Indian J. Plant Physiol., Vol. 11, No. 3, (N.S.) pp. 287-290 (Jul.-Sept., 2006)

SHORT COMMUNICATION

CHANGES IN ENDOGENOUS CYTOKININ ACTIVITY DURING SEED DEVELOPMENT IN TEA

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Received on 25 Oct., 2005, Revised on 25 Aug., 2006

The role of endogenous cytokinin activity was examined in tea seed during development that was divided into 6 stages. Cytokinin activity similar to zeatin (Z), zeatin ribosides (ZR) and iso-pentyl adenine (IPA) increased with advance in seed growth. Cytokinin activities (especially of Z and ZR) continued to increase from the liquid endosperm stage (i. e. stage 6) when histo-differentiation occurred to finally rise to a maximum at stage 8 or the early embryo maturation stage during which 90% of the endosperm was consumed by the growing embryos. Later, cytokinin activity similar to Z and ZR decreased from stage 9 (late embryo maturation stage). Although cytokinin activities declined during full maturity (stage 10), yet maximum activity was still detected in the form of cytokinin glucosides at this period.

***Key words*: Cytokinin activity, developmental stages, tea seeds.**

Tea [(*Camellia sinensis* L. (O.) Kuntze] seeds are recalcitrant (Berjak *et al*. 1993) and shown to lose viability very fast which makes their storage and transportation very difficult. An overall period of about 15 -18 months is required from anthesis to maturation for complete seed development (Bhattacharya *et al*. 2002). Generally, the external and internal factors during seed development affect the behaviour of mature seeds (Finch Savage 1995). While during 'histo- differentiation', rapid gain in fresh weight results from cell division and early expansion in *Avicennia mariana*, increase in dry weight due to cell enlargement in order to accommodate deposition of future reserves, characterize the phase of embryo maturation (Farrant *et al*. 1993). The state of continuum even at full maturity and lack of clear end point to seed development in tea as indicated by appreciable contents of soluble protein and total RNA (Bhattacharya *et al*. 2004) further confirmed the recalcitrant nature of this species.

Since the major type of plant growth regulators like auxin, gibberellins, and cytokinins are involved during histo-differentiation, detailed physio-biochemical studies on tea seed development were initiated in our laboratory. In the previous study (Bhattacharya *et al*. 2002), the vital role of ABA in the seeds during embryo maturation was ascertained. The level of endogenous free IAA in tea embryos continued to increase progressively up to embryo maturation phase, the period during which 90% of the endosperm was consumed by the growing embryo (Bhattacharya *et al*. 2004). Seeds are rich sources of cytokinins and a wide range of cytokinins has been isolated from seed tissues (Yang *et al*. 2000). It is believed that cytokinins play vital role in developing seeds as strong assimilate sinks (Brenner and Cheikh 1995). In the present study, the role of endogenous cytokinin activity in tea seeds during its different developmental stages was examined.

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IHBT publication no. 0564

Twenty bushes of tea (*C. sinensis)* growing at the Institute of Himalayan Bioresource Technology Experimental farm, Banuri, Palampur (36°N and 78.18°E and 1290 m above sea level), were selected randomly and ten branches of each bush were tagged. In tea, flower bud emergence takes place during May-June, (stage 1) and flowering starts during July-August (stage 2) followed by anthesis. While fruit set takes place during September-October (stage 3), fruit growth commences during November-December (stage 4). The seed development was followed from the first visible sign of seeds (stage 5, January-February) during which visible embryos, bathed in transparent liquid endosperm, were observed and subsequently seeds were collected for the study from this stage every month till November-December (stage 10- maturity) as described earlier (Bhattacharya *et al*. 2002*)*. Freshly harvested seeds (cotyledon + axes) from each of the stages were separately homogenized with chilled modified Bieleski's extraction buffer I (Bieleski 1964) comprising of chloroform: methanol: formic acid: water (5: 12: 12: 2, v/v, 10 ml/g fw), kept at 4^0C, centrifuged at 8,000 x g for 20 min at 5^0C to halt any enzyme action. The residue was re-extracted twice more, each time in chilled, modified Bieleski's extraction buffer II (methanol: formic acid: water: 6: 1: 4, v/v), vortexed and centrifuged. The two supernatants were pooled, filtered with Whatman no. 1 paper and evaporated *in vacuo* at 40^0C. This was re-suspended in 2.5 ml of 0.015 M K_2HP0_4 buffer (pH 5.8) and the suspension was loaded on to a column (35 x 2 cm) of insoluble PVP and eluted with 250 ml of the same buffer. The eluates were evaporated to minimum of 5 ml, adjusted to pH 3.0 with 1N HCl, centrifuged (8,000 x g for 15 min at 5°C) and passed through cation exchange Dowex 50 (H^+ form, 20-25 mesh) column (20 x 2 cm) at a flow rate of 40 ml/hr, as mentioned elsewhere (Nagar and Saha 1985). The columns were washed initially with 50 ml 80% ethanol and cytokinins were eluted with chilled 250 ml of 3M NH_4OH. The eluates were evaporated *in vacuo* at 40°C and taken up in 2 ml of 35% methanol. The relevant extracts were subsequently placed on a Sephadex LH-20 column (30 x 2 cm) and eluted with 35% ethanol at a flow rate of 20 ml/h. Fractions of 15 ml each were collected, dried *in vacuo* and assayed for cytokinin activity in 50 ml medium in each 100 ml flask using the soybean cotyledon callus test (Miller 1967). The fractions eluted as above were identified on the basis of elution patterns of the authentic cytokinins Z, ZR, ZG and iPA and all bioassays were repeated and means of the two are presented in the result.

Following Sephadex LH-20 column chromatography, two major peaks of cytokinin activity in seeds were detected at stage 5 (Fig. 1A), having elution patterns similar to zeatin riboside (ZR) and zeatin in fractions 7-8 and 12-13 respectively. However, two minor peaks were also detected, the most polar one eluting in fraction 4-5 similar to zeatin glucoside (ZG) and the other one eluting in fraction 16-17 similar to iso-pentyl adenine (iPA).

In subsequent stages of development (Fig.1B to Fig.1D), the cytokinin activities having elution pattern similar to Z, ZR increased slightly during stage 6 (Fig.1B). However, the increase in Z rose to a maximum only during the early embryo maturation stages 7and 8 and was predominant over the other two i.e. ZR and iPA (Fig.1C and 1D). Although the increase in ZR activity was maintained up to stage 7, it also rose to highest level at stage 8 (Fig.1D). A sharp reduction in activities of these three fractions was noticed during 'late embryo maturation phase' or stage 9 (Fig.1E). However, some detectable activity was still noticed in these three fractions even during full maturity (stage10; Fig.1F). Interestingly, with decrease in activities of all the three major fractions, the activity of the most polar fraction (4-5), i. e. the cytokinin glucoside tended to increase during subsequent stages of seed development especially from stage-8 (early embryo maturation phase) and predominated over others at final phase of seed maturity (stage-10; Fig.1F).

Seeds are considered to be a rich source of cytokinins and over the past 40 years, a wide range of different cytokinins has been described in these tissues (Lexa *et al*. 2003). High cytokinin activity during early stages of seed development occurs in most of the seeds and it is also accepted that the cytokinins are involved in embryo and endosperm development (Farrant *et al*. 1993, Lur and Setter 1993). In the present study, cytokinin activity similar to Z, ZR and iPA increased with progressive increase in seed growth, up to stage 8 (early embryo maturation stage), the stage during which 90% of the

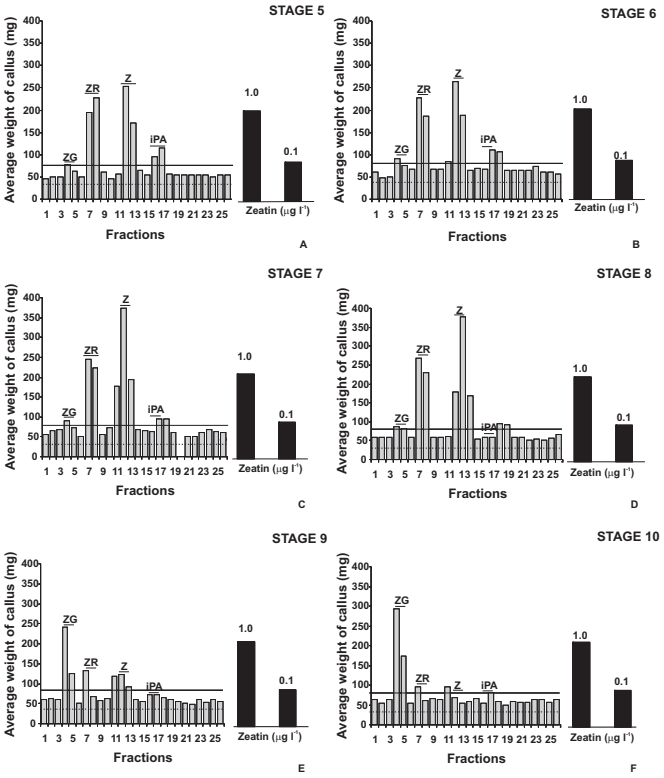

Fig. 1. Separation of cytokinin activity by Sephadex LH-20 column chromatography of the Dowex 50 purified extracts of tea seeds at different stages of development. A: stage 5; B: stage 6; C: stage 7; D: stage 8; E: stage 9 and F: stage 10. At each stage, 2.0 g fresh weight of seeds (cotyledon + axes) were analyzed. The broken and solid horizontal lines represent control and least significant differences at 5% level respectively. Z = Zeatin, ZR = Zeatin riboside, ZG = Zeatin-O-glucoside; iPA = Isopentyl adenine.

endosperm was consumed by the growing embryo (Bhattacharya *et al*. 2002). Although the increase in cytokinin glucoside activity was negligible during the stages 6 to 8 (Fig.1B to 1D) and increased from stage 9, maximum activity was noted only during the final stage of seed maturity (Fig.1F). This is in contrast to most orthodox seeds, yet in accordance with that of recalcitrant seeds like *Quercus robur* (Finch-Savage and Farrant 1997). Histo-differentiation stage in the tea embryos comprised of the liquid endosperm (stages 5 and 6) wherein the moisture content ranged between 80-82% (Bhattacharya *et al*. 2002) and the activities of cytokinins especially of Z and ZR were most prominent. This is not surprising since cytokinins control seed size by influencing the cell number in very young embryos of developing seeds and increased cell number would enhance storage capacity (Binus 1994). Studies in wheat and corn (Morris *et al*. 1993) show that cytokinin content is highest during developmental stages that encompass periods of the rapid nuclear and cellular division of the endosperm. The significance of greater increase in CK activity specially of Z and ZR during early embryo maturation phase of tea (Fig. 1 C & D) could be due to two possibilities, firstly reserve mobilization in the cotyledons by cytokinins and secondly stimulation of cotyledonary photosynthesis as in many vegetative tissues (Brenner 1987).

Although a fall in cytokinin activity similar to Z and ZR was evident in seeds from stage 9 (late embryo maturation stage), a steady increase in cytokinin glucoside activity was noticed from this stage onwards till seed maturity phase (stage 10, i. e. Fig. 1F). Cytokinin glucosides have been suggested to exist in storage and bound forms (McGaw and Burch 1995) and their levels have been found to fluctuate with maturity of fruits and leaves (Saha *et al*. 1985) which serve to regulate the levels of free forms of cytokinins in plants (Kaminek *et al*. 1997). In the present study, the cytokinins having elution pattern similar to Z and ZR, which are considered to be 'active forms' of cytokinins (Blintsov *et al*. 2001), appear to be the major ones. The decline in their activity from stage 9 (embryo maturation phase) may perhaps be due to their utilization during seed development or their conversion into their glucosides, which are needed as and when required. It has been suggested (Letham and Palni 1983) that the level of cytokinins and cytokinin glucosides, which are present within a particular tissue, may influence the initiation of cytokinin biosynthesis.

It has been proposed (van Staden 1983) that cytokinin activity in maturing seeds is regulated in such a way as to create a quiescent or dormant stage, which is essential for seed survival. Since a relative cytokinin activity was still found in seeds when it reached maturity it may not only be considered as a reservoir of cytokinins for developing seeds and as a sink for drawing nutrients (Brenner and Cheikh 1995) but also as reservoir of cytokinins for the germinating seedlings. The components and molecular mechanisms involved in cytokinin perceptions and signal transductions in tea seeds need further study.

ACKNOWLEDGEMENTS

The authors wish to thank Director IHBT for providing necessary facilities, Mr. Naseeb Chand for his help in carrying out the work, and Department of Science and Technology, New Delhi for the financial assistance in the form of a research project to the senior author.

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