flowering. The analysis of these cytokinins revealed that more Ribosides (RZ, IPA) than bases (Z, IP) as commonly found in plants (Dyson and Hall, 1972; Miller, 1975; Joseph, 1984). This can be explained by the higher ability of bases to bind to the receivers. Such fixed

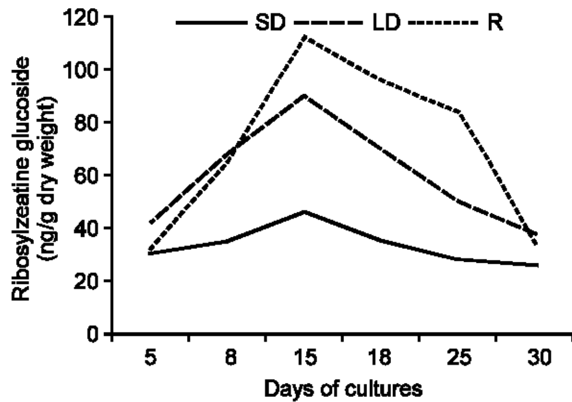

Fig. 5: Changes in ribosylzeatine glucoside levels versus photoperiodic conditions (SD = Short Day, LD = Long-Day and R = Exposure to Red Light)

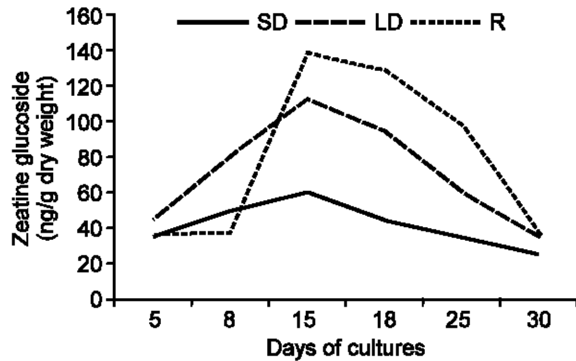

Fig. 6: Changes in zeatine glucoside levels versus photoperiodic conditions (SD = Short Day, LD = Long-Day and R = Exposure to Red Light)

Table 1: Effect of different photoperiodic treatments on *in vitro* flowering of fragment roots of *Cichorium intybus* L.

Photoperiodic conditions	Percentage of flowering (%)
Short day	00.00±0.00
Long day	55.75±0.91
Red light	64.28±3.21

molecules would become inaccessible by the alcoholic extraction used in this study and extracts would consequently be relatively poor in bases. Nucleotides were present in small quantities and in the form of ribosylzeatine only. Glucosides were present only in the form of glucoside of RZ and Z. Some authors have reported that these glucosides have a weak biological activity (Laloué and Pethe, 1982; Badenoch *et al.*, 1984b). In *C. intybus* L., favourable conditions for *in vitro* flowering and the formation of meristematic structures generator of floral buds are preceded by an important production of cytokinins, followed by a decrease during the floral expression's phase. On the contrary, the conditions putting obstacle to the flowering decrease strikingly these contents. Overall, we note 3 distinct phases of evolution of levels of endogenous cytokinins during *in vitro* flowering. (1) A pre-inductive phase, of a week approximately from the stake in culture. It is the phase of formation of meristematic nodules which get organized in buds. It seems to have a little dependence on photoperiod, and the contents of cytokinins in explants are low. (2) A photo-inductive phase also of a week approximately, where neo-formed meristems present a stage of development to which the susceptibility in the illumination is maximal. An important production of cytokinins is observed. Finally (3), a phase of initiation and floral development, with little sensitivity to day length and during which the quality previously acquired expresses itself outside by the elaboration of floral organs. Here, the contents of cytokinins fall again. It proves well that, the *in vitro* culture of explants of roots leads to a differentiation of tissues, to phenomena of embryogenesis and allows obtaining a plantlet (El Kbiach *et al.*, 2002; Azeqour *et al.*, 2002). The light modulates the morphogenetic process which leads to the regeneration this plantlet (Baaziz *et al.*, 1996; Majourhat, 2002). The only difference between the inductive and non-inductive conditions of flowering results in the intensity of production of cytokinins, which is raised in the conditions founders of the flowering. What lets augur the existence of a correlation between the cytokinins contents and the neoformation of the *in vitro* floral buds. These same relations of causality exist between the enzymatic activities and the primary events of the photoperiodic induction of the flowering (Attibayéba and Paulet, 2004). What shows that, the photoperiodic induction acts no only on cytokinins, but also on other metabolites and regulators which have an indisputable role on the flowering.

Conclusion: In this study, the measurable changes during the photoperiodic treatments are the successive increase and decrease of the contents of cytokinins as the experiments reach the switching point from photo-induction to floral expression phases. Levels of cytokinins are more important under conditions that are inductive to flowering than in non-inductive conditions. GC-analyses reveal that cytokinins are present mostly as nucleosides (isopentenyladenosine and ribosylzeatine), bases (zeatine and isopentenyladenine), glucosides (ribosylzeatine, zeatine), while ribosylzeatine was the only identified nucleotide. Photo-period appears to be a necessary condition for the stake with flowers bound to the long or short, daily duration of the illumination according to the sorts. Future work should (1) focus on the characterization of the physiological changes of the photo receiving systems, and biochemical sites involved in the perception of the stimulus in the floral induction and in the production of

cytokinins; particularly to bring to light put back Red Light-Far Red (R-FR) which allow to involve the phytochrom in the morphogenesis; (2) use short periods of illumination for photophase and (3) bring to light the possible role of blue, green and yellow lights. Such a comprehensive study would allow a better understanding of the modulation of morphogenetic process by light. This could have positive implications for increased quality and productivity in relevant farming activities.

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