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Protein Profiling during Mesocarp Development in Oil Palm Fruit

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ABSTRACT

This study was aimed at investigating the overall protein profiles of oil palm fruit during the mesocarp tissue development by means of isoelectric focusing (IEF) and two-dimensional gel electrophoresis (2-DE). Total protein was extracted from different stages of fruit development (namely, 5, 12, 15, 17, and 20 weeks after anthesis [WAA]) from *Elaeis guineensis* Jacq. Tenera and *E. oleifera* (17 WAA). The IEF separation was carried out on pH values ranging from 4.0-8.0. Changes in the patterns of protein after IEF were observed during mesocarp development and between the two species. The analysis of oil palm mesocarp gave rise to a protein map, comprising approximately 150 spots that were detectable by silver staining following high resolution 2-DE, with a pH range of 4.5-8.0 and a mass range of 8-100 kDa. Meanwhile, twenty five spots of protein showing variations in their intensity during the development of the mesocarp, with their p*I* ranging from 4.5-7.8 and M*r* 20-85 kDa, were analyzed. Continuous but non-uniform disappearance of some proteins and formation of new proteins were observed at the early stages of mesocarp development and during certain periods of oil synthesis and fruit ripening. The results of this study indicate that developing mesocarp revealed significant changes in the protein profiles during fruit development. However, further studies are still required to identify the proteins that are differentially expressed during fruit development.

Keywords: Two-dimensional gel electrophoresis (2-DE), mesocarp, *Elaeis guineensis, Elaeis oleifera*, oil palm

ABBREVIATIONS

WAA	:	Week after anthesis
IEF	:	Isoelectric focusing
SDS-PAGE	:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

INTRODUCTION

Palm oil is the second largest source of edible oil in the world and it has been recognized or accepted as one of the most usable consumer oil compared to other plant oils (Basiron, 2007; Basri *et al.*, 2005). In oil palm, there are two important species in the genus *Elaeis*, *E. guineensis* that has its centre of origin in Africa and the South American species, *E. oleifera.* During the fruit development in oil palm, oil

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synthesizes and accumulates predominantly in the mesocarp tissue. Although the exact time of the beginning of oil accumulation in palms is still uncertain, published results have suggested that the period of active oil synthesis starts at 12 weeks after anthesis (WAA), and the period of active oil synthesis is usually around 15 WAA and it ends when the fruit ripen at about 20 WAA (Aziz *et al.*, 1986; Oo *et al.*, 1986). In the mesocarp, the level of unsaturated oil in *E. oleifera* is 56%, whereas this is around 39% in *E. guineensis* Jacq. (Tenera) oil. The iodine value is also much higher in *E. oleifera.* However, the oil yield of pure *E. oleifera* is much lower, with oil to bunch ratio of 5%, as compared to the *E. guineensis* (Tenera) with oil to bunch ratio of more than 25% (Rajanaidu *et al.*, 1997; Rajanaidu *et al.*, 2000). It is likely that in the oil palm mesocarp, certain enzymes involved in fatty acid biosynthesis are present or abundantly present during the oil synthesis period. Regulatory proteins which are involved in switching on or increasing the level of expression of the genes coding for these enzymes may be present at the start or just before the period of active oil synthesis. Meanwhile, the variation in the gene expression has been shown by analyzing proteins synthesized *in vitro* from translated mRNA of mesocarp *E. guineensis* at different stages of oil synthesis (Abdullah *et al*., 1994; Cha and Shah, 2005). The results showed the presence of two proteins (namely molecular weight 68 kDa and 32 kDa) in greater abundance than the rest during 15 WAA and 20 WAA, respectively (Oo *et al.*, 1986). Budiani *et al.* (2002) reported that the expression of the two proteins with M*r* 31.0 kDa and M*r* 34.3 kDa increased sharply at the beginning and just before the period of active oil biosynthesis, respectively. Another study using the SDS-PAGE analysis of total proteins (Shah, unpublished results) showed that proteins of different sizes (namely, M*r* 68, 42, 37, 34, and 32 kDa) were differentially expressed during the development of mesocarp in *E. guineensis* (Tenera), while a protein with M*r* 29 kDa was expressed in *E. oleifera*, but not in *E. guineensis* (Tenera).

In this study, the two-dimensional gel electrophoresis (2-DE) was utilized to give a better resolution in the separation of the total protein during the mesocarp development in *E. guineensis* (Tenera), and it was also used to detect different proteins expressed in the mesocarp of *E. oleifera* and *E. guineensis* (Tenera) during active oil synthesis, as this might suggest a differential gene expression of oil synthesis between the two species. Thus, the present study provides an overview of the main oil palm *E. guineensis* and *E. oleifera* proteome variations during the precise stages of mesocarp development (oil synthesis) and ripening. The protein profile, using 2-DE, shall be helpful in understanding the biochemical and the molecular changes at different stages of development of oil palm fruit and during the oil synthesis.

MATERIALS AND METHODS

Plant Material

Oil palm of two species (*Elaeis oleifera*) and (*Elaeis guineensis,* Jacq.) of Tenera type inflorescences were tagged at anthesis and fresh fruit bunches were collected at different stages of development (5, 12, 15, 17 and 20 WAA). After the collection, the exocarp and kernel were removed from the fruits. The remaining mesocarp tissues were immediately frozen in liquid nitrogen upon collection and then stored at -80°C until further use.

Protein Extraction

Proteins were extracted essentially following the method proposed by Des Francs *et al.* (1985) with slight modifications. Frozen mesocarp from different WAA were homogenized in liquid nitrogen with a mortar and pestle, and the powder was resuspended in an extraction buffer (300 mM NaCl, 1 mM EDTA, 2% ampholyte pH 3.5-10 and 5-7 in the ratio of 1:4 and 10 µg ml^{-1} leupeptin), at the ratio of 1 to 2 (v/w). This mixture was incubated at room temperature (~ 27°C) for 15 min and then centrifuged at 13 000 × *g* for 5 min. Solid urea was added to

the supernatant to a final concentration of 9 M. Protein concentrations were estimated using the method proposed by Bradford (1976).

First-dimensional Gel Electrophoretic Analysis

Polyacrylamide-gel isoelectric focusing (IEF) analysis was performed as described by O'Farrell (1975). The IEF was carried out in 100 mm × 5 mm (ID) cylindrical tubes on a stand-alone casting device. The components of the gel tubes were 9 M urea, 2% ampholines Bio-lyte, 4% N-P40, 3.2% T, and 2.5% C acrylamide. The IEF linear pH gradient (about 4.0 to 8.0) was performed in the rod gels, with a 2% (v/v) mixture of Bio-lyte Ampholines of pH 5-7 and 3.5-10 (Bio-Rad, Richmond, Calif.) at a proportion of 4:1. When the polymerization of IEF gels was completed, 80 µg of protein sample in a volume up to 100 µl were loaded onto separate pre-focused tube gel. The first dimensional separation was performed at a constant voltage of 400V for 12 hr and of 800V for the final hour.

Coomassie Blue Staining

Gels were extruded from cylindrical tubes and placed in large test tubes containing a fixing solution (4% sulfosalicyclic acid, 12% trichloroacetic acid) and shaken gently for several hours, after which the fixative was poured off and replaced with staining solution (0.04% Coomassie Blue R-250, 0.5% $CuSO_4$, 27% isopropanol, and 10% acetic acid) and shaken gently over-night. Excess dye was removed with destaining I solution (12% isopropanol, 7% acetic acid, 0.5% $CuSO_4$). The gel was then incubated in destaining II solution (7% acetic acid, 5% methanol). After staining with Coomassie Blue, the total protein was expressed at each individual WAA in *E. guineensis* and at 17 WAA in *E. oleifera*.

2D-PAGE

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with IEF in the first dimension was performed using SE-600 system (Hoefer, CA, USA) in accordance with the laboratory manual adapted by Anderson (1991) with minor modifications. Briefly, the first-dimension, isoelectric focusing (IEF), based on the method of O'Farrell *et al.* (1977), was carried out as above, except that 160 mm × 1.5 mm rod gels were used. The samples containing 30 µg of protein in 25 µl were loaded after pre-focusing the gels for 1 hr at 400V. Electrophoresis was carried out at 400V for 12 hr followed by 1 hr at 800V. After the IEF, the gel rods were extracted and then equilibrated for 30 min in sodium dodecyl sulphate (SDS) sample buffer (Laemmli, 1970), and were either stored at –70°C or immediately loaded onto second-dimension SDS-PAGE. The second-dimension was performed in a vertical-slab gel electrophoresis (16 × 18 cm) using a 12% acrylamide resolving gel, without stacking gel, run at 30 mA per gel for about 4 hr. A high-molecular-mass marker (Pharmacia Biotech) that produced bands at 97, 66, 45, 30, 20.1, and 14.4 kDa were used. After the electrophoresis, the analytical gels were fixed overnight in ethanol:acetic acid:water solution (5:1:4, by vol.). The protein profiles were visualized by silver staining method according to Oakley *et al*. (1980), and modified by Hochstrasser *et al*. (1988). The experiments were performed in duplicate, and the representative gels were shown.

Gel Drying and Analysis

Fresh gels were soaked in 3% glycerol for 30 min and then placed between 2 dry sheets (Gel drying film, Promega) over-night. The dried gels were scanned with a GS-800 calibrated densitometer (Bio-Rad) and the gel images were analyzed using PDQuest 2-D software quantification (Bio-Rad). The gels were standardized by calculating the intensity of each spot as the percentage of the total intensity of the spots visualized on a gel, after which the differences of expression (induction or repression) of the spots between gels were statistically meaningful according to the PDQuest software.

RESULTS AND DISCUSSION

Protein Patterns in IEF during Mesocarp Development

Comparison of the IEF profiles of proteins during the development of mesocarp showed changes in the patterns of the total protein accumulated. The results of the IEF of proteins, extracted from mesocarp at different WAA from *E. guineensis* (Tenera) (5, 12, 15, 17 and 20) and at 17 WAA for *E. oleifera*, are shown in *Fig. 1*. The findings obtained indicate that the variations in the intensity of the expression patterns in the same p*I* region are due either to the synthesis or degradation of protein(s). However, at different p*I* regions, the variations in the intensity during the development of mesocarp in *E. guineensis* (Tenera) were observed. For example, a group of acidic proteins with *p*I ranging between 5.0 and 6.1 were abundantly expressed at 5, 12 and 15 WAA, while the expression level for these proteins at the different weeks remained unaffected. Four proteins, namely b, c, f and h (*Fig. 1*) with p*I* values of 4.5, 5.0, 6.5 and 6.8, respectively, were found to be expressed only at 12 WAA. A similar observation was made for Protein I (p*I* 6.9) but this occurred at 15 WAA. Two proteins, namely e and g (with p*I* values of 6.82 and 7.85, respectively), were only expressed in 15 and 17 WAA, while Protein g was observed to be expressed at 20 WAA. Protein j (p*I* 7.1) was expressed at a higher level at 15 WAA compared to the other weeks of development. Three major proteins with p*I* values of 6.9, 7.2 and 7.5 were highly expressed only at 17 WAA in *E. oleifera*. For a better resolution, these proteins were then separated by 2-DE.

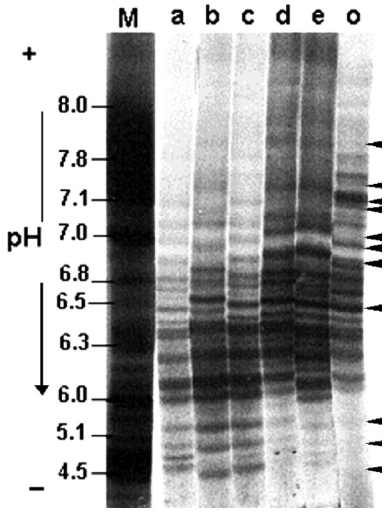

Fig. 1: IEF of the total proteins during the development of mesocarp. IEF was performed in pH 4.0-8.0 gradient using Ampholines mixture pH 3.5-10/5-7 in ratio of 4:1. A 80 µg protein loading was used for this purpose. Rod gels were stained with Coomassie Blue. Lanes a, b, c, d, e and o indicate 5, 12, 15, 17 and 20 WAA of E. guineensis *(Tenera) and 17 WAA of* E. oliefera*, respectively. Meanwhile, Lane M contained the IEF markers. Arrows show the observed variation of the proteins of different pI*

The earlier results by Abdullah *et al*. (1994), with the use of PAGE of *in vitro* translation products of mRNA, indicated that proteins with M*r* of 68 and 32 kDa had showed differential expression during the development of the oil palm mesocarp. The protein with M*r* 68 kDa molecular weight was observed at the start of oil synthesis, with the highest level seen at 15 WAA (Abdullah *et al.*, 1994). Extending this further, the researchers investigated the differential synthesis of total proteins during mesocarp and kernel development in the oil palm *E. guineensis* (Tenera) using the one-dimensional gel electrophoresis (Shah, unpublished data). Electrophoresis indicates the differences in the levels of proteins, as shown by the presence or absence of bands or the presence of bands with different intensities, showing that different proteins are synthesized at different week of mesocarp development corresponding to the different stages of oil synthesis. These results prompted the researchers to investigate the proteins using 2D-PAGE to give a higher resolution of the proteins of interest.

2-DE of Protein Analysis

In this study, the 2-DE patterns of the total and newly synthesized proteins during the development of mesocarp were also analyzed

at different weeks after anthesis. *Fig. 2* (*A, B, C, D, E*) show the overall patterns of the protein expression at the different stages of mesocarp development of *E. guineensis* (Tenera). Several proteins with molecular masses between 25 to 90 kDa showed changes in the level of expression, synthesized or disappeared during the five selected week of development.

The gels were divided into two squares (I) and (II) (4 × 2.5 cm) and amplified by computer scanning for mapping the total protein in the range of M*r* 25-90 kDa (*Fig. 3*). As observed, there were significant protein changes during mesocarp development in the Tenera. Meanwhile, twenty-five proteins showed variations during the development (*Figs. 3a, b, c, d, e*). The major protein changes noted during the development included the disappearance of some proteins, synthesis of new proteins, or an enhanced synthesis of preexisting protein (Table 1). For example, three proteins (1, 2, and 3) of M*r* 60, p*I* 6.0: M*r* 47, p*I* .6.0 and M*r* 47, p*I*

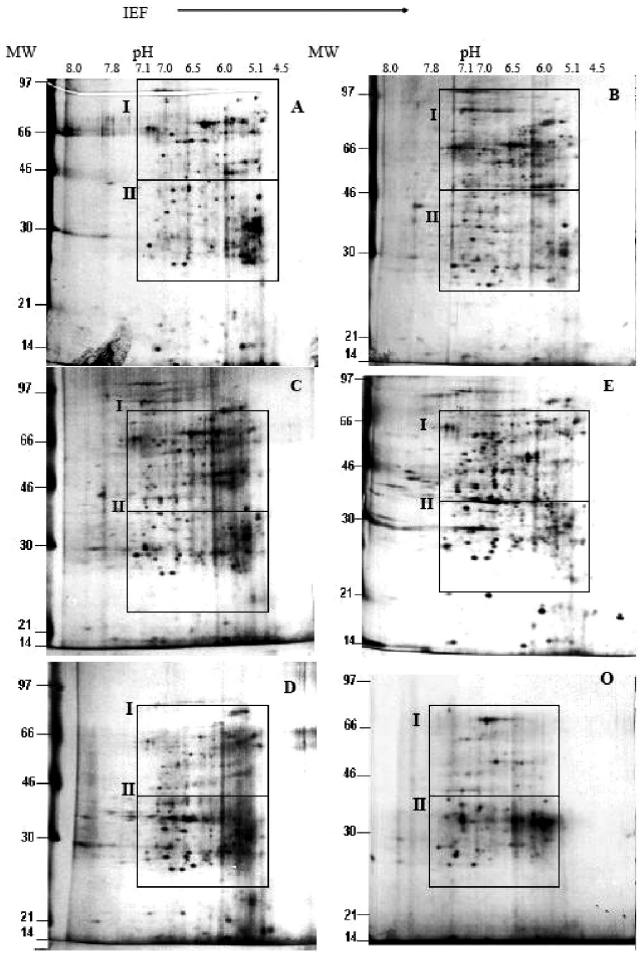

Fig. 2: Silver-stained 2-D gels of the total and newly synthesized proteins during mesocarp development. 2-DE patterns in E. guineensis *(Tenera) (A, 5 WAA; B, 12 WAA; C, 15 WAA; D, 20 WAA; E, 17 WAA) and 17 WAA for* E. oliefera *(O). The IEF was performed in pH 4.0-8.0 gradient. Mr standards are indicated for the analysis of gels which were divided into two squares I and II showing the variation in the protein patterns*

6.1 were observed only at 5 WAA, and were absent during the other stages of development. Four proteins (5, 6, 7 and 24) of M*r* 66, p*I* 7.3: M*r* 65, p*I* 7.0: M*r* 65, p*I* 7.1 and M*r* 28, p*I* 7.1, respectively, were highly expressed at 12 and 15 WAA, but were not detected at 17 WAA and they were found to be lower in their amounts at 5 and 20 WAA. One protein (4) of M*r* 71, p*I* 6.5 was abundantly present at 5, 12, 15 WAA, but it was not detected at 17 and 20 WAA. Three proteins (8, 11, 23) of M*r* 45, p*I* 6.2, M*r* 36, p*I* 5.1, and M*r* 29, p*I* 7.0 were observed to appear only at 12 WAA and were highly expressed at 15 WAA. These proteins were found to be either absent or present at low concentrations, at 17 and 20 WAA. Three other proteins (15, 16, 17) of

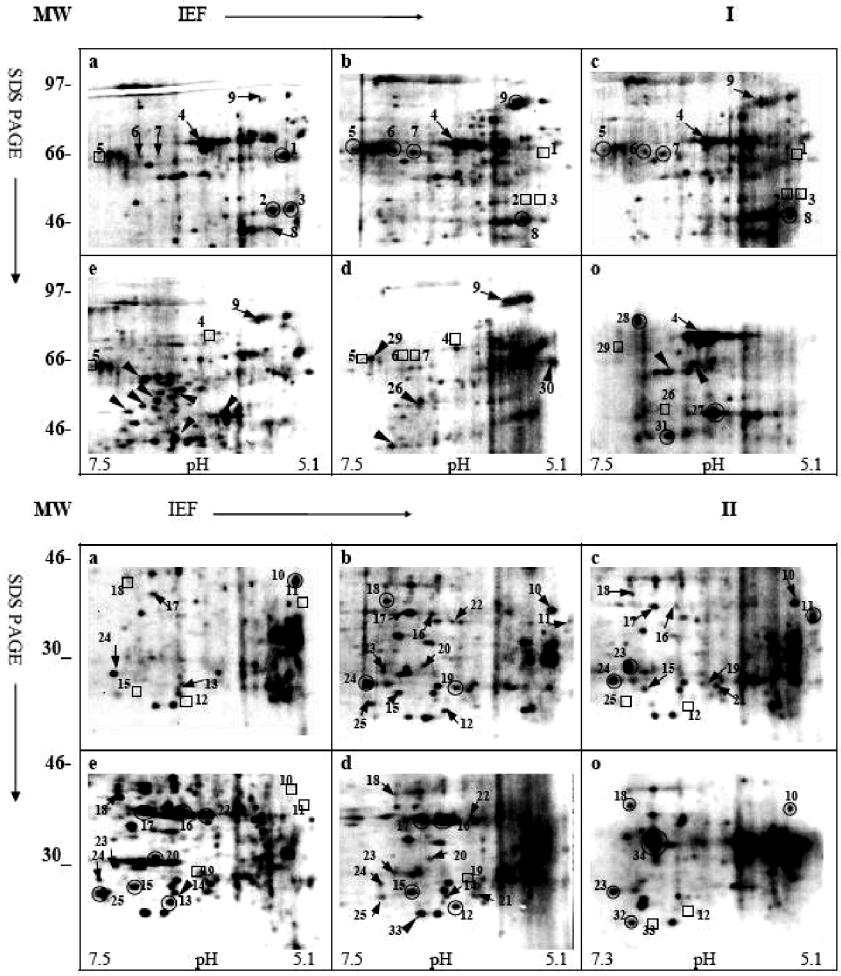

Fig. 3: Amplified 2-DE patterns of Squire I and II. The figure shows the locations of proteins in Square I and II from Fig. 2. The spots that disappeared or appeared (newly synthesized) in high amounts during the fruit development are indicated by the squares and circles, respectively. Arrows (►) point to the presence with enhanced expression during the fruit development protein; Arrow heads (—►) points to the proteins expressed during the fruit ripening stage. For E. oleifera *and* E. guineensis *(Tenera) at 17 WAA, the presence of highly expressed proteins is indicated by the arrowheads followed by the numbers and open circles, respectively*

TABLE 1
Protein changes during the development of mesocarp in *E. guineensis* (Tenera)

Protein	P*I*	Mr	5 WAA	12WAA	15WAA	17WAA	20WAA
1	6.0	60	H	N	N	N	N
2	6.1	47	H	N	N	N	N
3	6.0	47	H	N	N	N	N
4	6.5	71	H	H	H	N	N
5	7.3	66	N	H	H	N	P
6	7.0	65	L	H	H	N	N
7	6.9	65	L	H	H	N	N
8	6.2	45	N	P	H	N	N
9	6.2	82	L	H	P	P	P
10	5.6	38	H	P	P	N	N
11	5.1	36	N	L	H	N	N
12	6.6	26	N	P	N	H	N
13	6.7	26	P	N	N	N	H
14	6.7	27	N	N	N	L	H
15	6.8	27	N	P	P	H	H
16	6.7	37	N	P	L	H	H
17	7.0	37	L	P	P	H	H
18	7.2	38	L	H	P	L	P
19	6.6	28	L	H	L	N	N
20	6.9	32	N	L	N	P	H
21	6.4	27	N	N	P	P	L
22	6.5	37	N	P	N	P	H
23	7.0	29	N	L	H	L	N
24	7.1	28	P	H	H	L	L
25	7.1	27	N	L	N	L	H

Refer to *Fig. 2*, *Fig. 3* (I) and *3* (II) for the location of the different proteins on the two-dimensional protein map. M*r* data are the means of two gels. Protein intensity levels are expressed as H; high expression L; low expression P; present N; not present. Data were obtained through computer scanning of photos of the dried 2-DE gels at different stages during fruit development.

M*r* 27, p*I* 7.0, M*r* 37, p*I* 6.7, as well as M*r* 38, p*I* 7.2 at 12 and 15 WAA continued to be highly expressed even at 17 and 20 WAA. Meanwhile, two proteins (18, 19) of M*r* 37, p*I* 7.0, and M*r* 28, p*I* 6.6 were present in the low level at 5 WAA initially and were also abundantly present in high expression at 12 WAA, which later declined to undetectable levels at 15, 17, and 20 WAA. On the other hand, the protein (9) of M*r* 82, p*I* 6.2 was shown to be highly expressed at 12 WAA, and it continued to be present until 20 WAA. Three proteins (20, 22, 25) M*r* 32, p*I* 6.9, M*r* 37, p*I* 6.5, and M*r* 27, p*I* 7.1 were present at 12 WAA but absent at 15 WAA, which were expressed at 17 WAA and shown to be highly expressed at 20 WAA. The only protein that was expressed at the ripening stage (20 WAA) was protein 13 with a M*r* 26 and p*I* of 6.7.

A comparison of the 2-D protein patterns of *E. oleifera* and *E. guineensis* (Tenera) at 17 WAA was done by analyzing silver staining (*Fig. 2*) (O and D), respectively. The major differences between the two species, as shown in *Fig. 3* (I) and (II) (O and D), are tabulated in Table 2. Four highly expressed proteins (4, 27, 28, 32) of Mr 71, p*I* 6.5, M*r* 50, p*I* 6.4, M*r* 75, p*I* 7.0, and M*r* 25, p*I* 6.9, and one protein (10) of M*r* 38, p*I* 5.6 were found to be present in *E. oleifera* but not in *E. guineenses* (Tenera). Meanwhile, three proteins (18, 23, 31) of M*r* 38, p*I* 7.2, M*r* 29, p*I* 7.0, and M*r* 45, p*I* 6.8 were highly abundant in *E. oleifera* but these were found to be less in

TABLE 2
Comparison between the 2-D pattern of total proteins from 17 WAA of *E. guineensis* (Tenera) and *E. oleifera* mesocarp

Protein	P*I*	Mr	17 WAA *Oleifera*	17 WAA Tenara
4	6.5	71	H	N
9	6.2	82	N	H
10	5.6	38	P	N
12	6.6	26	N	H
18	7.2	38	H	L
23	7.0	29	H	L
26	6.8	53	N	P
27	6.4	50	H	N
28	7.0	75	H	N
29	7.2	66	N	P
30	5.5	64	N	H
31	6.8	45	H	L
32	6.9	25	H	N
33	6.8	25	N	H
34	6.8	37	H	P

Refer to *Fig. 2*, *Fig. 3* (I) and *3* (II), D and O, for the location of the different proteins on the two-dimensional protein map. M*r* data are the means of two gels. Protein intensity levels are expressed as H; high expression L; low expression P; present N; not present. Data were obtained through computer scanning of photos of the dried 2-D gels from active oil synthesis 17 week *E. guineensis* (Tenera) and *E. oleifera.*

E. guineenses (Tenera). Similarly, four highly abundant proteins (9, 12, 30, 33) of M*r* 82, p*I* 6.2, M*r* 26, p*I* 6.6, M*r* 64, p*I* 5.5, and M*r* 25, p*I* 6.8, and two proteins (26, 29) of M*r* 53, p*I* 6.8, and M*r* 66, p*I* 7.2, were present in *E. guineensis* (Tenera), but they were not found in *E. oleifera*.

The analysis by 2-DE confirmed the differential expression of the total proteins observed in the IEF during the mesocarp development and between *E. guineensis* and *E. oleifera* at the week of active oil synthesis (17 WAA). The changes in the lipid class and fatty acid compositions during the development of oil palm mesocarp and the variation in the lipid metabolism between *E. guineensis* and *E. oleifera* were reported by Oo *et al.* (1985) and Sambanthamurthi *et al.* (1987), respectively. Therefore, the differences observed between the total protein patterns during the period of active oil synthesis (17 WAA) may suggest the possibility of a different regulatory mechanism of oil synthesis between the two species. A previous work on the differential gene expression in different tissues and species, using mRNA differential display, revealed that one of the mesocarp-specific genes was specific for *E. oleifera* (Shah and Cha, 2000). Meanwhile, sequence homology showed that it codes for enzyme sesquiterpene synthase, which has a molecular weight ranging from 68-72 kDa. Therefore, it would be very interesting to analyze, in future studies, the protein obtained in this study from *E. oleifera* with molecular weight of 75 kDa, whether it could be similar to the protein observed in the other work or being involved in oil synthesis.

Development is characterized by the differential synthesis of gene products (transcripts) in time and space; few plant genes whose expression directly influences development have been identified. While these transcripts are produced in response to developmental signals (Davies and Robinson 2000), some proteins themselves associated with fruit maturity and in the development process (Abdi *et al*., 2002; Barraclough *et al.*,

2004). In the recent years, proteome analysis has been successfully applied to a range of plant tissues, such as grape skin ripening (Deytieux *et al.*, 2007), rip grape mesocarp (Sarry *et al.*, 2004), germinating tomato seeds (Sheoran *et al.*, 2005), and leaves, shoots, and roots of grapevine plantlets (Castro *et al.*, 2005). Enlargement of mesocarpic cells and differentiation of endocarp may not require very marked qualitative or quantitative changes at the molecular level. The former process, which is responsible for rapid increase of size and weight of the oil palm, may not involve the production of a different set of proteins but a more rapid synthesis of the existing ones. Meanwhile, developmentally regulated genes can be either codes for proteins with regulatory function or codes for proteins involved in tissue-specific function (Gasser *et al.*, 1988). A previous report on N-terminal amino acid sequences of proteins expressed during the period of oil synthesis in mesocarp tissues with p*I* range between 4.5 and 4.95 revealed a similarity to acetyl-CoA carboxylase (ACCase), enoyl-ACP reductase, and glyceraldehyde-3-phosphate dehydrogenase that are involved in oil biosynthesis (Budiani *et al.*, 2002; Harwood and Page, 1994). Therefore, proteins which were synthesized during or just before the active oil synthesis [12 WAA] might play significant roles in oil synthesis or regulatory mechanisms involved in oil synthesis. However, this would have to await further analysis on the eluted proteins. From the eluted proteins, DNA probes shall be designed according to the information from the amino acid sequence. The genes coding of the proteins of interest could be isolated and characterized in order to find out whether they have any important regulatory function/s or whether they code for functionally important proteins in oil synthesis.

CONCLUSIONS

To date, apart from the work published by Shah and Cha (2000), no other work has been done to investigate the production of different metabolites during mesocarp development and oil production in oil palm. The present study provides basic information on proteins map using 2-DE which is of utmost importance for an understanding of molecular and biochemical changes that happen at different stages of oil palm fruit development. The electrophoretic patterns/spots, obtained through the IEF and 2-DE of the developing mesocarp of *E. guineensis* proteins showed the variations in their intensity during precise stages of mesocarp development (oil synthesis) and ripening stage. Meanwhile, the disappearance of protein patterns/spots and loss of staining intensity appeared to be more abrupt during the early stages of fruit (5 WAA). Other proteins were observed to start synthesis at approximately 12 WAA (oil deposition in the mesocarp) and continue until fruit maturity at about 20 WAA. The findings of the present study also indicate that there are differences in the protein profiles during mesocarp development in oil palm between *E. guineensis* and *E. oleifera* at the accumulating oil stage (17 WAA). However, further analyses have to be done using MALDI-MS to identify the spots of the proteins of interest and to elucidate whether they contribute in any direct way to the mechanisms of oil synthesis or involve in repining process.

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