ANALYSING FATTY ACIDS CONTENT HALF SEED CHROMATOGRAPHY

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RESEARCH ARTICLE

ANALYSING FATTY ACIDS CONTENT IN WINGED BEAN (P. *tetragonolobus* (L) DC) THROUGH HALF SEED CHROMATOGRAPHY

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ABSTRACT

Indonesia, the second largest mega diversity in the world. It has a wide array of plants species including Winged bean (*Psophocarpus tetragonolobus* (L) DC). Here, this crop is cultivated in many areas though they are not consumed daily leads to very limited effort to develop it. Developing this crop may attract people to cultivate and so consumption like as source for industrial oil. Selections for high fatty acids content seeds, so far are done by selecting good quality of seedlings through selection of the whole seeds. Current study initiated the use of half seed analysis on gas chromatography (GC) to collect data of high fatty acids content of winged bean. Six genotypes were collected from Sumatera (SMT 1 and 2), Java (1 and 2), Bali and West Nusa Tenggara (WNT), and used as the research material. The SMT genotypes are long pod genotypes (27 and 20,5 cm), Java have either long or short genotypes (27 cm length and 10.3 cm). Bali and WNT genotypes were not clear as they were collected as seeds instead of pods. The long pod genotype has total 8-22 seeds but only 6-8 seeds for short type. The SMT seeds germinated 100% but other genotypes showed lower rate of germination (65% and 55%). of those fatty acids, the Sumateran seeds contain40-50% palmitic acids but only few numbers of seeds from other genotypes. Three individuals were high in palmitic and oleic acids content. The high palmitic acid content reflected the present of putative genes with with four or 3 dominant alleles (PPPP, PPPp) in few individuals. This data showed if half seed was able to be used as source for GC analysis.

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INTRODUCTION

Some annual and perennial crops are known to bear oil either in their seeds, fruits or mesocarp and nuts of up to 50% but contain only 15-30 % carbohydrates and 15-25% protein. FAO listed 21 primary oil crops (FAO, 2018). But not winged bean (*Psophocarpus tetragonolobus* (L) DC) which might due to limited data. In Indonesia, winged bean has long time been being cultivated in many areas from Western to Eastern parts and so have various local names/genotypes (Rukmana, 2000), however, they consume this plant as vegetable only which leads to no breeding efforts, for example by analysing its nutrition contents. Breeding needs a wide array of genetic variations, (Poespodarsono, 1988) suggested to collect various germ plasmswhihc might obtained from (Fitriani *et al*., 2015) either local, national or overseas. Indonesian farmers produced 0.7 tons winged bean dry seeds which are much smaller than any Asean countries (Rismunandar, 1983); thus needs to change customers' preferences from utilizing it as vegetables to become as raw material in industrial oil (Rukmana, 2000); (Yeates *et al*., 2014) stated the matured winged been seeds contain 17% fat consists of 35% MUFA (mono unsaturated fatty acids). The MUFA might reduce cholesterol in blood thus reduce the possibility of coronary vascular blockage (Fresee, 2001; Velasco *et al*., 1999). Winged bean seeds have also reported as raw material for "soy sauce", youghurt, and "soy-milk" (Haryoto, 1995); these industries require huge number of winged bean seeds; this might be obtained through breeding. Plant composes fat/oil as food reservation and utilizes it during the initial phase of growth; started from producing fatty acids with different numbers of carbon (Cramer, 1990). Of this, the first product is palmitic acid C16:0 which might get 2 extra units carbon to form stearic acid (C18:0). The stearic acid might further undergo desaturation to form C18:1, C18:2 and C18:3 or to get another 2 carbon units to form C22:0 and C22:1 (Downey, 1987; Stumpf, 1980). Those steps run in four different phases and enzymes: *ß-ketoacyl-CoA-synthase (KCS)* condenses Malonyl CoA to form *ß-ketoacyl-CoA*; *ß-ketoacyl-CoA reductase* to form *ß-hydroxyacyl-CoA*. The *ß-hydroxyacyl-CoA* reductase dehydrate it to form *enoyl-CoA*; last the *trans-2,enoyl-CoA reductase* changes e*noyl-CoA*and form *acyl CoA* (Millar and Kunst, 1997); and (Puyaubert *et al*., 2000). Current study was done to select parental plant candidates based on their fatty acids content as reflected by half seed gas chromatography (GC) results, and plant another half as planting material (Sasongko, 2003). The GC, separates the material into 2 different phases (Day and dan, 1980); the first phase forms a stationer layer with wide surface, and the second forms a liquid phase which passes through the first phase (Montgomery *et al*., 1983). Stated the compounds are split through processes like adsorbtion, absorbtion or both to pass the layer and might be used to analyse closed to complex compounds or very small amount of samples but more precise than those previous methods (Roth *et al*., 1981).

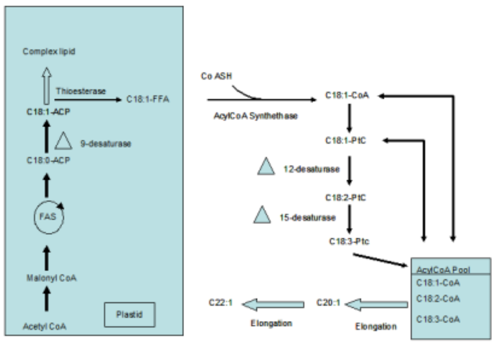

Figure 1. Simplified biosynthesis of fatty acids [Slabas, 2001]

MATERIALS AND METHODS

Research Material: Current study applied 6 genotypes winged beans from Sumatera (1 and 2), Java 1 and 2, Bali and West Nusa Tenggara/WNT) to be analysed for their morphological characters like pod's length, total number of seeds per pod, seeds' colour, form and weight prior to GC analyses. The seeds of each genotypes was germinated and half of the seed/ one cotyledon was subjected for GC. The GC machine is Shimadzu type 2010 with a silica *capillary column* of DB-23, 0.25 x 30 m x 0.25 film thickness of (7%-W Scientific) Agilant Technology USA. The N2UHP (nitrogen ultra high pressure) gas was chosen as delivering gas material. Temperatures were set up as follows: *oven, detector,* and *injection* 90°C, 250°C, and 260°C consecutively and split to 1:50 at 100 kPa.

Research protocol

Seeds germination: 10 seeds of each genotype were selected based on their physical performances and placed in multi tray pot filled with smooth sand in the dark room. The seeds were positioned in a sequential number and sprayed with water daily. At the 10th days, the non-germinated seeds were categorized as rotting and replaced with the new ones. In contrast, prior to GC analyses, the cotyledon of germinated seeds will be dissected and placed in plastic clip then stored in minus 80°C compartment. Another half seed will be observed for their germination time, length of shoots and roots. Following to this, another half seed/one cotyledon was returned to the growing media for another 10 days before planted on the cultivation area.

Half seed's preparation for GC analyses: The dissected-cotyledone was put into 1 mL injection vial, added with100 µl iso octan:iso propanol (9:1) and vortexed for 3 minutes and left over night. The solvent was dried in warm air and added with 50 µl 0.5 N NaOH methanolic and vortexed for 3 minutes, left for 5 minutes. 100 µl BF3 20% methanol was added, vortexed for 3 minutes and left for 30 minutes to allow methylation. Following to this, 200 µl N hexane p.a, was added and vortexed for 3 minutes then added with 200 µl NaCl and re-vortexed. The top layer was taken by a hamilton syringe to be injected into the gas chromatography apparatus. Data of this step were fatty acids content showed in the GC machine.

Seed's planting: The left half sed/cotyledon was aclimatized in a polybag filled with catlle's manure for at least 2 weeks to reach approximately of 20 cm height prior to planting in the soil. Five plants were planted on each row which was set as follows: 3.5 m length, 50 cm within row and 70 cm between rows. At both edges, border crops of land-water spinach were planted. Each plant was given 3 m high pole to allow the plant twist to the pole and grow well. The plants which were not grow well then replaced with new seedlings. Data of this step were total number of plants grew well to harvesting time.

Plant's care: The growing plants were cared out by giving one garden-scoop organic fertilizer of cattle's manure weekly until approaching its generative then changed to one spoon urea per plant per week. Watering however, was done daily from planting to harvesting. Weeding was done weekly by pulling out the weeds.

Harvesting: Harvesting was done when the plants showed at least having one dry-cracked pod winged bean, this particular plant then pulled out. Data were obtained from observing the plants length, root length, fresh and dry weight, total number of pods, pod's length, number of seeds per pod, seed's weight, total number of root nodules, and tubers.

RESULTS

Sampling siteS: From some environemtal factors were observed , all sampling sites were noticebly fit for cultivating winged bean. The temperature and rain falls of all sampling sites were quite similar but not Java which have more rain falls (Table 1). Those environemtal factors, however, do not parallel to winged bean production level from those particular areas due to small areas spent for cultivating it. Data of fatty acids compound (Table 3) obtained from individual half seed GC analysis then calculated and shown as group. The Java genotypes (1 and 2) contain the mediocre content of Palmitic acid (C16:0) of approximately of 35% and erucic acid (C22:1) approximately of 1.2%, but they were showing low level in Oleic (C18:1) of about 10.1% and Linoleic acid (C18:2) of 20% only. The Bali and WNT genotypes however, had the highest amount of palmitic which varied from 35 to 46% and oleic acids of 23 and 15.86%. The high palmitic acid content of 35 -60% in winged bean seeds genotypes Bali and WNT showed the presence of putative genes for palmitic acid as many as 4 or 3 dominant alleles dominant alleles (PPPP or PPPp) (Fig.2).

DISCUSSION

Environmental data of the sampling sites proofed that winged beans are cultivated in many areas of Indonesia from West to Eastern part thus indicating that Indonesia is quite favourable to cultivate them.

Table 1. Sampling sites parameters of rain falls, air temperature, and total cultivation area

Sites	Rain falls (mm)	Temperature (°C)	Total Area(Ha)
SMT 1	100-350	26-33	<1
SMT 2	100-350	26-33	<1
JAVA 1	>750	26-33	<1
JAVA 2	> 750	26-33	<1
Bali	600-900	25-33	<1
WNT	150-400	28-34	<1

Table 2. Average value of number of pod's length, seeds per pod, 100 seed's weight, number of seeds germinate, dormantion period and shoot's length

Genotypes	Pod's length (cm)	Number of seeds pod^{-1}	100 seed's weight (gr)	Number of seeds germinate (unit)	Time to germinate (days)	Shoot's length (cm)
SMT 1	27.00	14.39	47.7	10	15	13.1
SMT 2	20.50	12.77	40.7	10	14	12.5
JAVA 1	27.00	15.95	41.6	9	21	11.8
JAVA 2	10.63	6.95	43.2	4	21	12.0
BALI	NA	NA	39.2	5	27	17.3
WNT	NA	NA	37.4	6	28	9.25

Table 3. Genotype Winged bean plants containing high palmitic (C16:0) and other fatty acids

Source	C16:0	C18:1	C18:2	C22:1
Java 1	35.72	9.85	20.46	1.27
Java 2	35.51	10.54	20.2	1.28
Sumatra 1	40.90	7.77	26.88	2.32
Sumatra 2	40.30	5.28	18.24	2.32
Bali	35.26	23.12	6.20	2.33
WNT	45.85	15.86	13.23	2.03

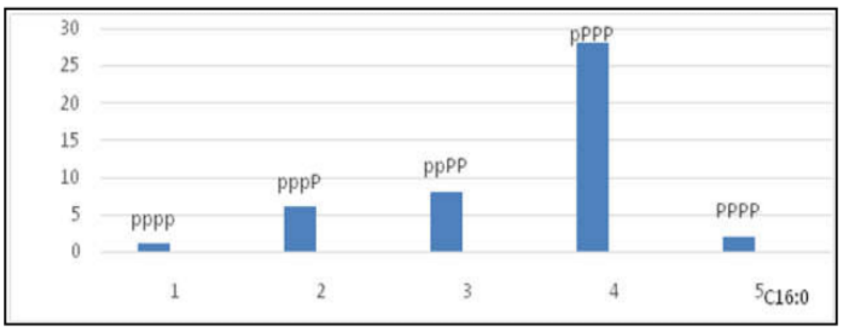

Figure 2. Distribution of winged bean's Palmitic acid (C16:0) with their putative gene for palmitic acid

The production rate, however, does not parallel with the enviromental data but more related with the consumer's preferences in utilizing them and reflected in small cultivation area spent for (Table 1). The small area of winged bean plantation were might due to the famers' preferences to cultivate more higher economic value and so called as cash-crops e.g.: corn, peanuts, soy bean. Corn for example, in the mean time the Indonesian government put it as a new primadona and expected to export up to 1 million tons in the 2019. The Indonesian farmers also grow soy beans in a massive and extensive way to fullfill hgh deman on derivative products; currently it is cultivated in approximately 648,000 Hectare land with 2.75% incrase p.a. Increasing of the winged bean production, therefore, supposed to be started from changing the preferences in utilizing it, for example by utilizing mature seeds instead of young/immature pods (Rukmana, 2000). Stated that mature winged bean seeds contain all nutrition including fat/oil. Interestingly, the fat/oil contains in the seeds comprises of unsaturated fatty acids which is known as important compound in edible oil\ industries. The demands on edible oil is always increase parallel with people's conciousness on healthy food (Murphy, 1994). (Fresee, 2001) stated the utilization of unsaturated fatty acid oil in daily intake will reduce the possibility of hearth coronary disease since this fatty acid might lowering cholesterol content in blood (Velasco *et al*., 1999). Observations on morphological characters (Table 2) showed there were some variations among those genotypes collected. Three genotypes (Sumatera 1, 2, and Java 1) were classified as long pod's type, however, they still varied in length (27 and 20.5 cm length). These genotypes also bearing different numbers of seeds (14.29 and 12.77 seeds for long and short pods consecutively). It was predicted that the farmers treated their plants in different ways. More interstingly the Javanese winged beans, the farmers also cultivated two different genotypes (27 and 10.63 cm length) which produced 15.95 and 6.95 seeds. From 100 seeds' weight point of view, the Sumatera 1 genotype produced the largest seeds of 47.7 gr whereas the smallest came from WNT (37.4 gr). These diferences might due to lesser rain falls in this area (Table 1) as this crop require 600-1,500 mm p.a.as stated by (Davis, 1945), amount, distribution and intensity of rain falls affecting the peas pod formation. Current data were different from that reported by (Rukmana, 2000) stated the long pod genotype supposed to bear 8-22 seeds/pod and 6-8 seeds/pod for the short type. The difference in seeds bearing per pod might due to planting space applied in this tudy or long period to generative phase of 60-140 days after planting (DAP) and another 30 days for seed's filling. (23) stated that spacing took an important role in affecting the plants to stand in their generative phase. Spacing of 12.5 cm within row took longer time to form the first flower than 75 cm, whereas current study applied 50 cm apart. Space within row also important factor in green pods and seed's formation, however, winged beans are mostly cultivated in small areas in narrow-space (<15 cm). Alternatively, it might probably due to some environmental factors which were not conducive along the plant's growth as well as seeds filling in the pods (Sastrapradja *et al*., 1981). Stated that winged beans have 2-10 flowers which will turn to pods per influorenscence. But in most of the cases, not all flowers turn to pod and not all pods filled by seed on each of its seta. Moreover, (Lambeth, 1950) reported that the low yield of lima beans was strongly correlated with high temperature, low relative humidity and soil moisture defficiencies. These three factors affecting total number of dropping flowers and young pods. The seed's filling in the pod might be affected by both external and internal factors. External factors such as pollinator's insects, as well as predators like acaro, and grasshopers are commonly noted in the plantation's areas. In line to this study, the low seeds formation in angiospermae was also quite low (Stephenson, 1981; Bawa and Buckley, 1989; Champbell and Halama, 1993).

There are three steps of fruit's formations namely: 1. evocation 2. Inisiation, and 3. Anthesis (Elisa, 2018).The longer time for seeds' filling the more possibility of pods abortion, because of one or combined-factors like: lack of soil nutrients, exceeding water during seeed's filling, environment stress or pest and diseases attack (Asamoah *et al*., 1981). Moderate to severe soil moisture stress affect pods formation and seeds filling. However, (Liu, 2004) suggested drought stress might also responsible to pod's filling. Apart from those factors, (Bennett *et al*., 2011) suggested the following phytohormones like salicylic acids, ethylene, absisic acids, giberellin, and cytokinin take a role in pod's development (Habekottè, 1[illegible]). Stated that seed's formation much related with branch's number and the time of pod initiation during flowering. The seeds will germinate when their embryo release giberellin actively, and push out aleuron of the endosperm, synthesis amilase, maltase and protease and changes reserve food to energy as well as cell's formation. The Sumatera genotypes needed shorthest time (14 days) to germinate, indicated the pods collected from Sumatera genotypes were obtained from the newest harvesting time in this area. Whereas, the Balinese and WNT genotypes required double time to germinate, and Javanese in the middle (Table 2). The Sumateran genotypes had 100% germination rate while Bali and WNT showed only 55% germinated, indicated if they were obtained from different harvesting period. Alternatively, the differences in germination rate might due to level of seed's maturity as it affect dormantion period. Others are total amount of water, temperature, light and soil. The seed's maturity level is important because indicating seed's physiological maturity as well as enough food reservation prior to germinate (Tianna, 2012) and so having higher viability than the younger seeds.

Apart from that, the author suggested also seed's size and dormant period as the factors affecting germination. Some seeds undergo a resting time and germinate when the enivronment are favour to them. Dormant period might also affected by thinnes or thickness of the seed's coat thus needs physical or chemical break to allow the seed start to germinate (Nawwaf, 2018). The low germination rate of the seeds collected from Java and Bali as well as WNT were probably because of those single or combined-factors. GC analyses of winged bean cotyledon (Table 3) showed some individuals of the Sumateran seeds containing high palmitic acids of (40-60%), followed by the Javanese whereas the lowest content was noted from both Bali and WNT seeds. These data were strongly against of those reported by (Rukmana, 2000) who stated that winged bean seeds contain <10% palmitic acid. This discrepancy might due to different methods of analysis, soxhleation against half seed gas chromatography. The previous methods of soxhleation, which is based on a serial steps of sieving, is the most common method on analyses of fats but this method takes much longer time and more labourious than that of gas chromatography. (Kramer *et al*., 2010), however, reminded the use of a correct detector and standard solution, as moving phase in gas chromatography are two important factors of GC analysis.

This paper noted high palmitic acids content in winged bean seeds was closely to that of contain in palm oil plant. This suggested the importance effort on breeding activity to increase customers preferences of other oil sources crop. Selecting high in palmitic acid content seeds which might then used further as the plants candidate for either direct oil production or cross breeding. By doing so, the hybrids will produce high palmitic acid since this compund is important in either edible oils and oleochemical industries like:soap, cosmetics, and release agents following saponification of sodium palmitate (Fresee, 2001). Recently, paliperidone palmitate, a long-acting antipsychotic medication, has been used in the treatment of sciophrenia through injection intravenously (Bennett *et al*., 2011). Apart from that, current data confirmed if single cotyledone contains fatty acids as much as in the whole seeds leads to a further seeds' analysis without discarding the germplam which is mostly being applied in the previous time for seed's analysis. These data allow the breeders to select which seeds would be treated further planting as the parental cadidates or discarding. This report, showed also the presence of putative dominant genes of P which caused high palmitic acid (C16:0) content of 40-60% with at least 3 dominant alleles but only 1 allele for recessive. In considering to this situation, winged bean oil might then be applied as raw material in oleochemical industries than that of edible oil industries such as: soaps, cosmetics, paintings, drugs, and chocolates.The utilization of palmitic oil as edible oil, need a serial steps like degumming.

Conclusion

Plant mass-selection which is basedd on their fatty acids content might be done by applying half seed (single cotyledon) analysis and another single seed is planted and used as candidate for particular interest. The sumatera Winged bean genotypes containhigh palmitic acid.

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