BIOLOGIA PLANTARUM 47 (4): 581-584, 2003/4

BRIEF COMMUNICATION

Lipid composition of *in vitro* developing seeds of *Brassica campestris* L.

N. SHARMA, A. PHUTELA, S.P. MALHOTRA and R. SINGH*

Plant Biochemistry and Molecular Biology Laboratory, Department of Biochemistry, CCS Haryana Agricultural University, Hisar-125 004, India

Abstract

A liquid culture technique has been developed to study lipid metabolism in seeds of *Brassica campestris* L. grown *in vitro* from terminal inflorescences detached 4 to 46 days after anthesis. Seeds developed under these conditions exhibited pattern of growth, deposition of storage products and lipid composition similar to those from intact plant.

Additional key words: fatty acids, liquid culture, non-polar lipids, polar lipids.

Brassica oils are used not only for edible purposes but also as raw materials for industrial purposes. Accordingly, there is considerable interest in the possible manipulation of *Brassica* plants either to increase the production of oil or to alter its quality (Topfer *et al.* 1995). Hence, any concerted effort aimed at modification of lipid composition would require the detailed study of biochemical changes occurring during seed development which on the intact plant is complicated by the interaction of pods and seeds with the mother plant. To overcome these complications, a liquid culture method to study the physiology of early flower and/or fruit development (Nitsch 1951) has been used for cereals (Donovan and Lee 1977, 1978, Williams *et al.* 1998) and legumes (Karamdeep *et al.* 1998, Singh and Kaushal 2000). However, a similar method is not available as yet for the growth of inflorescence of oil seeds. We report here the growth of explants bearing inflorescence from *Brassica campestris* L. in liquid culture containing MS medium. To assess the success of the technique, an attempt has been made to compare the results on growth pattern and lipid composition of seeds grown under *in vitro* liquid culture medium with those of seeds grown *in situ* in greenhouse (Archana *et al.* 1999).

Brassica campestris L. cv. Toria was raised in pots filled with soil, in the greenhouse of the Department of Biochemistry, CCS Haryana Agricultural University, Hisar. Fully opened flowers were tagged on the day of anthesis during active flowering phase. The inflorescence in three replicates bearing uniformly developing pods were detached from the plants at 4 days after anthesis (DAA) until 46 DAA at 7 d intervals and were aseptically cultured for 10 d in culture tubes. Explants consisting of a segment of stem were cut under water from the basal region of the terminal inflorescence bearing only four to six floral organs at the uniform stage of development. The stems were surface sterilized with aqueous mercuric chloride solution (0.1 %, m/v) containing 4 - 5 drops of *Teepol* detergent for 10 min and subsequently rinsed repeatedly with sterile distilled water. The stems were then inserted through a cotton plug in 60 cm^3 culture tubes containing 30 cm^3 of Murashige and Skoog (MS) liquid culture medium. Five stems were inserted in each tube. All transfers were carried out in a laminar flow cabinet to reduce microbial contamination. The culture tubes were then immersed in a low temperature water bath (maintained at 2 - 4 °C) kept in a growth chamber (irradiation of 200 μmol m^{-2} s^{-1} for a 14-h photoperiod and day/night temperature of 25/18 °C). Culture medium was replaced every 3rd d to prevent microbial growth. The

Received 19 November 2001, *accepted* 11 April 2003.

Abbreviations: DAA - days after anthesis; DAG - diacylglycerol; DAGAT - diacylglycerol acyl transferase; DGDG - digalactosyl diacylglycerol; GC - gas chromatography; GL - glycolipids; MGDG - monogalactosyl diacylglycerol; MS - Murashige and Skoog; NPL - non-polar lipids; PA - phosphatidic acid; PC - phosphatidyl choline; PE - phosphatidyl ethanolamine; PG - phosphatidyl glycerol; PL - polar lipids; PLP - phospholipids; TAG - triacylglycerol; TL - total lipids; TLC - thin layer chromatography.

Acknowledgement: This investigation was supported by ICAR, India. Dr. S.K. Gupta is thanked for providing GC facilities.

*Author to whom correspondence should be addressed; fax: +91-1662-34952; e-mail: sudhir@hau.nic.in

stem explants were grown in liquid culture for 10 d. Throughout culturing period, stems were made to dip in the liquid medium. After culturing, pods were weighed, freeze dried and analyzed.

Oil content in the seeds was determined using nuclear magnetic resonance (*MK-III A*, *New Port Analyzer*, New Port, UK) equipped with 2 cm^{-3} coil assembly (Collins *et al.* 1967). Protein was estimated by conventional micro-Kjeldahl method, while the method of Clegg (1956) was employed for quantitative estimation of starch. Extraction, fractionation and analysis of lipids by thin layer chromatography and fatty acid analysis by gas chromatography (*Hewlett Packard* model *5130A*) were carried out exactly by the methods described in our earlier communication (Archana *et al.* 1999).

Preliminary experiments revealed that MS medium supplemented with glutamine at a concentration of 0.25 g(N) dm^{-3} was the best liquid medium for the growth of *Brassica* inflorescence *in vitro*. In this medium, the inflorescence could be successfully cultured for 10 d. Explants bearing inflorescence, detached from field grown plants at 4, 11, 18, 25, 32, 39 and 46 DAA after 10 d of culture gave the seeds (14, 21, 28, 35, 42, 49 and 56 DAA, respectively).

As in field grown seeds (Archana *et al.* 1999), the *in vitro* grown seeds also exhibited a sigmoidal pattern (Fig. 1*A*) of oil deposition typical of oil rich seeds. The initial phase of 0 - 14 DAA involves rapid cell division but no deposition of stored material with dry mass only 0.3 mg $seed^{-1}$ and oil content only 98 mg g^{-1}(d.m.) at 14 DAA. The second phase up to 49 DAA exhibited a dramatic increase in oil and in dry mass while the third phase from 49 DAA until maturity is characterized by dehydration and desiccation. The protein content increased continuously from 14 DAA to 49 DAA, remaining almost constant until maturation. A similar increase in protein content in *in vitro* grown seeds has been observed earlier in chickpea pods grown under liquid culture for 5 d (Karamdeep *et al.* 1998, Singh and Kaushal 2000) and could be attributed to the deposition of oleosin in oil bodies (Murphy and Commins 1989). Starch content of the *in vitro* grown seeds decreased continuously during development indicating that at initial stages, oil synthesizing machinery is either absent or incomplete diverting the excess photosynthates for starch accumulation. During the second phase of active oil deposition, there is a metabolic shift from starch to oil, which leads to the mobilization of starch serving as a source of carbon and energy for oil biosynthesis (Singh 1998). Transient storage of starch has previously been observed in field grown *Brassica campestris* (Archana *et al.* 1999) and *Brassica napus* (Eastmond and

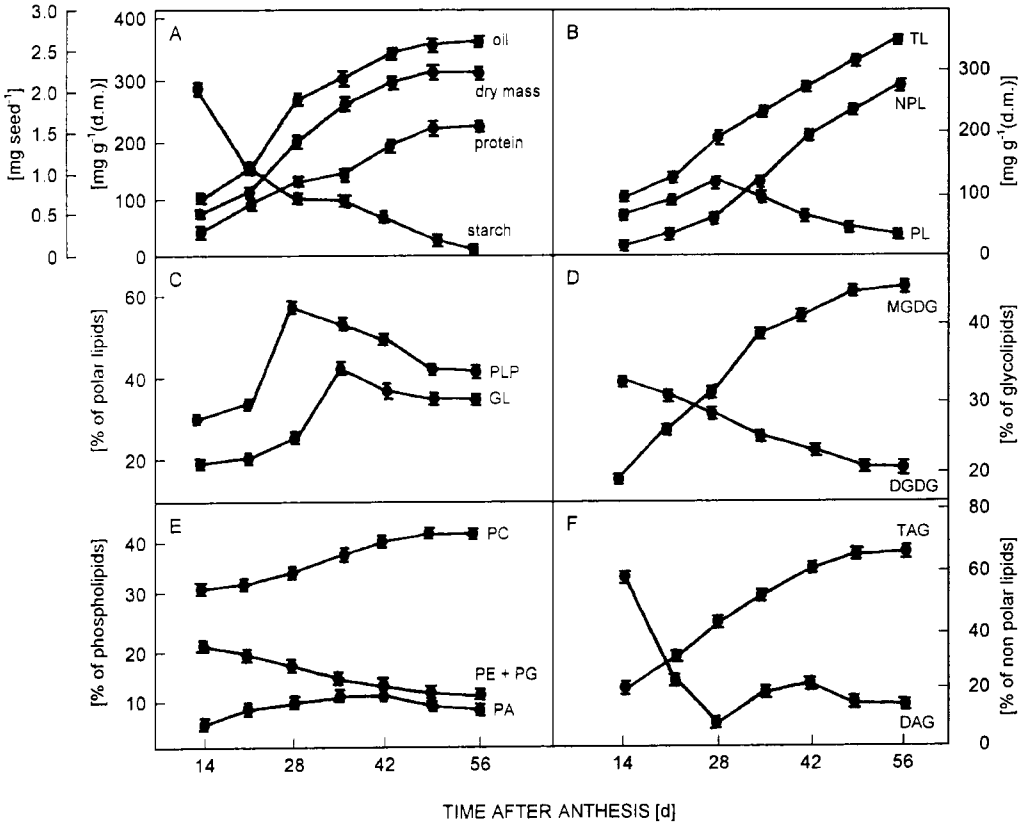

Fig. 1. Changes in storage products and lipid components in *in vitro* grown seeds of *Brassica campestris* at different days after anthesis. The means of four replicates ± SE

Table 1. Fatty acid composition of total, polar and non-polar lipids of *in vitro* developed seeds of *Brassica campestris* during development. Means of four replicates ± SE.

Lipids	DAA	16:1	18:0	18:1	18:2	18:3	22:1
Total lipids	14	20.4 ± 1.3	14.1 ± 0.1	18.0 ± 0.5	25.3 ± 0.5	10.8 ± 0.3	9.2 ± 0.5
	21	16.0 ± 0.5	8.2 ± 0.3	25.2 ± 0.3	23.8 ± 0.6	14.2 ± 0.6	12.1 ± 0.5
	28	8.2 ± 0.8	6.7 ± 0.6	22.1 ± 0.2	22.5 ± 0.5	17.3 ± 0.5	19.8 ± 0.5
	35	7.7 ± 0.4	4.2 ± 0.2	18.9 ± 0.5	19.2 ± 0.6	21.1 ± 0.4	26.4 ± 0.3
	42	5.9 ± 0.4	3.5 ± 0.2	15.1 ± 0.4	17.2 ± 0.6	24.3 ± 0.2	30.4 ± 0.8
	49	3.9 ± 0.2	2.9 ± 0.1	10.9 ± 0.4	16.7 ± 0.7	22.7 ± 0.4	38.3 ± 1.2
	56	3.0 ± 0.1	2.6 ± 0.1	9.9 ± 0.4	17.0 ± 0.5	20.4 ± 0.6	44.2 ± 0.7
Polar lipids	14	28.0 ± 1.1	8.3 ± 0.3	25.0 ± 0.9	20.5 ± 0.9	18.0 ± 0.9	-
	21	19.7 ± 1.0	7.6 ± 0.3	25.2 ± 0.7	23.0 ± 0.7	22.6 ± 0.9	-
	28	18.3 ± 0.7	6.6 ± 0.4	26.1 ± 0.6	24.2 ± 0.7	23.0 ± 0.7	-
	35	14.3 ± 0.7	4.3 ± 0.2	28.3 ± 0.7	27.1 ± 0.6	25.8 ± 1.0	-
	42	13.9 ± 0.5	4.1 ± 0.2	23.4 ± 0.8	27.4 ± 0.8	28.9 ± 1.0	-
	49	10.7 ± 0.4	4.1 ± 0.2	23.7 ± 1.1	29.7 ± 1.0	29.7 ± 1.1	-
	56	10.4 ± 0.5	3.7 ± 0.1	21.8 ± 1.0	28.9 ± 1.1	33.8 ± 1.0	-
Non-polar lipids	14	34.4 ± 1.0	21.5 ± 0.9	26.1 ± 0.6	7.6 ± 0.3	5.1 ± 0.2	4.1 ± 0.2
	21	22.0 ± 0.9	13.2 ± 0.7	20.3 ± 0.7	14.4 ± 0.8	16.6 ± 0.9	10.9 ± 0.5
	28	10.3 ± 0.5	10.0 ± 0.9	17.1 ± 0.9	20.3 ± 1.0	21.7 ± 1.0	20.0 ± 0.9
	35	8.1 ± 0.3	7.4 ± 0.6	13.5 ± 0.9	19.2 ± 0.9	24.9 ± 1.1	26.6 ± 0.9
	42	7.7 ± 0.4	5.5 ± 0.3	10.7 ± 0.4	19.0 ± 1.0	23.9 ± 1.1	30.2 ± 1.3
	49	6.1 ± 0.3	5.0 ± 0.5	8.6 ± 0.3	18.8 ± 1.0	20.7 ± 1.0	36.0 ± 1.2
	56	5.8 ± 0.3	4.2 ± 0.2	8.1 ± 0.2	18.4 ± 0.8	18.9 ± 1.1	38.8 ± 1.0

Rawsthorne 2000, Rawsthorne *et al.* 1996), whereas castor bean has been reported to possess no transitory storage prior to oil accumulation.

Non-polar lipid (NPL) fraction accounted for only 20 % of total lipids at 14 DAA and increased dramatically with high rate of deposition attaining 84 % of total lipids at 56 DAA. In contrast, the polar lipid (PL) fraction was about 76 % of total lipids at 14 DAA and increased slightly up to 28 DAA and then decreased progressively to 11 % at 56 DAA (Fig. 1*B*). Perry and Harwood (1993) and Archana *et al.* (1999) have reported the PLs to be relatively more important during early stages of seed development, while the NPLs at later stages of seed development. PLs are mainly the components of membranes, whereas, NPLs constitute storage lipids. Accordingly, the decrease in PL fraction at later stages has been linked with the chloroplast breakdown (Perry and Harwood 1993), while the increase in NPL is linked with the deposition of storage lipids (Norton and Harris 1975) until complete maturation of seeds.

The content of PLPs was always higher than that of glycolipids (GLs) (Fig. 1*C*). As has been reported earlier (Archana *et al.* 1999) maximum content of PLPs was observed at 28 DAA, while GLs were maximum at 35 DAA. Since the GLs are the main components of chloroplast membrane, their reduction may again be linked to the chloroplast breakdown at later stages, which is indicated by the disappearance of chlorophyll (Perry and Harwood 1993). Higher amount of PLPs throughout development suggests that besides being the components of membrane lipids, they have a role to play in Kennedy pathway for triacylglycerol (TAG) synthesis in seeds of oil crops (Stobart *et al.* 1998).

Phosphatidyl choline (PC) accounted for 36 to 46 % of the total phospholipids (Fig. 1*E*). Phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) together accounted for 24 % of PLPs in young seeds and their content decreased to 13 % at maturity. Only a minor fraction of total PLPs (8 %) was present as phosphatidic acid (PA) at 14 DAA, which increased to 13 % at 42 DAA and then showed a slight decrease up to 9 % of total PLPs. PA and PC have been reported to play an important role in Kennedy pathway for TAG synthesis in many oil storing seeds (Stymne and Stobart 1987). Lower concentration of PA indicates that the third stage of Kennedy pathway involving phosphatidic phosphatase exerts little flux control over the overall pathway. The significant accumulation of PC indicates its involvement in the production of highly unsaturated linoleate- and linolinate-enriched oils (Schultz and Ohlrogge 2000).

Digalactosyl diacylglycerol (DGDG) accounted for 20 % of total GLs in young seeds and increased during development to 43 % at 49 DAA after which the content of DGDG remained constant (Fig. 1*E*). On the contrary, monogalactosyl diacylglycerol (MGDG) concentration was maximum at 14 DAA (32.5 % of GLs) and decreased to 21 % at 49 DAA, after which it remained constant.

This also supports the earlier argument that during development, MGDG is converted to DGDG. As expected, TAG being the main component of neutral lipids followed a pattern similar to that followed by NPL fraction (Fig. 1*B*). Active synthesis of TAG started at 14 DAA and the content increased dramatically reaching maximum at maturity. On the other hand, DAG showed a decline upto 28 DAA, increased upto 42 DAA and then declined subsequently. It could be suggested that at later stages, diacylglycerol acyl transferase (DAGAT) was unable to utilize its substrate fully thus exerting some flux control over the Kennedy pathway (Perry and Harwood 1993).

Critical examination of the data on fatty acid composition of TLs (Table 1) revealed that during phase 1 of cell division, it resembled that of photosynthetic tissues (high in palmitic and linoleic acid). In phase 2 of active deposition of storage material, the fatty acid composition resembled that of storage TAG (erucic acid and linolenic acid increased; linoleic, palmitic and stearic acid decreased, while the oleic acid increased transiently and then decreased). During phase 3 of desiccation and dehydration, the amount of all fatty acids remained almost constant, while that of erucic acid further increased. Presence of erucic acid only in NPL fraction and its complete absence from PL fraction suggests that it is the component of storage lipids and not that of structural lipids. Furthermore, the content of oleic acid remained almost constant in PL fraction, while it decreased continuously in NPL fraction, suggesting that erucic acid in NPL fraction is synthesized from oleic acid precursor. These results on compositional changes in the proportion of different fatty acids in PL and NPL fractions of *in vitro* grown seeds were in consonance with those reported earlier for field grown *Brassica campestris* (Archana *et al.* 1999, Hobbs *et al.* 1996) and *Brassica napus* (Norton and Harris 1975).

Since the changes in growth, storage products and lipid components of *in vitro* grown seeds followed patterns similar to those observed earlier for the seeds developing on the intact plant (Archana *et al.* 1999), it could be inferred that culturing of detached inflorescence was successful.

References

Archana, Sharma, N., Malhotra, S.P., Singh R.: Changes in fatty acid composition in developing seeds of *Brassica campestris* L. - J. Plant Biol. **26**: 165-172, 1999.

Clegg, K.M.: The application of anthrone reagent to the estimation of starch in cereals. - J. Sci. Food Agr. **1**: 40-44, 1956.

Collins, F.I., Alexender, D.E., Rodgers, R.C., Silvela, L.: Analysis of oil content of soybean by wide line NMR. - J. amer. Oil Chem. Soc. **44**: 708-710, 1967.

Donovan, G.R., Lee, J.W.: The growth of detached wheat heads in liquid culture. - Plant Sci. Lett. **9**: 107-113, 1977.

Donovan, G.R., Lee, J.W.: Effect of the nitrogen source on grain development in detached wheat heads in liquid culture. - Aust. J. Plant Physiol. **5**: 81-87, 1978.

Eastmond, P.J., Rawsthorne, S.: Co-ordinate changes in carbon partitioning and plastidial metabolism during the development of oilseed rape embryos. - Plant Physiol. **122**: 767-774, 2000.

Hobbs, D.H., Hume, J.H., Polph, C.E., Cooke, D.T.: Changes in lipid composition during floral development of *Brassica campestris*. - Phytochemistry **42**: 335-339, 1996.

Karamdeep, L., Singh, R., Bhullar, S.S.: Accumulation of starch, protein and activities of sucrose cleaving, transaminating enzymes in chickpea pods raised through liquid culture. - Plant Physiol. Biochem. **36**: 675-681, 1998.

Murphy, D.J., Cummins, I.: Biosynthesis of seed storage products during embryogenesis in rapeseed, *Brassica napus*. - Plant Physiol. **135**: 63-69, 1989.

Nitsch, J.P.: Growth of excised ovaries *in vitro*. - Amer. J. Bot. **38**: 566-577, 1951.

Norton, G., Harris, J.F.: Compositional changes in developing rapeseed (*Brassica napus* L.). - Planta **123**: 163-174, 1975.

Perry, H.J., Harwood, J.L.: Changes in the lipid content of developing seeds of *Brassica napus*. - Phytochemistry **32**: 1411-1415, 1993.

Rawsthorne, S., Eastmond, P.J., Kang, F., DaSilva, P.M.R., Smith, A.M., Hutchings, D., Emes, M.J.: Carbon partitioning in plastids during development of *Brassica napus* embryos. - In: Williams, J.P., Khan, M.U., Lem, N.W. (ed.): Physiology, Biochemistry and Molecular Biology of Plant Lipids. Pp. 152-174. Kluwer Academic Publishers, Dordrecht 1996.

Schultz, D.T., Ohlrogge, J.B.: Biosyntheis of triacylglycerol in *Thunbergia alata*: Additional evidence for involvement of phosphatidyl choline in unusual monoenoic oil production. - Plant Physiol. Biochem. **38**: 169-175, 2000.

Singh, R.: Carbon and energy sources for fatty acid biosynthesis in non-photosynthetic plastids of higher plants. - Proc. indian nat. Acad. Sci. **B64**: 335-354, 1998.

Singh, R., Kaushal, B.: Response of sugar interconversion, starch and protein accumulation to supplied metabolites during pod-filling in chickpea. - Indian J. exp. Biol. **38**: 51-55, 2000.

Stobart, A.K., Stymme, S., Shewry, P.R., Napier, I.: Seed lipids. - In: Harwood, T.L. (ed.): Plant Lipid Biosynthesis: Fundamentals and Agricultural Applications. Pp. 223-245. Cambridge University Press, Cambridge 1998.

Stymne, S., Stobart, A.K.: Triacylglycerol biosynthesis. - In: Stumpf, P.K., Conn, E.E. (ed.): The Biochemistry of Plants. Vol. 9. Pp. 175-214. Academic Press, New York 1987.

Topfer, R., Martini, N., Schell, J.: Modification of plant lipid synthesis. - Science **268**: 681-686, 1995.

Williams, M., Robertson, E.J., Leech, R.M., Harwood, J.L.: Lipid metabolism in leaves from young wheat (*Triticum aestivum* cv. Hareward) plants grown at two carbon dioxide levels. - J. exp. Bot. **49**: 511-520, 1998.