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LIPID BIOSYNTHESIS IN DEVELOPING KERNELS OF ALMOND (*PRUNUS AMYGDALUS*)

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Key Word Index—*Prunus amygdalus*, Rosaceae, kernel development, lipid biosynthesis, acetate incorporation, glucose, glycerol

Abstract—An active process of oil filling which was initiated at 50 DAF (days after fertilization) reached a maximum level at 70 DAF, as revealed by [1-^{14}C]acetate incorporation into lipids of developing almond kernels A several-fold incorporation into total lipids was recorded with [1-^{14}C]acetate, compared with [U-^{14}C]glucose Labelled glucose was incorporated mainly into the polar lipid fraction Glycolipids were more effectively synthesized with [U-^{14}C]glucose than with [1-^{14}C]acetate with a partial loss of label in phosphatidylinositol (PI) and diphosphatidylglycerol (DPG) fractions Addition of 0 5 mM glucose and glycerol, either singly or in combination, enhanced [1-^{14}C]acetate incorporation into lipids at 50 DAF while at 70 DAF these compounds decreased the label The possible role of cofactors like NADPH and/or ATP and glycerol originating from glucose has been discussed

INTRODUCTION

The *in vivo* flow of carbon from sucrose, the primary physiological precursor in plants, to the ultimate formation of triacylglycerols, which accumulates as oil bodies in the cytoplasm of the storage cells of oil-rich seeds, is not understood, although a large amount of work has already been done on oil seeds [1–3] Using acetate as the substrate much work has been reported on the lipid synthesizing capacity of the tissue both *in vivo* [4, 5] and *in vitro* [3, 6, 7] In a very few reports [8, 9], [^{14}C]glucose was used as a labelled precursor The choice of glucose as a precursor for such a study is dictated by three factors firstly, it is much closer to sucrose on the metabolic path than acetate and, therefore, should serve as a better precursor in delineating the pathway, secondly, it permits the channelling of hexose carbons into both the glycerol and fatty acid moieties of triacylglycerols, and thirdly, it can supply energy in the form of ATP and/or NADPH The role of the glycerol moiety in the biosynthesis of lipids has hitherto remained unexplained The present study has indicated that glucose serves as a potential source of energy in the form of ATP and/or NADPH, and the carbon skeleton as glycerol for the synthesis of lipids in the developing kernels of almond

RESULTS AND DISCUSSION

The kernels of almond were initially assayed for their capacity to incorporate [1-^{14}C]acetate into lipids at different stages of development (Fig 1) Up to 50 DAF, the ^{14}C-incorporation into total lipids was low, increased incorporation was observed thereafter, and the maximum value was attained at 70 DAF The oil content showed an accumulation in the almond kernel only after 60 DAF Furthermore, visual examination has revealed that the internal contents of the kernels, which were initially a gelatinous mass, started solidifying only after 60 DAF These observations indicated that the active period of lipid synthesis is initiated at 50 DAF with a maximum rate at 70 DAF Therefore, all subsequent experiments were

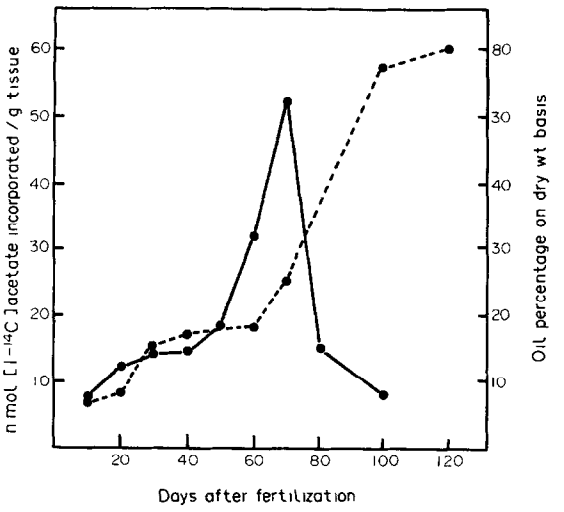

Fig 1 Incorporation of [1-^{14}C]acetate into lipids and the oil percentage of developing kernels of almond (*Prunus amygdalus*) Almond nuts were harvested on the cited days after fertilization The kernels were taken out and sliced Incubation medium contained 0 1 M potassium phosphate buffer, pH 7 4, 10 μCi (217 nmol) of [1-^{14}C]acetate and 1 g tissue in a final volume of 3 ml The conditions of incubation and extraction of lipids were as described in the Experimental A portion of tissue slices was extracted for total oil content Each value represents an average of two replicates with duplicate observations in each replication (●——●) Pattern of acetate incorporation, (●----●) oil percentage on a dry wt basis

conducted at 50 and 70 DAF as these stages represent the period of onset of rapid lipid synthesis and maximum lipid synthesizing capacity of the tissue, respectively

Uptake of radioactivity recorded with [1-^{14}C]acetate was several-fold higher than with [U-^{14}C]glucose (Table 1) This difference was obviously expected as the glucose is channelled into many more metabolic paths than acetate In our studies on developing sunflower seed [5] and in those on castor bean [8], it has been observed that nearly 50% of the ^{14}C-label found in the lipids was present in the fatty acid component The remaining label was assumed to be primarily incorporated into the glycerol of glycerolipids Gurr *et al* [9] reported that, in crambe seeds, labelled glucose was incorporated only into the glycerol, and not at all into the fatty acid moiety This would leave the source of acetyl-CoA for fatty acid biosynthesis in oilseeds as an open question Since the size of the glycerol-3-phosphate and acetyl-CoA pool at any one time has not so far been determined, quantitative interpretations on this aspect would be rather meaningless

Compared to acetate, labelled glucose was incorporated preferentially into polar lipids (Table 1), confirming that a part of the glucose must have been incorporated as glycerol into the carbon skeleton of lipids This was further indicated by the presence of larger amounts of radioactivity in the partial glycerides with labelled glucose than with acetate The incorporation of glucose into the lipids as glycerol has previously been reported in developing sunflower from our laboratory [5] and in *Crambe abyssinica* seeds by Gurr *et al* [9] The incorporation of [U-^{14}C]glycerol into glycerol-3-phosphate has been demonstrated by Gurr *et al* [9] who attributed this to the operation of the glycerol phosphate pathway in the seed tissue

Labelled glucose was more effectively incorporated into glycolipids, viz MGDG + SG, ESG and DGDG, than labelled acetate at both stages of development Glucose was possibly utilized via UDP-glucose into the glycosyl moiety of glycolipids In support of this view, Ongun and Mudd [17], who reported on the presence of an epimerase system in plants, observed that in spinach chloroplasts [^{14}C]glucose was incorporated via UDP-[^{14}C]galactose, at first into MGDG and, subsequently, into DGDG and steryl glycosides Furthermore, increased synthesis of glycolipids with labelled glucose at the cost of various

Table 1 Incorporation of [1-^{14}C]acetate and [U-^{14}C]glucose into total lipids and distribution of radioactivity into various lipid fractions of 50 and 70 day old kernels of almond (*Prunus amygdalus*) (values are means ± s e)

	50 DAF		70 DAF	
Incorporation into lipids	[1-^{14}C]Acetate	[U-^{14}C]Glucose	[1-^{14}C]Acetate	[U-^{14}C]Glucose
Total lipids				
nMol/g fr wt	269±08	24±02	396±05	43±01
% uptake of label in lipids	250±06	36±01	368±04	64±01
Polar lipids*	449±12	631±05	461±05	621±05
Non-polar lipids*	551±12	369±05	539±05	379±05
Non-polar fractions (% relative radioactivity)				
Triacylglycerols	353±04	167±03	363±02	214±07
Partial glycerides	64±02	216±06	39±02	152±06
Free fatty acids	27±01	54±03	41±01	53±05
Sterols + sterol esters	556±06	563±02	557±06	581±06
Polar fractions (% relative radioactivity)				
LP	09±01	09±01	12±01	12±01
PI	122±11	53±13	188±02	76±02
PC	143±09	138±17	236±10	218±08
PG + SL	80±07	61±01	64±01	73±07
PE	21±01	17±00	30±02	21±02
PA	16±05	16±05	16±01	09±03
DPG	588±20	453±03	452±05	479±01
Un	08±03	13±00	04±01	08±02
MGDB + SG	04±02	164±22	01±00	82±09
DGDG	06±01	14±01	01±00	07±01
ESG	03±01	63±12	05±01	19±02

Conditions of incubation as in the Experimental Figures are average of two replicates with duplicate observations in each replicate

* % relative radioactivity

LP, Lysophosphatides + phosphatidylserine, PI, phosphatidylinositol, PC, phosphatidylcholine, PG, phosphatidylglycerol, SL, sulpholipids, PE, phosphatidylethanolamine, PA, phosphatidic acid, DPG, diphosphatidylglycerol, Un, unknown lipids, MGDG, monogalactosyldiglyceride, SG, steryl glycosides, DGDG, digalactosyldiglyceride, ESG, esterified sterylglycosides

Table 2 Incorporation of [1-^{14}C]acetate into total lipids and various lipid fractions of developing kernels of almond in the presence of glucose and glycerol

Treatment	nMol/g fr wt	% uptake* of radioactivity into lipids	Relative % radioactivity		Relative % radioactivity in various polar lipids				
			Polar	Non-polar	PI	PC	DPG	Other phospholipids	Total glycolipids
50 DAF									
[1-^{14}C]acetate (control)	269±08	250	449±02	551±02	122±11	143±09	588±20	134±03	13±01
[1-^{14}C]acetate + glucose (0 5 mM)	297±04	276	472±06	528±05	116±10	155±07	596±07	124±02	08±01
[1-^{14}C]acetate + glycerol (0 5 mM)	340±12	316	461±08	539±07	120±02	202±11	538±16	133±04	08±01
[1-^{14}C]acetate + glucose (0 5 mM) + glycerol (0 5 mM)	339±16	314	413±06	587±06	74±05	162±18	587±27	169±04	09±01
70 DAF									
[1-^{14}C]acetate control	396±05	368	461±03	539±03	188±03	236±10	452±05	116±01	07±00
[1-^{14}C]acetate + glucose (0 5 mM)	313±29	292	475±08	525±06	138±08	179±07	555±24	118±03	09±01
[1-^{14}C]acetate + glycerol (0 5 mM)	364±24	340	436±02	564±03	110±10	153±20	615±20	116±03	07±00
[1-^{14}C]acetate + glucose (0 5 mM) + glycerol (0 5 mM)	271±14	252	476±02	524±03	145±06	170±02	515±17	149±04	11±01

Figures are the average of two replicates with duplicate observations in each replication Values are means ± s e

PI, Phosphatidylinositol, PC, phosphatidylcholine, DPG, diphosphatidylglycerol (cardiolipin), other phospholipids, all phospholipids as shown in Table 1, excluding PI, PC and DPG

*Indicates the percentage of labelled substrate incorporated into lipids out of total labelled substrate added in the reaction mixture

phospholipid moieties, mainly PI and DPG (cardiolipin) was observed at 50 DAF (Table 1) But for the lower incorporation into PI and DPG with labelled glucose, no marked difference between the incorporation of labelled acetate and glucose into other phospholipids was observed Several reasons can be ascribed to account for these differences (1) the contribution of [U-^{14}C]glucose for supplying inositol to be incorporated into PI may be restricted, (2) the inositol in some way may be utilized for the synthesis of various glycolipids, (3) decreased incorporation into DPG with labelled glucose than with acetate may result from the removal of phosphatides from DPG for the synthesis of galactolipids, and/or (4) there may be decreased availability of labelled glucose for both the components of triacylglycerols, i e fatty acids as well as glycerol

Addition of glucose (0 5 mM) and glycerol (0 5 mM) increased the incorporation of [1-^{14}C]acetate into lipids of almond kernels at 50 DAF (Table 2) At 70 DAF, both the metabolites resulted in decreasing the acetate incorporation At 50 DAF, the enhancement of radioactivity was greater with glycerol than with glucose Correspondingly, the decrease was less at 70 DAF with exogenous glycerol as compared to glucose at equimolar concentration The effect of using glucose in combination with glycerol was similar to the treatment where only glycerol was used at 50 DAF However, at 70 DAF, an additive decrease was recorded when both the metabolites, viz glucose and glycerol, were used together

The observed stimulation of lipid biosynthesis with glucose and glycerol could be due to a restricted supply of glucose, glycerol and other derived metabolites in the tissue during the early stages of development (50 DAF) In fact, exogenously supplied glucose and glycerol probably enhanced fatty acid biosynthesis, apart from diluting the label in the lipids at 50 DAF Glucose furnishes glycerol and acetate in the cell [18, 19] to provide a carbon skeleton of lipids, mainly triacylglycerols and NADPH and/or ATP, respectively, thus initiating a chain reaction to stimulate fatty acid biosynthesis and increase their level in the metabolic pool The presence of glycerol kinase in plant tissue [9] may enable exogenous glycerol to enter into the metabolic path leading to the synthesis of lipids, mainly triacylglycerols [20]

The observed decrease of label into total lipids at 70 DAF with added glucose and glycerol may suggest that the cofactors, which were limiting at 50 DAF or earlier, may no longer be so, and may in fact dilute the label during the later phase of optimal synthesis The excess ATP derived from exogenous glucose could inhibit phosphofructokinase, a control step of the glycolytic pathway, thus blocking the supply of glycerol-3-phosphate The finding that glycerol has relieved this inhibition partially at 70 DAF may provide indirect evidence for such a blockage at the level of phosphofructokinase It may be pointed out that the observed decrease of label, amounting to 30%, into lipids in the presence of both glucose and glycerol is quite dramatic though we have no definite explanation for it at present It may be worthwhile mentioning that dilution of radioactivity with glucose and glycerol has been taking place at both 50 and 70 DAF, although the enhancement of fatty acid synthesis at the former stage overcomes the effect of dilution The dilution of label at 70 DAF would be expected to be greater because of the larger content of oil in the seed It is further speculated that glycerol enters the glycolytic pathway to synthesize fatty acids through acetyl-CoA, thus providing a sparing action on acetate at later stages of kernel development Incorporation studies with [U-^{14}C]glycerol into fatty acids may be helpful in understanding the pathway from glycerol through acetyl-CoA to fatty acids

At 50 DAF, polar lipid synthesis was increased slightly by the addition of cold glucose and glycerol, possibly making available the precursors as discussed above, and was decreased, due to effect of dilution, when both metabolites were added together However, no such effect was recorded at 70 DAF It is interesting to note that DPG (cardiolipin), which acts as a temporary reservoir of phosphatidyl moieties [21], constitutes the major fraction of polar lipids synthesized at these stages The addition of glucose and glycerol did not significantly influence ^{14}C-incorporation into DPG at 50 DAF, while the amount of label in DPG was increased by reducing the PC and PI fractions at 70 DAF Since the label in DPG with respect to PC and PI appears interdependent, the present data do not allow us to distinguish whether exogenous glucose or glycerol decrease the synthesis of PC and PI or dilute the label in these phospholipid moieties

The absence of any significant effect of exogenous glucose and glycerol on the glycolipid content was most likely due to their lower rate of synthesis in the seed as compared to neutral and phospholipids

EXPERIMENTAL

Materials Almond (*Prunus amygdalus*) trees of H-15 cultivar, 5 years of age, with normal health and dense foliage were selected from the new orchard of the Department of Horticulture, Punjab Agricultural University, Ludhiana (India) [1-^{14}C]Acetate (sp act 46 15 mCi/mmol) and D-[U-^{14}C]glucose (sp act 74 mCi/mmol) were obtained from the Isotope Division of BARC, Bombay (India)

Pollination The flowers being self-incompatible, were pollinated by taking pollen from tree No 29 grown in the almond collection and known for being compatible with the H-15 cultivar Pollination was carried out on a sunny day when the day temp recorded 25–30° in February–March Twigs of the pollinated flowers were tagged to ascertain the exact age of the kernel The unopened and the fertilized flowers were removed on the day of pollination to retain homogeneity in the tissue sampling Fertilization of the ovule takes place within 48 hr and the period of 2 days after tagging was termed as zero days after fertilization (DAF)

Incorporation studies The fruit (nuts) was collected in the morning on appropriate days in polythene bags in an ice box and immediately brought to the laboratory for incubation on the same day The nuts were opened and the kernels cut into slices of 0 2–0 3 mm thickness against the cotyledonary divide with a scalpel Batches of 1 g of tissue slices were incubated in 0 1 M KPi buffer, pH 7 4 and 5 μCi [1-^{14}C]NaOAc or [U-^{14}C]glucose in a final vol of 2 ml (expt 1) while in other case cold glucose and/or glycerol (0 5 mM) was added to the reaction mixture containing 5 μCi [1-^{14}C]NaOAc (expt 2) The reaction was run at 25° for 6 hr with continuous shaking in a SEW laboratory shaking incubator (Dubnoff type) The reaction was stopped by removing the tissue and transferring to $CHCl_3$–MeOH (2 1) for lipid extraction [10]

Lipid extraction and analysis The tissue was homogenized in $CHCl_3$–MeOH (2 1) and filtered through sintered funnel grade 3 The H_2O soluble impurities from the filtrate were removed by using 20% of the vol of 0 9% NaCl soln The $CHCl_3$–MeOH phase was subsequently evaporated at low temp to near dryness

and the residue was again dissolved in 10 ml of Analar grade $CHCl_3$ A suitable aliquot of total lipid extract was taken into a scintillation vial and the solvent evaporated at low temp To the lipid residue in the vial was added 10 ml of PPO–POPOP–toluene scintillation soln [4 g PPO (2,5-diphenyl oxazole) and 200 mg POPOP (1,4-bis-2,5-phenyloxazolyl benzene] dissolved in 1 l of toluene) The radioactivity was measured using a liquid scintillation spectrometer

For determining radioactivity in different lipid classes, polar and non-polar lipids were separated by solvent partition method [11], followed by TLC using Si gel G as the adsorbent For the separation of various non-polar lipids, the TLC plates were developed, after having applied the sample as a fine spot, in a solvent system containing petrol–Et_2O–HOAc (80 20 1) by ascending chromatography [12] The polar lipids were separated by 2D-TLC using $CHCl_3$–MeOH–H_2O (65 25 4) in the first direction [13] and MeCHO–HOAc–H_2O (100 2 1) in the second direction [14] The spots were visualized with I_2 vapour and marked Identification of individual lipid components was achieved using different spray reagents and by comparison of R_f values with those of standard compounds [15] After removing the I_2 from the oven at 40°, the Si gel from each zone containing the adsorbed lipid was scraped off separately into a scintillation vial The scintillation fluid (10 ml) containing 0 4% PPO and 0 02% POPOP in toluene for non-polar lipids and fluid used by Bray [16] for polar lipids was added to each vial The contents of the vials were thoroughly mixed and counted in a liquid scintillation spectrometer as above

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