Incorporation of ^{14}C 18:2 into Different Lipid fractions of *Glycine max* Cotyledons

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Abstract: a pulse-chase study of radiolabled ^{14}C linoleoyl-CoA was carried out at an early and late stage of embryo triglyceride (TG) synthesis of in order to investigate factors that contribute to fatty acids content of normal oilseeds such as *Glycine max* (Soybean). Data indicate that ^{14}C-18:2 is rapidly removed from PC and incorporated directly into TG. Also no significant changes occurred in ^{14}C -18:3 levels in soybean. This indicates that desaturase activity decreased during the late stage in soybean embryos and most of ^{14}C -18:2 was released from PC and incorporated into TG without being converted into 18:3.

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1. Introduction

In developing soybean cotyledons, the accumulation of radioactive polyunsaturated fatty acids into phospholipids results from acyltransferase activity, also further desaturation of oleic acid incorporated in phospholipids was found in developing soybean cotyledons (Wilson et al., 1980). In flax seeds, incorporation of ^{14}C from acetate into lipids was very rapid and with phospholipids and 1, 2-diacylglycerols it was within as short as 5 min. Triacylglycerols were labeled much slower (Dybing and Craig, 1968). In the biosynthesis of triacylglycerol the acyl groups are esterified at the *sn*-1, 2, and 3 positions of the glycerol back bone by the activities of the acyl-CoA: *sn*-glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA:1-acyl-*sn*-glycerol-3-phosphate.

Therefore the production of high levels of specific fatty acid in transgenic or wild type plants is important for an efficient channeling of this fatty acid from membrane lipids into the TAG. However, the mechanism for such selective channeling of certain fatty acids into the TAG is not understood (Dahlqvist et al., 1998). Another TG synthesis route has been discovered in animal (rat) and in plant (safflower) systems. Lehner and Kuksis (1993) and Stobart et al. (1997) presented evidence for a non-glycerol-3-phosphate pathway for transacylations of glycerol back bone using diacylglycerol transacylase (DGTA) that catalyzes the transfer of fatty acid between two molecules of DG resulting in the production of TG and MG. Additionally in animal systems evidence for Acyl-CoA: glycerol acyltransferase (AGAT) catalyzes the direct acylation of glycerol by acyl-CoA producing MG were found in addition to the acylation of glycerol-3 phosphate by GPAT in the glycerol-3-phosphate pathway (Lee at al., 2001).

The first desaturation step takes place in the chloroplast to form oleic acid (18:1 Δ9 [where Δ9 indicates that the position of the double bond is between carbons 9 and 10]); this is catalyzed by the Δ9-stearoyl-ACP desaturase using 18:0-ACP as substrate. The oleic acid is then release from its ACP by a thioesterase converted to a CoA thioester and transported into the cytoplasm, where the subsequent desaturation steps to linoleic acid (18:2 Δ9,12) and linolenic acid (18:3 Δ9,12,15) are catalyzed by Δ12 desaturase (also known as ω6-oleate desaturase) and ω-3 desaturase (also known as Δ15-linoleate desaturase).

Desaturation and TAG synthesis take place in the endoplasmic reticulum (ER). Fatty acids synthesis starts in the plastids and the resulting FA are delivered to the cytosol as thioesters derivatives (fatty acyl-CoA pool) (Browse and Somerville, 1991; Ohlrogge et al., 1991; Topfer et al., 1995). Different fatty acyl-CoAs are deposited into membrane and storage lipids in the ER by different types of acyltransferases. ER desaturases modify the fatty acids esterified to lipid membranes specially phosphatidylcholine (PC) (and possibly other phospholipids) (Ohlrogge and Browse, 1995; Weselake, 2000). The fatty acid distribution in TAGs is widely varied, and it is species and variety specific (Roughan and Slack, 1982). acyltransferase (APAT), and acyl-CoA:1,2 diacylglycerol acyltransferase (DGAT), respectively (Roughan and Slack, 1982; Frentzen, 1986; Frentzen, 1998). Moreover, the fatty acid composition in position 2 of the glycerol back bone can be modified through the reversible acyl-CoA: lysophosphatidylcholine acyltransferase reaction (Stymne and Stobart, 1984), and CDP-choline: diacylglycerol choline phosphotransferase reaction (Ichihara, 1984; Slack et

al., 1985). As reviewed by Bernerth and Frentzen (1990) the properties of some different acyltransferases, such as DGAT from *Tropaeolum majus* and *Limnanthes douglaii* , have already investigated especially for their fatty acid specificities and selectivity and they fund to be highly specific toward their substrates and this wieldy vary from plant to plant. The synthesis of C18 polyunsaturated fatty acids from oleic acid and their incorporation into TAGs in oilseeds are better understood (Stymne and Stobart, 1987). It has been shown for many species that diacylglycerols (DAG) are the precursors of phosphatidylcholine (PC) and TAG. During the formation of triacylglycerols through glycerol-3-phosphate or the Kennedy pathway, (Kennedy, 1961), there is an equilibrium between the DAG pool, PC pool, and glycerol backbone (Slack et al., 1985; Stobart and Stymne, 1985).

Linoleate incorporated into TAG from the acyl-CoA pool occurs through an acyl exchange between oleoyl-CoA and linoleate at position *sn*-2 of PC. Then oleoyl position in the *sn*-2 position of PC has been fund to be important in regulating the level of polyunsaturated fatty acids availability for TAG formation. Also in castor, DAG acyltransferase (DGAT) is highly selective for DAG species and utilizes both medium and long-chain acyl-CoA species (Griffiths et al., 1988; Bafor et al., 1990; Lin et al., 1998). In addition, the conversion of PC to free fatty acids by phospholipase A_2 is strongly dependent on the enzyme itself and different phospholipases have strong specificity toward specific fatty acids (Lin et al., 1998). In addition, other acyltransferases such as glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) from different organisms have different specificity toward different fatty acids (Lohden and Frentzen, 1992; Pillai et al., 1998; Sharma et al., 2012). The regulation and the metabolic factors of different oil accumulation levels and their wide range of fatty acid contents in oilseeds are currently unknown (Ohlrogge and Jaworski, 1997). However, sufficient acyltransferase activities and glycerol-3-phosphate levels are not the only limiting factors in TAG synthesis rates in vivo, but the amount of fatty acid produced in plastids may in part determine the amount of TAG synthesized in oilseeds (Bao and Ohlrogge, 1999).

Linoleoyl phosphatidylcholine is the main substrate for microsomal ω3 desaturase, but the linoleic acid ester with galactolipids or sulfolipids is the main substrate for plastid isoforms (Kinney, 1994). The linolenate is synthesized much less at the *sn-1* position of PC than that synthesized at the *sn-2* position. In addition there are two genes that control linoleoyl-PC desaturation during the early stages of the high accumulation of TAG in linseed (Stymne et al., 1992). It is now evident that humans need to increase their dietary ω-3 fatty acid levels. Oils high in ω-3 fatty acids are also more useful renewable resources such as drying oils used in products such as printing inks. So in order to be able to engineer seeds oil to produce such nutritionally and industrially valuable compounds in a relatively lower commercial production expense, it is important to understand the factors that affect high 18:3 accumulation in TAG in high producer plants.

Soybean seeds contain 20% oil and 8% of it is 18:3 (Dahmer et. al., 1991). Pulse chase studies were carried in order to investigate factors that contribute to fatty acids content of normal oilseeds such as *Glycine max* (Soybean)

2. Material and Methods

2.1. Plant material

Seeds of two different cotyledons developmental stages; were TG is mostly synthesized; from soybean (7-9 mm& 9-11 mm) were taken, and seed coats were removed and cotyledons were separated.

2.2. Pulse-Chase

Radiolabled 18:2 CoA (0.0033 μCi) / mg fresh seed tissue, 1 μL/mg fresh weight of embryo of 0.1 M potassium phosphate buffer, pH 7.2, 0.1% Tween 20 (Buffer A) were added to clean test tubes. Mixed together, and the seed tissues were added to the test tube that corresponds to their weight, and then incubated for 90 min. in the presence of fluorescent light. At the end of the 90 min. all tubes were washed 2 times with 0.1 M potassium phosphate buffer, pH 7.2, 0.1% Tween 20, each time with 1 mL. Two replicates, from each stage of soybean were taken for lipid analysis (zero time), and the rest were incubated for further chasing. Samples were collected for lipid analysis after 30, 60 (1h), and 120min. (2h), from zero time.

2.3. Separation of lipid classes using TLC

Total lipids were extracted in chloroform: methanol (2:1 v/v). To analyze the incorporation of radiation in different lipid classes, individual lipids were separated by one-dimensional thin layer chromatography 20x20 cm TLC plate (LK60 silica gel 60 Å, Whatman) by the method of (Kumar, 1983). TLC plates were developed in two solvent systems. Phospholipids and glycerolipids were separate in chloroform: methanol: water (65:25:4, v/v) for 10 cm ascending. Neutral lipids were separated in hexane: diethyl ether: acetic acid (100: 100: 2, v/v) for 19.5 cm ascending (Miquel and Browse, 1992). Lipids were located by spraying the plates with solution of 0.005% primulin in 80% acetone, followed by visualization under UV light. The silica gel from each lipid band were scrapped, transferred to a tube containing 2 mL of

2% (V/V) H_2SO_4 in methanol, and fatty acid methyl esters were prepared as described above.

2.4. Reverse phase TLC

To separate linoleate (18:2) and linolenate (18:3) a reverse phase TLC approach was applied. Fatty acids methyl esters, for each lipid classes, obtained from the previous steps were loaded into TLC plates (KC18 silica gel 60 Å, Whatman) plates were developed in acetonitrile: acetic acid: water (70: 10: 10 v/v) for 19.5 cm ascending. This plate was stained with iodine. 18:2 and 18:3 bands were scrapped; activity was counted using a liquid scintillation analyzer (Packard, 15000 Tri-Crab).

3. RESULTS & DISCUSSIONS

3.1. ^{14}C incorporation into different lipid fractions of soybean

Figure 1A shows that the incorporation of ^{14}C -18:2 in soybean PC at the first stage is increased at ½h of the chase time until the end of the chasing period, while ^{14}C -18:2 level in PC of the second stage decreased dramatically after 1/2h. The ^{14}C - 18:3 level in PC slightly increased by the end of chasing period. Figure 1B data showed that ^{14}C -18:2 levels in TG is increased dramatically in the first stage seeds after 1/2h and reached a maximum level at 2h. Also data showed that the ^{14}C -18:2 level in TG is rapidly increased after 0h and reached the maximum level at 1/2h and remained mostly steady till the end of the chasing time (2h), while ^{14}C -18:3 level is slightly increased in both stages. Data from Figures (1A&1B) highly suggest that ω-3 desaturase activities are low in both stages and convert a low amount of ^{14}C -18:2 from PC into ^{14}C -18:3. Also the data suggest that most of ^{14}C -18:2 is transferred from PC into TG which emphasizes high activity of acyltransferases enzymes and their specificity toward the incorporation of 18:2 in TG over 18:3 which also is not available at a high concentration. This data agree with Rubel et al. (1972), Carver and Wilson (1984), Wang et al (1987), Dahmer et al. (1989), and Suryadevara et al, (2008). The increase in lipid content in the soybean seed is accompanied by a shift in lipid composition from PC to TAG accumulation (Privett et al., 1973; Suh et al., 2002). Figure 1C data showed that^{14}C incorporation level in soybean DAG is at a very low level in both stages but the incorporation of both ^{14}C -18:2 and ^{14}C -18:3 levels is dramatically decreased in DAG of soybean seeds at the first stage, while ^{14}C -18:2 and ^{14}C - 18:3 level is almost steady at the second stage during the chasing time. These may be due to the rapid conversion of DAG into TAG indicating that DGAT is very active and has high efficiency transferring 18:2 into TG (Figure 1B). From Figures 1D& 1E it is apparent that the ^{14}C incorporation level in MAG and free FA is very low. Also the ^{14}C -18:2 levels in both the first and second stages is much higher than ^{14}C -18:3 levels (2 fold). Moreover Figure 1D shows that the^{14}C -18:2 level in MAG of first stage seeds is increased after 1/2h of chase time and ^{14}C -18:2 level in MAG of second stage increased till 1/2h and remain steady till the end of chasing time (2h). However Figure 2A shows that the percentage of ^{14}C incorporation in both TG and PC of the first stage; of soybean cotyledon developmental stages; is increased, while the ^{14}C percentage in both DG and FFA pools is dramatically decreased during the chasing time while ^{14}C level in MAG slightly increased. However Figure 2B shows that the ^{14}C level in PC of the seeds at the second stage decreased after 1/2h while it increased in TG of the same stage toward the end of the chasing time. Also Figure 2B shows that ^{14}C level in all other lipid fractions (MAG, DAG, and FFA) is almost steady during the chasing time, and there were only slight increases in FFA levels toward the end of the chasing time, this may be due to that both PC and TG reaching a steady state by the end of the chasing time and therefore there is no use for the FFA pool. MAG showed a slight decrease toward the end of the second stage. Data from both Figures 1& 2 emphasize that the lipid metabolism in soybean is in favor of accumulating 18:2 into TG, also these data highly suggest that ω-3 desaturase activities is low compared with *D. moldavica*. Figure 3 illustrates a suggested pathway in favor of accumulating 18:2 into TG in soybean seeds. Soybean data indicate that ^{14}C-18:2 is rapidly removed from PC and incorporated directly into TG.

No significant changes occurred in ^{14}C -18:3 levels in soybean. This indicates that desaturase activity decreased during the late stage in soybean embryos and most of ^{14}C -18:2 was released from PC and incorporated into TG without being converted into 18:3. The soybean data showed that linoleoyl-CoA gets incorporated into PC and transferred to TG (Wilson, 1987; Wang et al., 1987; Dahmer et al., 1989; and Suryadevara et al., 2008) without or with limited desaturation which reflects that ω -3 desaturase activity in soybean is relatively lower pulse chase data emphasize the role of acyltransferases in TG and could roll out that more than one pathway may exist for storage lipid biosynthesis in plants (Slabas et al., 2001). Figures 3 illustrated the pathway of the biosynthesis of triacylglycerols soybean since ^{14}C -18:2 incorporation and its conversion into ^{14}C -18:3 were chased in different lipid classes; PC, TG, DG, MG; in both plants.

Figure1(A): ^{14}C-18:2 incorporation in soybean PC

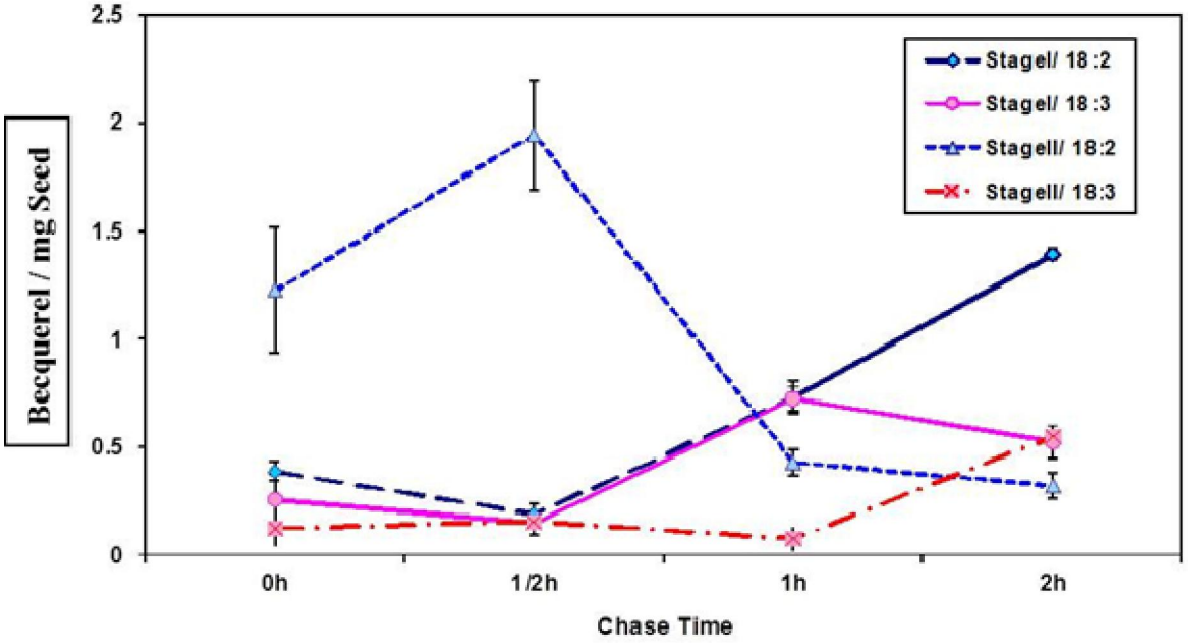


Figure1(B): ^{14}C-18:2 incorporation in soybean TAG

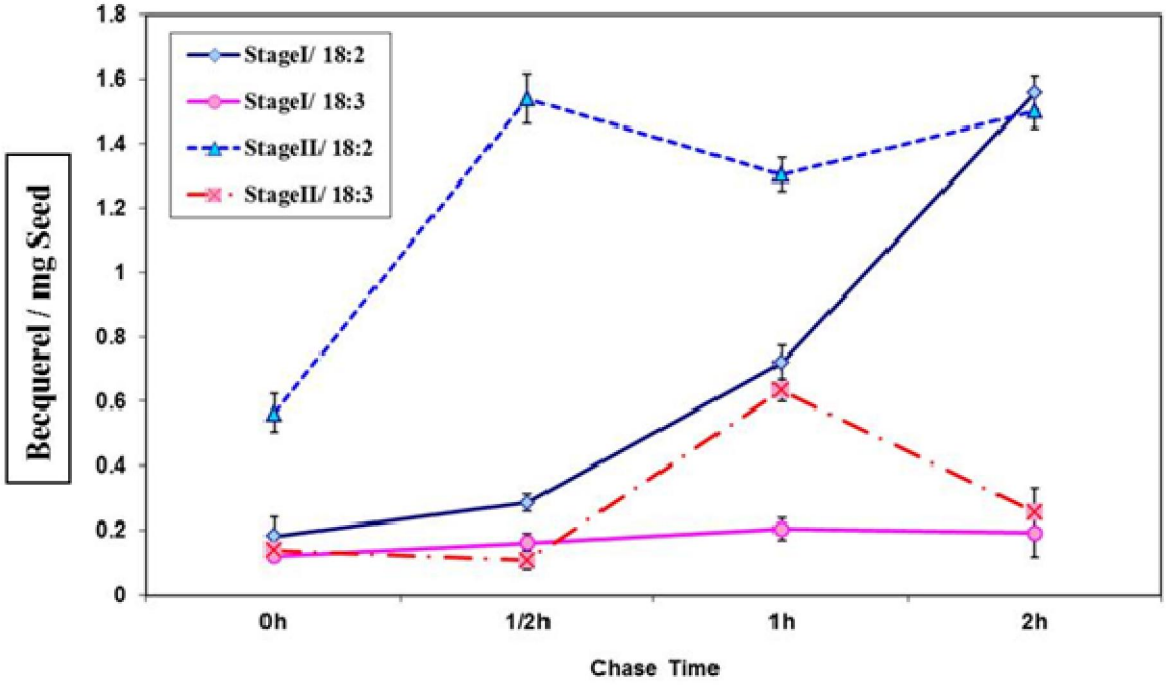


Figure 1 (C): ^{14}C-18:2 incorporation in soybean DAG

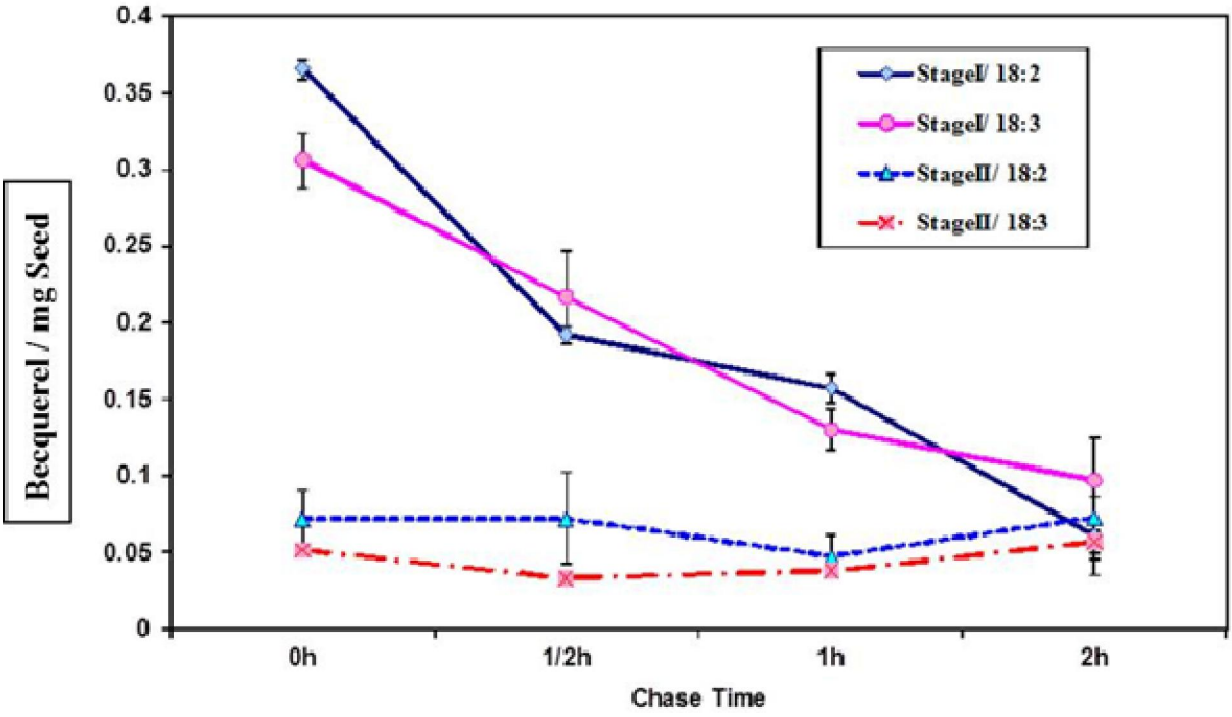


Figure 1 (D): ^{14}C-18:2 incorporation in soybean MAG

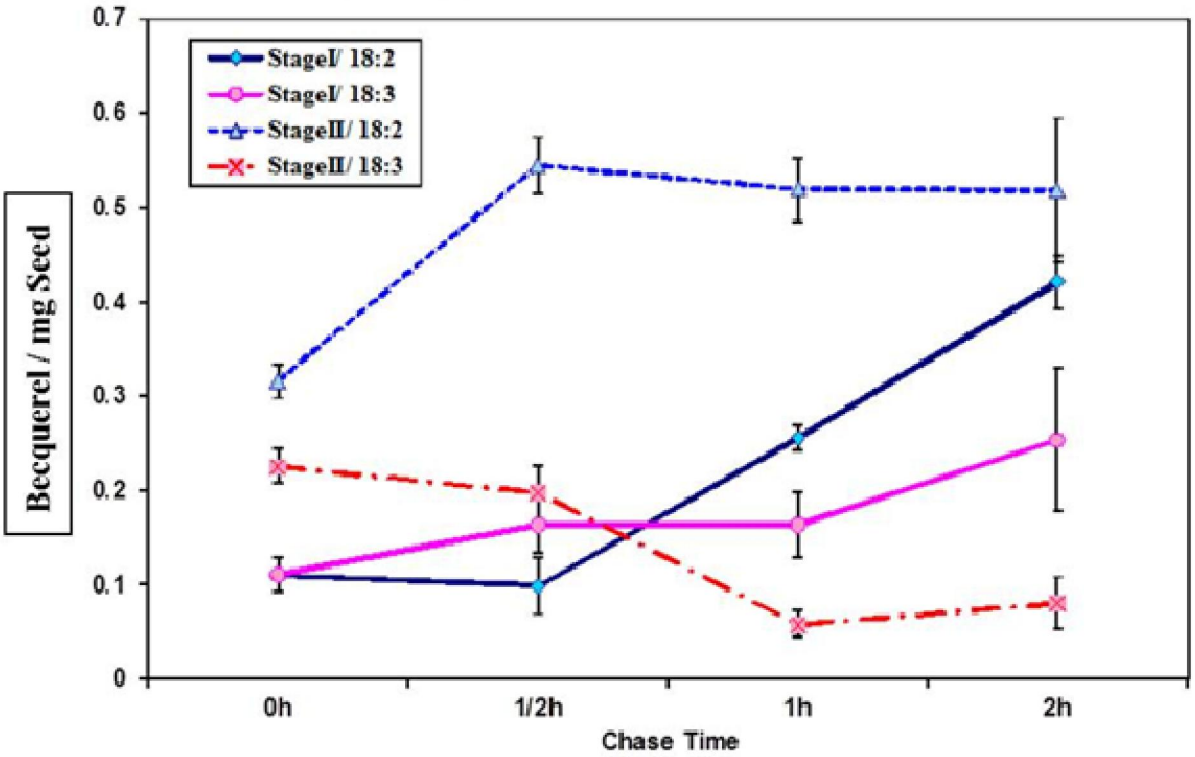


Figure 1(E): ^{14}C-18:2 incorporation in soybean free FA

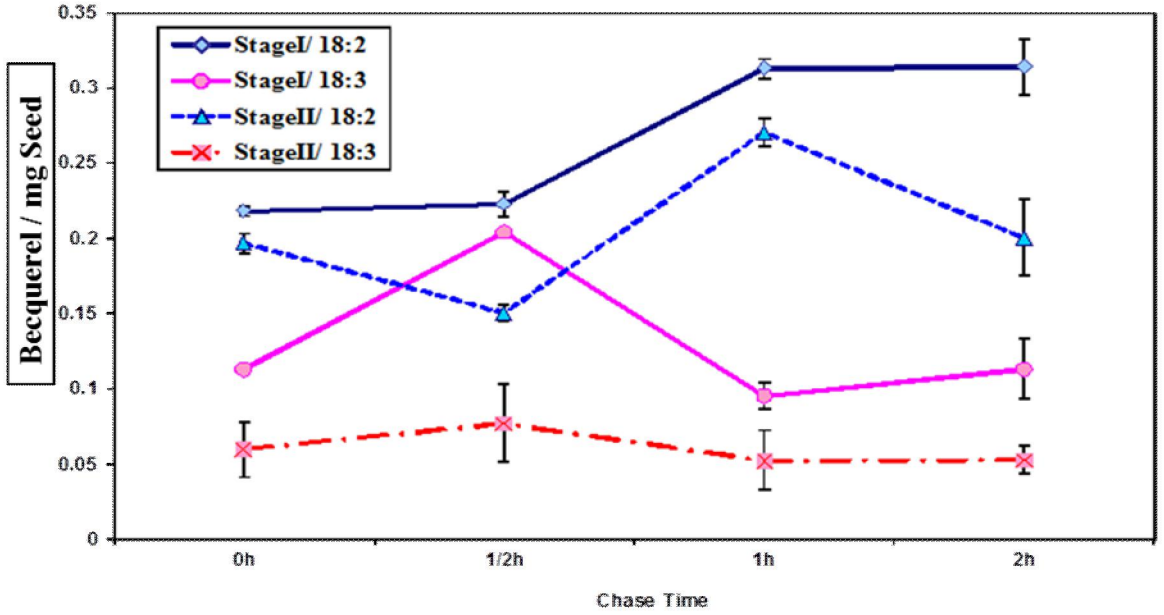
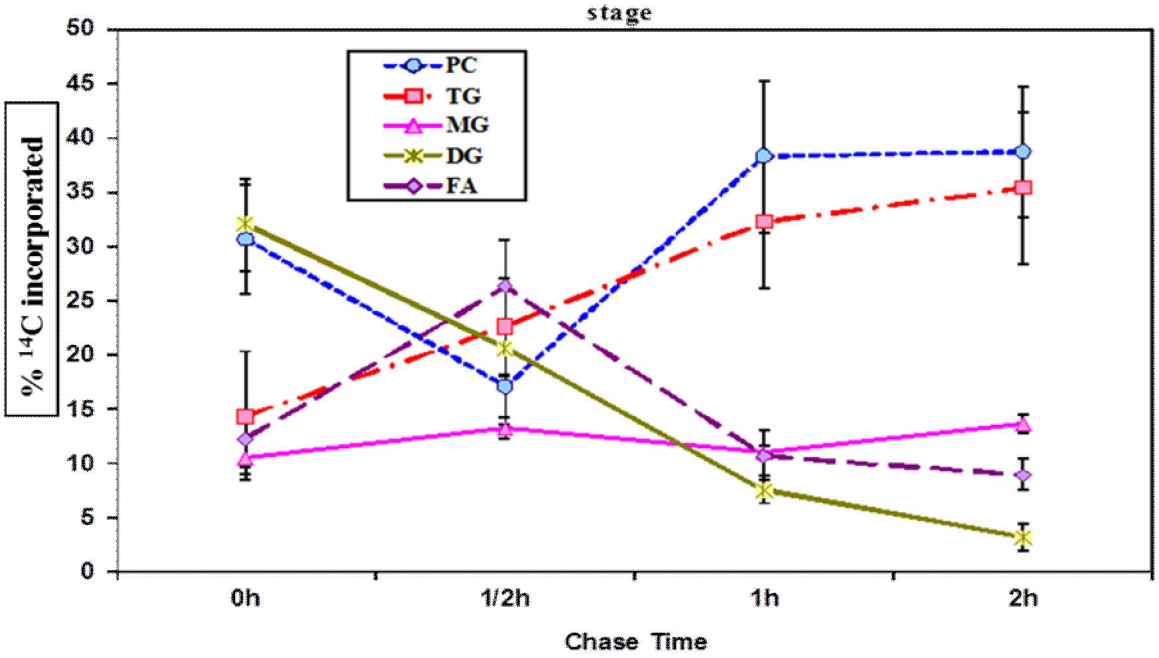


Figure 1: Soybean seeds from 2 different cotyledon developmental stages were fed ^{14}C -18: 2CoA for 90 minutes, the incorporation of ^{14}C into both 18:2 and 18:3 were chased in the different lipids fractions at 0h, 1/2h, 1h, and 2h. (A) ^{14}C incorporation in phosphatidyl choline, (B) ^{14}C incorporation in triacylglycerol, (C) ^{14}C incorporation in diacylglycerol, (D^{14}C incorporation in free fatty acids, and (E) ^{14}C incorporation in monoacylglycerol.

Figure 2 (A): % ^{14}C-18:2 incorporation in lipid fractions in soybean first stage



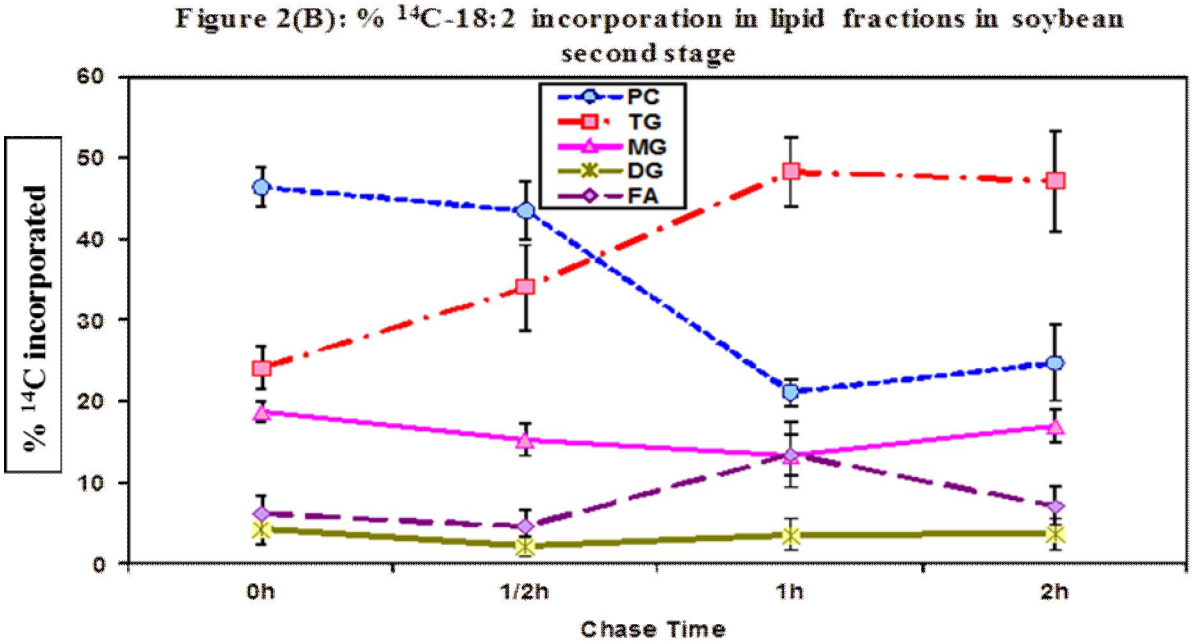


Figure 2: The percentage of ^{14}C incorporation in lipid fractions in soybean cotyledon at two different developmental stages, (A) First cotyledon developmental stage, (B) Second cotyledon developmental stage. Both stages were fed ^{14}C -18: 2 CoA for 90 minutes, the incorporation of ^{14}C into both 18:2 and 18:3 were chased in the different lipids fractions at 0h, 1/2h, 1h, and 2h

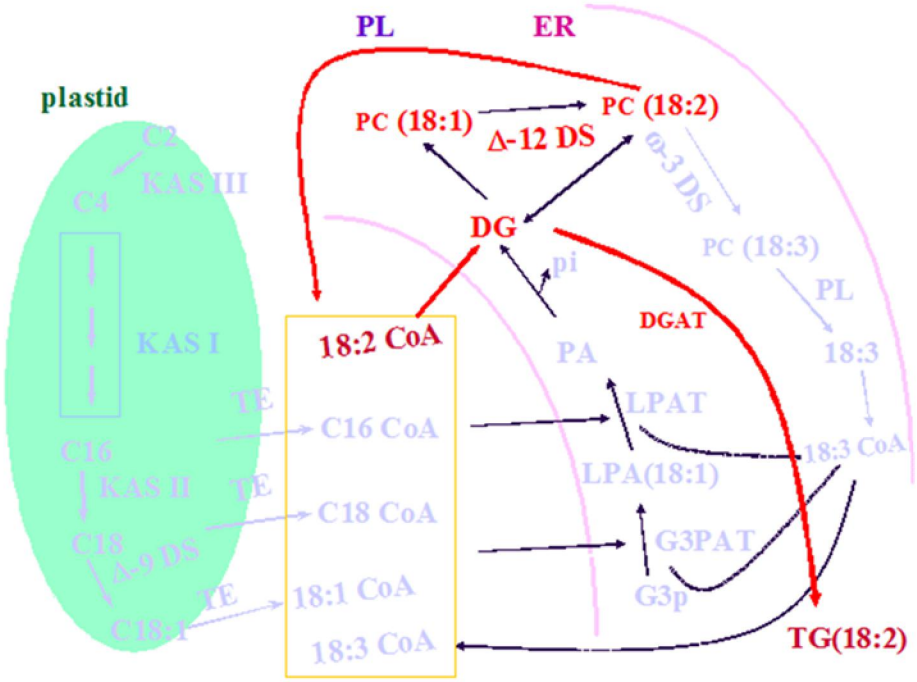


Figure 3: Suggested illustration for TG synthesis in soybean seeds. As ^{14}C -18:2 incorporation and its conversion into ^{14}C -18:3 were chased in different lipid classes; PC, TG, DG, MG, and FFA; in soybean seeds

This Figure is based on Kennedy pathway (Kennedy, 1961)

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