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HALF SEED CHROMATOGRAPHY –A NON DESTRUCTIVE METHOD IN SEED'S MASS SELECTION

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ABSTRACT

Winged bean (*Psophocarpus tetragonolobus* (L) DC) has long time been cultivated in Indonesiautilized mainly asvegetable thus less favour than other grain crops to make low productivity of 0,7-tonnes only. Increasing in production might be reached by utilizating in various ways, for example: its monounsaturated fatty acids content to reduce coronary vascular blockageby reducing cholesterol level. So far, there is no scientific report on winged beans breeding efforts. Selections, if any, are most likely done by utilizing the whole seeds not half one as in the current study, initiates half seed analysis and gas chromatography (GC). Samplings were done in Sumatera, Java, Bali and NTB. The Sumateran pods, are both long types (27 over 20,5 cm). Java either long or short types (27 cm length and 10.3 cm). The long pod type has total 8-22 seeds but only 6-8 seeds for short type. The Sumateran seeds had germination rate of 90-100% but other seed's showed lower rate of germination. Seeds then germinated in a dark room in a multitray pots filled with smooth sands sequentially. Single cotyledone or half seed was dissected from germinated-seedsfor its fatty acid analysis using gas chromatography and another single cotyledone was planted. The gas chromatography is completed with a silica *capillary column* DB-23, 0.25 x 30 m x 0.25 film thickness of (7%-W Scientific) Agilant Technology USA.The nitrogen ultra high pressure gas was chosen as delivering gas material. Temperatures were set up as: *oven*, *detector*,and *injection* 90^{o}C, 250^{o}C, and 260^{o}C consecutively and split to 1:50 at 100 kPa. Most of the seeds collection containe oleic acid (C 18:1) content 8-20%, except for B1, 2 and NTB 5 36.15% and 33.50% cosecutively. This study noted that most cotyledoneae have putative genes of oleic acid as recessive alleles (oooo), and dominant allele of (O) in few individuals only, but different from previous report with oOOO alleles, but allele for palmitic acid was dominant (P) to allow pPPP=46% of the total fatty acids. Total mono unsaturated fatty acids content (MUFA) and (PUFA)varied from 59-65% and 18-30% which fit to lowering cholesterol content and in keeping lear blood's circulation due to Omega 6(C18:2) and Omega 9 (C18:3). Plant mass-selection might be done by applying half seedanalysis. Winged bean contains more palmitic acid than oleic except for 2 individuals which show high content of oleic acid but has high MUFA and PUFA.

Keywords : mass selection, half seed chromatography, MUFA, PUFA

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INTRODUCTION

Winged beans (*Psophocarpus tetragonolobus* (L) DC) have long time been known and cultivated in Indonesia,Rukmana (2000) stated if this crop is predicted to be is indigenous plant of Indonesia new Guineae,In Indonesia, therefore, they have different local names like: Jaat (West Java), Kecipir (Central Java) Balimbing nuts (West Sumatra), biraro in Ternate and Kalongkang in Bali,In the mean times, however, these plants are utilized asvegetable only, where people consume their young pods thus less favoour than soy beans, ground nuts, and corn which are cultivated in much larger area and more serious way,

In compared with other Asean countries, which cultivate them in a more intensive way, the Indonesian farmers produce in much smaller numbers,Rismunandar (1983), stated if those Asean countries, produced 4,5 tonnes dry seeds, but Indonesia produced 0,7-tonnes only,

This production level needs to be improved by utilizating them in more various ways, Apart from their high fiber in the young pods, the winged bean seeds contain nutritious compounds such as protein, fats, and carbohydrates,Rukmana, 2000; Yeates,, et, al, 2014) mentioned if matured winged been seeds contain 17%fat which consist of 35% monounsaturated fatty acids (MUFA) and 20% poly ones,The fats contained on the matured seeds are much higher than that of in young pods (BOSTID, 1981) which consist of only 2% fats,These two fatty acids are important sources in both edible and industrial oils,Edible oil which comes from unsaturated fatty acids may reduce the possibility of coronary vascular blockage (Freese, 2001) by reducing cholesterol (Velasco, dkk,, 1999),Whereas, polyunsaturated fatty acids might be useful in biochemical industries (Piazza and Foglia, 2001), Both industries require huge number of winged bean seeds wiht specific fatty acids compounds, Winged bean seeds have also reported as the raw material for "soy sauce", youghurt, and "soy-milk" (Haryoto, 1995; Dewi, 2005),

During seed's development, embryo forms food reservation as fat and protein which is important during the initial phase of growth, Cramer (1990), stated that in this phase seed compiles ftty acids with different number of carbon and so their double bonds to form either unsaturated or saturated fatty acids, Of these fatty acids, the first product is palmitic acid C16:0 which might got extra unit of carbon to form stearic acid (C18:0), Stearic acid will be dasaturated to form C18:1, C18:2 and C18:3, alternatively this compound will get additional unit of Carbon to form C22:0 or C22:1 (Downey, 1987),Stumpf(1980), Millar and Kunst (1997) and Puyaubert et,al, (2000) reported that all of those processes might run in 4 different phases as follows:

a) Malonyl CoA with the help of *ß-ketoacyl-CoA-synthase (KCS)*will be condensed to form *ß-ketoacyl-CoA*,
b) *ß-ketoacyl-CoA reductase*enzyme will reduce *ß-ketoacyl-CoA*to form *ß-hydroxyacyl-CoA*

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c) *ßhydroxyacyl-CoA*with the help of *ß-OH-acyl-CoA dehydratase* enzyme will be dehydraed to form *enoyl-CoA,*

d) The enzim *trans-2,3-enoyl-CoA reductase*enzyme will reduce adalah e*noyl-CoA to* form *acyl CoA,*

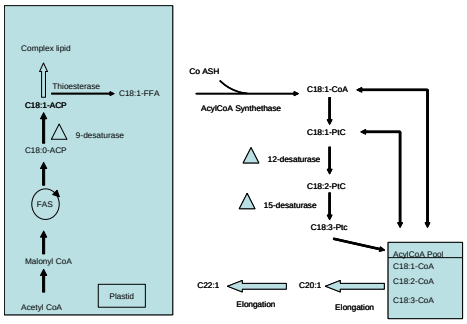

Figure 1,Simplified biosynthesis of fatty acids Slabas et,al, (2001),

Limited number in utilizing the winged beans might also cause limitation in the availability of good winged beans seedlings,So far, there is no scientific report on winged beans breeding efforts available, Breeding acitivities, however, always initated by selection of the parental plants based on their phenotypic traits like the colours of the flower, plant's height, fruit size, etc**but not** based on their nutritious content,

In the mean time, plant's selection which is based on analyse of seed's nutrition contents applies to whole seed, thus the breeders lose their planting material,Information aboout nutritious content contained in individual seed becomes prerequisite to be obtained without discarding potential seed to be planted,

Current study initiates analternative way on selecting parental plants which based on nutritious content in one cotyledone instead of the whole seed applying gas chromatography, since it needs such a series of study as well as tremendous numbers of breeding material Sasongko et al,, (2003), Data of nutritious content in one cotyledone were then used as data bank in selecting the candidates for parental plants, and another cotyledone of the seed was then planted as normal plant(Sasongko, dkk,, 2003),Data of this study might also be used as the latest information of fatty acids content contined in winged bean seeds before planting as candidate parental plants to obtained such hybrids fit to aparticular purpose through vvarious breeding steps like cross breed, back cross or test cross as well as through genetic engineering,Finding of good quality winged beans for industrial purposes may attract farmers to cultivate them since demand of these plants,

Gas chromatography, is one among those chemical analyses based on separation of material being analysed,Day and Underwood (1980) stated if gas chromatography split compounds being analysed into 2 different phases, one of those phases will form stationer layer with wide surface, while another phase is a liquid which passing the first phase,

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(Montgomery et, al,, 1993) stated that components being analysed are split through processes like adsorbtion, absorbtion or both, Meanwhile gas, functions as a factor whch takes compounds being partitioned, Gas chromatography is possible to be used to split gas components, volatilized, without forming its derivate, This characteristics allow gas chromatography to analyse such component like fat, sterol, amino acids, or pesticieds,,

This method known as more precise and shorter than those previous methods as destillation, cristalization or extraction,Furthermore, Roth and Blaschke (!981) stated if gas chromatography might also be used to analysed closed compounds, complex compounds or even very small amount samples,

Gas chromatography processes, howevver, depend on other factors such as:

1,the succeess of quantitative partition method by appling such compound fit to component will be analysed

2, easy to handle

3, fast, and accurate in quantitative determination

4, sensitive detector

METHODS

Current study was initiated by collecting winged bean germ plasms from Lampung-Sumatera, Java, Bali and West Nusa Tenggara, The germ plasms were then obeserved for their phenotypic traits like pods' length, total number of seeds per pod, seeds' colour, form and weight, Following to this observation, seeds were selected individually for colour, and size, The selected seeds then placed in a multitray pots filled with smooth sands sequentially to allow germination in the dark by covering the multitray pots with aluminum foil at room temperature, When the seed germinated, it was taken out from its growing medium for dissection, Dissection was done by cutting one cotyledon after measuring the stem length, The dissected cotyledone was put in a clip plast6ic bag and stored in -80^{o}C freezer, since gemrination did not run simultaneously for all seeds, Preseving the cotyuledone in the -80^{o}C was purposed to minimise metabolism runs while waiting for the later cotyledone,

Analysis of fatty acids was done following the prpearation of cotyledonaein a Shimadzu tyoe 2010,This machine is completed with a silica *capillary column* 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 upt as follows: *oven, detector*,and *injection* 90^{o}C, 250^{o}C, and 260^{o}C consecutively and split to 1:50 at 100 kPa,

Cotyledone was prepared as follows: the dissected cotyledone was put into an injection vial, then added with100 µl iso octan:iso propanol (91:1) and vortexed for 3 minutes and left over night

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to allow extraction of the fatty acids from cotyledone, Following day, iso octan : iso propanol (9:1) was dried in a warm air, The dired cotyledone was added with50 µl 0,5 N NaOH methanolic and vortexed for 3 minutes then left for 5 minutes, Following to this, 100 µl BF3 20% methnol was added and vortexed for 3 minutes then left for 30 minutes to allow methylation,After methylation process, 200 µl N hexane p,a, was added and vortexed for 3 minutes then added with 200 µl NaCl and vortexed, The top layer was taken by a hamilton syringe to be injected into the gas chromatography apparatus.

RESULTS AND DISCUSSION

Current study noted if all winged bean populations are cultivated in almost similar environmental condition like adequate rain falls, favourable temperature but they are in small area only (Table 1), Most of them produced yellow seeds though the Sumatera and NTB produced black ones.

Table 1. Sampling sites parameters

Parameters	Rain falls (mm)	Temperature (^oC)	Area(Ha)
Bali	600-900	25-33	<1
NTB	150-400	28-34	<1
Sumatera 1	100-350	26-33	<1
Sumatera 2	100-350	26-33	<1
PWT 1	>750	26-33	<1
PWT 2	> 750	26-33	<1

Table 1, Shows PWT (Purwokerto) and its surrounding areas has high rain falls rate and warm temperature are suit for cultivating winged beans. Out data, however, do not reflect on its production level since total area spent for cultivating this crop is much lower than those area spent for others with higher economic values e.g.: corn, peanuts, soy bean. The last three are cultivated massively either due to they might be consumed directly, or indirectly. Apart from that, the Indonesian government also support much on corn cultivation, so Indonesia was able to export to some countries and expect toreach 1 million ton in the 2019 year (Republika co.id. 18/09/04 *accessed on* tgl 28/09/18). Similar situation happens to soy beans which grow extensivelly in term of cultivation areas as well as its production. Currently Indonesia spends about 648,000 Hectare areas with increasment of approximately 2.75% per annum (Bussiness tempo.co.id 26/07/12 *accessed on* 28/09/18).

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Table 2. Morphological characters of winged bean pods and seeds

Source	Average pod's length (Cm)	Average total seeds/pod(unit)	100 Seeds weight (Gr)	Germ length (cm)
SMT 1	27	14.39	47.7	13.08
SMT 2	20.5	12.77	40.68	12.5
PWT 1	27	15.95	41.60	11.84
PWT 2	10.63	6.95	43.2	11.96
BALI	NA	NA	39.17	17.26
NTB	NA	NA	37.47	9.25

Table 2. shows variation in morphological characters of winged beans pods and seeds though they come from closed-areas (closed regency). Pod's length of the SMT (Sumatera), for example, though both types are long pod types but they are different on length (27 to 20.5). More interstingly, the Purwokerto has two different types of winged beans (long and short pod) types. The first type was collected from Purwokerto 1 (27 cm) length, but another type has only 10.3 cm in average without have any particular reason. Rukmana (2000) stated the long pod winged bean has different colour of flower to the short type one. The long pod type has a white flowers with total seeds of 12-22 seeds, meanwhile the short has purple flowers and 4-8 seeds per pod. Current study noted some slight discrepancies to that of reported earlier, where the long pod has 8-22 seeds per pod but only 6-8 seeds for short pod type. The above diffetences might be due to differences in their flowering times. Winged bean will approach reproductive period after 60-90 days after planting (DAP) followed by long period for seed's filling which takes another 30 days after flower's formation. It is also predicted some environmental factors were not conducive to the plant's growth as well as seeds filling in the pods. Another, might due to their diffewrences in flowering time which also affecting the seed's filling indirectly. 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 so not all pods filled by seed on each of its seta. 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, nad grasshopers are commonly noted in the plantation's areas. Stephenson (1981), Bawa and Buckley (1989) stated that the fruit's formation within the angiospermae were quite low rate, and some of those fruits form little number of seeds (Campbell and Halama (1993). According to Elisa (2004) plant has some steps to form fruits, i.e.: 1. evocation (fruit induction) to change the vegetaive cells to become reproductive ones. 2. Inisiation, the floer starts producing reproductive shoots. 3. Anthesis,a process of flower's bloom and maturation of reproductive organs. Long period of time to form and pod's filling causes some

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young pods abort before seed's filling or even the flower aborts before fertilization, might be because of one or combined-factors like: lack of soil nutrients, exceeding water during seeed's filling, environment stress or pest and diseases attack.

Seeds collections were weighed for their weight (Table 2) and selected for 20 seeds to be germinated as parental plant candidates. For this purpose, seeds were germinated on the smoothy sand and placed in the dark room to minimise photosynthesis processes due to diffusion of auxin hormone. Seeds germinate in 14-28 days after depend on their dormantion period and on total amount of water surrounding the seed to stimulate germination. Germination starts when the embryo releases giberellin actively, pushes out aleuron of the endosperm, synthesis amilase, maltase and protease and changes reserve food to energy as well as cell's formation.

Table 2. shows germination rate of the seeds collection, both Sumatran (1 and 2) seed populations show the germination rate of 90-100% but other showed lower rate of germination. This discreapancy on germination rate was might be due to different harvesting time.

Table 3 shows that the oleic acid (C 18:1) content in winged bean cotyledone of three (Bali 1,2 and NTB 5) contain over 30% which is similar or higher than that of reported by Rukmana (2000), to end these indivdual plants are potential to be selected as the candidate plants for high oleic acid content. These data proofed also if single cotyledone contains fatty acids as much as in the whole seeds which is mostly being applied in the mean time for seed's analysis.

During its biosynthetic pathway, plant initially produces palmitic acid (C16:0) and due to the presence of *palmytoil-ACP elongase,* it will be elongated to form stearic acid (C18:0; Fig, 1) by addition of 2 carbon units. The desaturase enzyme will react to this compound to form mono unsaturated fatty acid with the same number of Carbn units (C18:1, oleic acid) and further to become linoleic and linolenic acids (C18:2 and C18:3). Alternatively, stearic oil might get addition of 2 Carbon unit to form long chainfatty acid C22:0 or even C22:1 (Downey, 1987).

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Table 3. Winged bean individuals with high palmitic and oleic acids content (%).

Source	C16:0	C18:0	C18:1	C18:2	C18:3	C22:1
Bali 1	**40,34**	9,67	**33,52**	12,39	4,13	4,31
Bali 2	**40,39**	7,75	**36,15**	10,26	5,47	2,98
NTB 5	**40,33**	9,67	**33,50**	12,39	4,12	1,29
PWT 1:4	**46,38**	13,78	8,66	12,40	4,51	0
PWT 1:8	**42,18**	13,92	5,59	17,68	6,91	1,45
PWT 1:9	**46,04**	16,47	3,78	14,06	5,28	0
PWT1:10	**48,53**	15,86	2,40	12,79	5,08	2,73
S1:2	**41,55**	14,53	6,76	26,32	6,66	1,86
S 1:3	**40,28**	11,42	2,91	28,07	12,55	4,16
S1;:4	**41,31**	11,95	20,31	18,87	4,21	2,35
S 1:5	**54,51**	15,85	7,44	12,68	3,30	1,27
S 1:6	**41,91**	13,80	5,16	24,30	8,26	4,27
S 1:7	**44,12**	14,27	5,10	26,83	6,43	2,47
S1:9	**41,30**	11,95	20,31	18,86	4,20	1,17
S 2:1	**42,13**	13,17	6,21	19,95	14,12	1,88
S 2:4	**47,16**	14,97	5,51	19,75	10,29	2,24
S 2:5	**44,00**	12,11	9,48	21,26	8,31	2,87
S2:6	**41,92**	13,80	5,12	24,31	8,27	2,40
S2:7	**48,94**	13,34	8,41	20,56	5,,20	1,92
S2:8	**48,83**	12,51	18,85	13,19	3,18	0,97
S 2:9	**48,49**	15,48	5,48	16,57	6,69	4,16
S2:10	**48,50**	15,40	5,49	16,58	6,70	2,14
Bali 3	**46,21**	13,55	12,56	16,17	8,40	3,13
Bali 7	**49,35**	15,09	5,45	16,46	7,84	1,24
Bali 8	**51,56**	14,10	9,04	13,45	4,43	2,32
NTB 1	**46,70**	14,80	11,87	22,40	4,26	1,24
NTB 2	**49,35**	15,09	5,45	16,46	7,82	2,31
NTB 3	**46,20**	13,56	12,56	16,17	8,40	2,32
NTB 6	**46,10**	11,25	27,71	8,81	2,37	2,32

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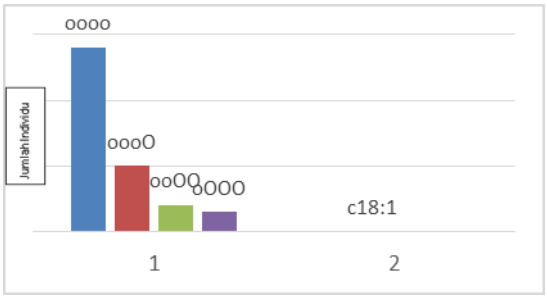

Figure 3. Distribution of oleic acid (C18:1) on winged bean's cotyledoneae with their putative genes for Oleic

Figure 3. shows most of the winged beans cotyledoneae have putative genee of oleic acid as recessive alleles (oooo), and dominant alle of (O) presented in few individuals only. These data were different from that of reported by Rukmana (2000) who stated if winged bean seeds contain 39% oleic acid (C18:1) and furtherly became the main refernce of this study to tailoring high olei acid winged bean. This discrepancy might due to method of analysis, in most of the cases seed's anaylsis require the whole seed instead of single cotyledone. The use of whole seed, might double the fatty acid content but this method might not be applied as seed's selection method. Alternatively, the previous seed's analysis method applied maseration and soxhletation, which is different from current study which apply half seed (single cotyledoneae) and furtherly analysed by gas chromatography method. In many food industries, 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 chormatography. Nawwaf (2011), however, reminded the use of a correct detector and standard solution, as moving phase in gas chromatography. The presence of putative dominant gene of P which caused high palmitic acid (C16:0) content comes to a suggestion if winged oil do not fit for food industries since Palmitic oil is categorised as saturated fatty acid which might lead to coronary diseaase to the customers

If the high content of palmitic acid is assumed with the presence of dominant allel P,the winged bean seeds collection of those 6 population show that winged bean are high in palmitic acid content (pPPP=46% of the total fatty acids) Table 3 and Figure 4 Table 3. show some winged bean cotyledoneaes contain palmitic acid (C16:0) between 40 and 50%.

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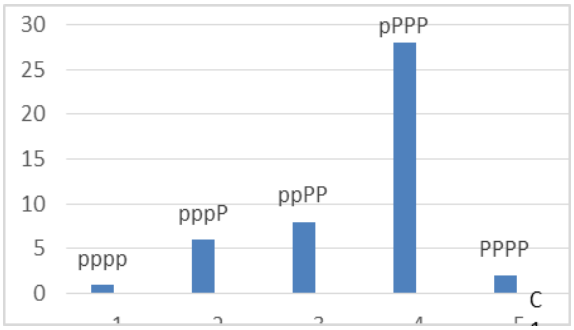

Fig 4. Distribution of winged bean's Palmitic acid (C16:0) with their putative gene for palmitic acid.

Current study is then suggested to utlise the winged bean fatty acids for further industries based on their total mono unsaturated fatty acids content (MUFA) which varied from 59-65% or poly unsaturated Fatty acids (PUFA) 18-30% (Figure 4). This mono unsturated fatty acid are strongly suggested to be consumed by people with high cholesterol content in their blood, by lowering cholesterol content. These data however, disagree to Rukmana (2000) who stated if MUFA in winged bean is less than 35% and 19% PUFA. The PUFA of C18:2 (linoleic acid) is the source of Omega 6, while C18:3 (Omega 9). These tweo components are important for human's health in keeping blood's circulation and present in many different types of seed plant. Ideally, an adult male consume about 17 gram and 12 gram for female on their daily intake.

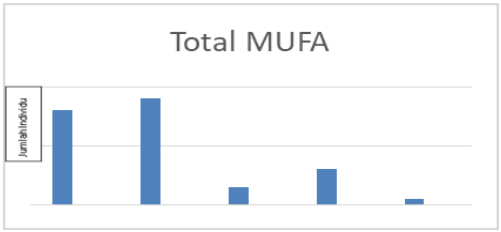

Figure 5. Distribution of MUFA (C16:1+C18:1+C22:1) on winged bean seeds of 6 populations

CONCLUSION

Plant mass-selection which is basedd on their fatty acids content might be done by applying half seed (single cotyledoneae) analysis and another single seed is planted and used as candidate for particular interest. Winged bean contains more palmitic acid than oleic except for 2 individuals which show high content of oleic acid

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