BUKTI KORESPONDENSI JURNAL

RASĀYAN Journal of Chemistry

[Vol. 12. Nomor 3 (July-September) Tahun 2019]

Sebagai:
Kum B. Syarat Khusus
(Lampiran IIIB.1.c.1.2)

Pengajuan Kenaikan Jabatan
dari Lektor Kepala ke Guru Besar

Oleh
Dr. Ir. Marlin, M.Sc.
NIP. 197003141994032002

Fakultas Pertanian
Uniersitas Bengkulu
2023

Judul	:	Secondary Metabolites Change Under Vernalization and Its Relation to Flowering Competency in Shallot (*Allium cepa* Var. *Aggregatum*)
Vol. No. Tahun	:	Vol. 12. Nomor 3 (July - September) Tahun 2019
Nama Jurnal	:	RASAYAN Journal of Chemistry. ISSN: 0976-0083 (Online),
Reputasi Jurnal	:	Terindeks di: Scopus (Q4) = **1,8** & SJR = **0,26**

Timeline Publication / Korespondensi

No	Tanggal	Proses dan Materi Korespondensi	Halaman bukti
1.	22 April 2019	Penulis melakukan Submission/ Editors menerima Manuscript	1
2.	24 April 2019	Editors mengirimkan Permintaan Copy right Form	2
3.	25 April 2019	Editor mengirimkan Revision 1 : perbaikan sesuai format dan permintaan nama dan alamat email dari 3 potensial reviewers	3-4
4.	26 April 2019	Penulis mengirimkan perbaikan naskah sesuai format dan mengirimkan 3 nama potensial reviewers	5
5.	30 April 2019	Editor merespon dan menyetujui 3 nama potensial reviewers yang dikirimkan penulis	6
6.	13 Mei 2019	Editors menyetujui naskah untuk diproses dan direview oleh tim reviewers RASAYAN Journal, editors mengirimkan invoice biaya proses publikasi	7-11
7.	17 Mei 2019	Editors mengirimkan Reviewer Report, RJC-5356_1	12-22
8.	18 Mei 2019	Penulis meengirimkan bukti transfer processing charges	23
9.	19 Mei 2019	Penulis mengirimkan hasil revisi naskah sesuai koreksi dari reviewers (Answers to reviewer's comments)	24-30
10.	21 Mei 2019	Editors mengirimkan a Proof of payment Editors mengirimkan acceptance letter. Editors meminta bukti Copyright of transfer dan Letter of Original Work	31
11	24 Mei 2019	Penulis mengirimkan copyright of transfer dan Letter of Original Work, sesuai permintaan editors	32-33
12	25 Mei 2019	Penulis menerima laporan dan koreksi dari reviewer jurnal	34-39
13	29 Mei 2019	Editor menerima da menyetujui formulir copyright of transfer dan Letter of Original Work yang dikirimkan	40
14.	1 Juni 2019	Editors mengirimkan hasil cek Plagiarism (Plagiarism Status) dan laporan koreksi dari reviewer	41-62

15.	21 Juni 2019	Penulis mengirimkan jawaban dari pertanyaan dan saran yang disampaikan oleh reviewer Penulis mengirimkan hasil perbaikan sesuai saran dan koreksi dari reviewer	63-79
16	21 Juni 2019	Editors menerima hasil revisi yang dikirimkan	80
17	27 Juni 2019	Editors berjanji akan mengirimkan Galley Proofs dari naskah yang akan diterbitkan dalam jurnal RASAYAN	81-82
18	16 Juli 2019	Penulis menanyakan ke editors tentang perkembangan galley proofs dari naskah yang ditelah direvisi sebelumnya	83
19.	22 Juli 2019	Editors menyatakan bahwa galley proofs naskah sedang disiapkan	84-85
20.	22 Juli 2019	Editors mengirimkan formulir untuk verifikasi galley proof	86-95
21.	28 Jui 2019	Penulis mengirimkan hasil revisi dan verifikasi galley proofs	96-104
22	29 Juli 2019	Editors merespon hasil revisi galley proofs	105
23.	3 Agustus 2019	Editors mengirimkan pemberitahuan bahwa naskah telah dipublikasikan online pada RASAYAN Journal of Chemistry , Vol.12, No.3, July - September, 2019. Melalui website : www.rasayanjournal.com	106-108
24.	27 September 2019	Editors mengirimkan informasi tentang penerbitan hardcopy	109
25.	26 Oktober 2019	Editors mengirimkan bukti penerimaan biaya publikasi	110-112

Bengkulu, April 22nd 2019

Prof.(Dr.) Sanjay K. Sharma

Editor-in-Chief

RASĀYAN Journal of Chemistry

Dear Editors,

I am pleased to submit an original research article entitled "The Secondary Metabolites and Its Relation to Flowering Competency in Shalot (*Allium cepa* var. *aggregatum*)" by Marlin, Awang Maharijaya, Agus Purwito, and Sobir for consideration for publication in RASĀYAN Journal of Chemistry. This manuscript builds on our study to determine the metabolites composition in shallot and its relation to flowering competency.

The non-targetted metabolites were identified using GC-MS and assigned by matching their mass spectra with those available in the WILEY7 LIB. In this manuscript, we show a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The metabolites composition was visualized in heatmap. Metabolites composition from 5 genotypes were difference and suggested correlated to flowering competency.

The submission has not been previously published, and is not under consideration for publication elsewhere. I certify that this article is new, and very appropriate to be published in RASĀYAN Journal of Chemistry. All authors have made substantial contributions to the concept and design of the research, acquisition of the data and its analysis, and drafting of the manuscript. We have no conflicts of interest to disclose.

Thank you for your consideration. We are looking forward to hearing from you soon.

Yours Sincerely,

Dr. Marlin

Department of Crop Production, Faculty of Agriculture, University of Bengkulu

Jalan WR. Supratman Kandang Limun Bengkulu 38121, Indonesia

Email : marlin@unib.ac.id

THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (*Allium cepa* var. *aggregatum*)

Marlin[1], A. Maharijaya[2], A. Purwito[2], and Sobir[2]

[1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu. Jalan WR. Supratman Kandang Limun Bengkulu 38121, Indonesia

[2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University. Jalan Raya Dramaga Bogor 16680, Indonesia

*Corresponding author, email : marlin@unib.ac.id

Mobile No. +6285368227265

Address : Jl. WR. Supratman No. 23 RT 9 Kandang Limun Bengkulu 38123, Indonesia

ABSTRACT

Shallot extract contains a great quantity of essential oil, volatiles, and other compounds. The composition of secondary metabolite can be regarded as the ultimate reactions of biological and physiological to genetic and environmental conditions. This study was objected to identify metabolomic composition and its relation to flowering competency in 5 genotypes of shallot. The non-targetted metabolites were identified using GC-MS and assigned by matching their mass spectra with those available in the WILEY7 LIB. Data matrix was analyzed by using metabolomic package of the R software. Heatmap was visualized for a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The composition metabolites from 5 genotypes were difference and suggested correlated to flowering competency. The 3 genotypes of flowering type of shallot produced highest concentration of phytol (ditherphene alcohol) and low concentration of nitrogen compound. Whereas, 2 genotypes of non-flowering types contained high concentration of organosulfur and nitrogen sources. Metabolite profile of 5 genotypes contained of volatile and non-volatile phytonutrients, vitamin, saturated fatty acid and organosulfur compounds.

Keywords : dissimilarity matrix, GC-MS, non-targetted metabolite, organosulfur, phytol

INTRODUCTION

Shallot (*Allium cepa* var. *aggregatum*) is an important *Allium* plant that been used for food, ornamental, natural alternatives to food synthetic preservatives and medicinal for curing various diseases. *Allium* plants contain different sugars, amino acids, vitamins, sulfurous compounds, enzymes, flavonoids, saponins and minerals[1]. *Allium* are also valued for their therapeutic properties; they are active as diuretics and laxatives and have been used to treat headaches and parasitic worms[2]. Onion has beneficial effect as their prebiotic, and their ability to decrease levels of insulin, cholesterol, triacylglycerol and phospholipids for human health[3], and considered an important vegetable and medicinal plant prescribed as an effective therapy for several complaints in healing treatment such as natural cancer treatment[4]**.** These beneficial effects will become an important target in shallot breeding and management practices.

Shallot genotypes may vary in flowering pattern, naturally flowering, inducible flowering, and non-inducible flowering shallot[5]. Flowering in shallot can be induce by an exogenous treatment such as vernalization. Vernalization is an important adaptation of plants to initiate flowering in response to a prolonged exposure to low temperatures[6,7]. The principles derived from vernalization are likely to be widely relevant to epigenetic reprogramming in many organisms[7]. External condition plays important regulation in plant bioactivity and influences the metabolomic composition. Metabolomic composition in plant is various and specific, that depends on genetic and plant growth environment. Metabolomic describes plant phenotype differentiation.

Metabolomic technologies have revealed a new insights in biological systems through metabolic dynamics[8]. The metabolomic composition determines biological and physiological function of the plants. Metabolite compounds are observed in the 6 *Alliaceae* family[9], in *Allium cepa* (onion) extracts[10,11], in *Allium rotundum* L[12], and in *Allium sativum* (garlic) formulations[13,14]. Metabolomic defined as a comprehensive analysis in which all the metabolites of a biological system are identified and quantified[15]. Metabolites are the end products of cellular regulatory processes. Metabolites profiling in plant can be directly extracted from a part of plant to find new molecules contents. Profiling of the metabolite composition is an important way to evaluate the contents of the medical plants, as well as their safety and efficacy[16].

Mass spectrometry is an analytical method, which allows the determination of the mass formula and structure. With mass spectrometry, individual molecules can be measured based on their molecular peak (m/z) and characteristics spectra of fragment ions[17]. Mass spectrometry technology is high sensitivity to identify the unknown and unexpected of the components present in the complex biological samples[18]. These fragments often provide a clue to the molecule's structure, but without a reference compound, the difficulty of making an identification increases markedly[17]. The analytical strategy *gase chromatography mass spectrometry* (GC-MS) used to analyze the volatile compounds, and selected compounds were structurally measured by mass spectrometry transposing the method to GC-MS[19]. The mass analyzer separates the molecules or/and fragments according to their masses and the detector detects and quantifies the separated ions[17]. GC-MS analysis is performed on

single-quadrupole mass spectrometers, which provide nominal-mass information. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories databases[18].

This study was objected to identify secondary metabolite composition and its relation to flowering competency in 5 genotypes of shallot.

EXPERIMENTAL

Plant materials

Local genotypes of shallot with different pattern of flowering were selected as plant material. Shallot genotype 'Bentanis' is used as the naturally flowering, while genotypes 'Bima Brebes Ilokos, Tajuk and Sumenep' are selected as the induced flowering of shallot (Marlin *et al.*, 2018). Each genotypes were treated with vernalization in 8 ^{o}C temperature for 5 weeks, and no vernalization treatment as control. The bulbs were then planted in 45 cm diameter polythene bags containing 8 kg of growing media (soil: manure: husk = 2: 1: 1). Each polybag was planted with three bulbs. Before planting, shallots bulbs were soaked in a fungicide benomil 50% at a concentration of 2 g.L^{-1} for 15 minutes. The bulbs were then soaked into solution of plant growth promoting rhizobacteria of 5 g.L^{-1} for 15 minutes. NPK fertilizers with a ratio of 15:15:15 were applied in a dose of 600 kg.ha^{-1} or 2.4 g per polybag.

Metabolomic analysis

The samples were the leaves of shallot plants at 4 weeks after planting. GC-MS unit was carried out on an GCMS-QP2010 system

(Shimadzu Corporation, Japan) coupled to mass spectrometer detector. The samples were inserted into the quartz chamber in the GC-MS unit. Helium was used as a carrier gas in a constant flow mode at 0.85 ml/min. The chamber was heated in an oxygen-free environment at a temperature of 400 ^{o}C for 0.2 minutes. The reaction will produce heat-mediated cleavage of chemical bonds in the macromolecular structure and produce low molecular weight with a chemical composition that identify specific compound of metabolite. Compound mixtures were then passed through the column GC-MS analysis. The column is Rt x 5 MS, with length 60.0 m, thickness 0.25 µm, and diameter 0.25 mm. The initial temperature of the column was 50 °C, which was gradually increased by 10 °C up to 280 °C. At the end of this period, the column oven temperature was 50 ^{o}C raised up to 280 °C. Injection port temperature was ensured as 280 °C and helium flow rate as 0.85 ml/min. Mass spectrometer detector was employed to detect compounds when they were vented from the column. Temperature of the detector was 200 °C. The volatile compounds of the plant samples were then identified for each treatments.

The identification of the components of non-targetted metabolite was assigned by matching their mass spectra with those available in the WILEY7 LIB. The volatile compounds of the plant samples were then identified for each genotypes. Spectra were compared with National Institute of Standard and Technology (NIST, 2005 v2.1) library to identify the unknown compounds. The data were confirmed using PubChem online database in National Center for Biotechnology Information (https://pubchem.ncbi. nlm.nih.gov/), chemicalbook database (http://www.chemicalbook.com), and NIST webbook

database http://webbook.nist.gov/. The datasets were then log-transformed in order to acquire the normalized data. Data matrix based on non-targetted metabolomic of shallot in each vernalization treatments were analyzed using Metabolomic package of the R software (R version 3.2.2 http://www.r-project.org/). Metabolomics package was operated to construct HCA (*hierarchical cluster analysis*) and visulized heatmap with dendrogram. HCA was performed using Euclidean distance method and complete linkage agglomerative method.

RESULTS AND DISCUSSION

Metabolite compounds in 5 shallot local genotypes were affected significantly by vernalization treatments. A total of 130 of metabolomic compound of non-vernalized bulbs, and 122 of metabolites compound of vernalized bulbs of 5 shallot local genotypes were identified by GC-MS. Metabolite compound obtained where then visually described by metabolite heatmap (Figure 1). Heatmap is widely used in metabolomic research because it is able to visualize large amounts of metabolite data and its differences in sample groups[20]. Heatmap of both non vernalized and vernalized bulbs clustered 5 shallot local genotypes into three groups (Figure 1). First group was G2 (Bima Brebes genotype) and G4 (Tajuk genotype), second group was G1 (Bentanis genotype), and the third group was G3 (Ilokos genotype) and G5 (Sumenep genotype). Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by different

colour of each heatmap coloumn. Specific metabolite compounds were visualized on each genotype column with a bright green color. Hierarchical clustering analysis showed that the genotype had a significant effect on the metabolites composition of the shallot. Each genotype of shallot produces different metabolites that associated to the flowering competency. Hierarchical clustering analysis of shallot metabolites grouped shallot genotypes into three distinct genotypes, in both vernalization treatments (Figure 1 and 2). The result indicated that the presence of specific compounds produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability.

Shallot genotypes located in the same group showed that the genotype had the same metabolite profile, and showed significant metabolite diversity with other groups. Visualization with heatmap also confirmed in the presence of different colors from each of the heatmap columns. The genotypes included in the first group were the Bentanis genotype (G1). In the second group there was the Bima Brebes (G2) and Tajuk genotype (G4), while in the third group there was Ilokos (G3) and Sumenep genotype (G5). The Bentanis genotype separates on its own group, and has close proximity to the Bima Brebes and Tajuk groups. These three genotypes are genotypes that have the ability to generate interest (sensitive to flowering).

Visualization with heatmap is widely used in metabolomic research because it can visualize large amounts of metabolomic data and classify it in sample groups[20]. Specific metabolite compounds of each sample of onion are visualized on each genotype column in bright green.

Mass spectrometry methods can accurately detect compounds including identification of unknown and unexpected compounds[18]. Identification of the molecular structure is often given without reference compounds, making it difficult to make a real identification[18]. The genotypes of shallot that have the ability to produce flowers will produce the specific metabolite compounds. Metabolite compounds produced in the transition process can be an indicator to determine the ability of flowering in shallot. The distribution of metabolomic compounds produced in non-vernalized shallot genotypes is presented in Figure 3.

The results showed each genotype produced different metabolite compound. In Bentanis genotype showed the presence of monomethyl ester (41.82%) higher than Sumenep genotype (2.12%). The monomethyl ester compound, in the form of Mg-protoporphyrin IX mono-methyl ester cyclase is the catalyst required for the oxidation process of chlorophyll formation in plants[21]. The Bentanis genotype grows faster than Sumenep genotype. Bentanis genotype has robust maximum vegetative growth compared to Sumenep genotype at 5 weeks after planting. The results in Table 1 show that Bentanis genotype has higher plant height (36.96 cm) than of Sumenep (29.82 cm). The presence of monomethyl ester (Mg-protoporphyrin IX mono-methyl ester cyclase) compounds can increase the formation of chlorophyll more optimally, thus increasing the photosynthesis process for the Bentanis genotype growth.

Low concentration of proline (3.55% and 5.56%) was produced in Bentanis and Tajuk genotypes (Figure 3). Proline compounds are generally produced by plants as their response to stress condition.

Bentanis genotype was also produced succinimide compounds at about 4.78%, those can not be found in other genotypes. Succinimide compounds are used as intermediate compounds in the process of forming organic compounds that stimulate plant growth.

The presence of high concentrate of ammonium carbamate compound is found in Bima Brebes genotype. Ammonium carbamate is an important compounds in the formation of carbamoyl phosphate and the formation of ATP into ADP[22]. While the presence of disulfide (dipropyl disulfide) compounds was found higher in non-sensitive flowering genotypes, such as Ilokos and Sumenep (1.69% and 5.43%), compared with other genotypes (0.8–0.14%). The content of dipropyl disulfide is an important component of activity as an antimicrobial and antioxidant and gives a distinctive aroma to shallot plant. The sulfur compounds in dipropyl disulfide is important the components of these metabolite compounds[23]. The sulfur compounds are known to have important activities as antimicrobials and antioxidants[24,25]. Derivative products of allicin compounds such as diallyl disulfide, diallyl trisulfide found in garlic essential oil indicate activity as a good antimicrobial[26].

Onion bulbs had the highest concentrations of propanethiol compounds having an aroma 20 times higher than dipropyl disulfide compounds[27]. It also found in non-flowering shallot plant, Ilokos and Sumenep genotypes, they produce higher acetic acid compounds (1.04% and 5.98%) compared to the flowering type, the Bentanis genotype (1.02%).

Utilization of sensitive instruments in metabolomic analysis is an important way to detect the content of plant essential compounds.Plant metabolites consist of highly diverse chemical compounds ranging

from inorganic compounds to hydrophilic, volatile alcohol and ketone compounds, amino and non-amino acids, hydrophobic lipids, and complex natural products[18]. The distribution of metabolomic compounds produced on 5 shallot genotypes with vernalization treatment is presented in the form of diagrams in Figure 4.

Utilization of mass spectrometry technology is a great way to solve research and analysis problems in the metabolomic field[18]. Metabolomic analysis was succesfully to identify potential compounds extracted directly from shallot genotypes. The results showed higher concentrations of acyclic ditherphene alcohol (phytol) compounds in flowering type shallot, Bentanis genotype (8.23%), Bima Brebes (2.2%) and Tajuk (7.52%) than the Ilokos genotype (1.15%) and Sumenep (2.19). The phytol compounds is an indication that vernalization treatment has a positive effect in inducing flowering of shallot genotypes, explaining that these three genotypes are sensitive to vernalization treatment to induce flower formation. Phytol compound is a compound that serves as a precursor of vitamin E and K. The content of these vitamins plays an important role in the plant body as an enzymatic cofactor in the process of metabolism that stimulates reproductive response. The content of vitamin E in the form of tocopherol is an important compound as one of the factors in the context of a cellular antioxidant system[28]. The differences of metabolite profile might play a key role in flowering initiation in shallot.

In flowering type of shallot genotypes, Bentanis, Bima Brebes and Tajuk also showed the formation of nitrogen oxide and semicarbazid hydrochlorid which are relatively lower than the non-

flowering type, Ilokos and Sumenep. Nitrogen compound is a compound that plays an important role in the vegetative phase of the plant. Once the plant enters the reproductive phase, the nitrogen compounds in the plant body will decrease. In contrast, the Ilokos and Sumenep genotypes show the formation of high nitrogen compounds ie ammonium compounds ammonium carbamat, and nitrogen oxide. High content of nitrogen compounds in Ilokos and Sumenep genotypes explains that these two genotypes are not sensitive to vernalization treatment, thus not encouraging the flowering formation.

The results also showed that the Sumenep genotype contained high organosulfur compounds compared with other genotypes, namely thiophene (2.31%) and trisulfide (4.25%). The organosulfur is an important compound in giving aroma and spiciness upsurge in shallot, and play an important role as an antioxidant. Essential compounds in *Allium* have strong antioxidant ability. Tests on antimicrobial activity showed that all essential oils on *Allium* were able to inhibit the activity of gram-positive and gram-negative bacteria[9].

Vernalization treatment triggered in the formation of proline compounds in the Sumenep genotype. Vernalization treatment provides a stress condition to the plants that increase the plant mechanism of adaptation by producing proline compounds. Proline accumulation in the plant body is a physiological response to stress, and as a generative tissue development[29]. Secondary metabolite is the cellular response in plant that receives external pressure.

The metabolite profile of 5 local onion genotypes is also rich in non-volatile phytonutrients, especially the content of phenolic compounds (phenol, 2-propenamide, 3-pyridinamine, N-furfurylidene

2-phenylethyl amine, pyrrolidinedione), amide compounds aziridine-2-carbothioic acid amide) and protein compound (pyroglutamic acid). The essential components, mono and sesquiterpenes, carbohydrates, phenols, alcohols, ether, aldehydes and ketones, are responsible for biological activity and also for their fragrance[30]. Phenols and polyphenol compounds play important roles as antioxidants in plants[31,32,33]. Research on *Allium rotundum* showed the presence of antioxidant activity of phenol compounds with high concentrations of up to 4%[12]. Onion extract contained the highest of phenol content, at 17.18 mg GAE per gram of fresh weight[34], and a phenol content of 114.70 mg GAE per 100 g of different onion samples[35].

Metabolite compound is difference among shallot genotypes. Each plant genotype performs metabolic activities that depend on their genetic conditions and the ability to adapt to its environment. The plant biological activity may imply to proportion of metabolite containing in a plant.

CONCLUSIONS

Secondary metabolite is associated to the flowering competency in shallot genotypes. GC-MS analysis in shallot of 5 genotypes detected 130 metabolites compounds in non-vernalized treatment, and detected 122 metabolites in vernalization treatment. Each genotypes produce different specific metabolites which is clustered according to the ability to flower. Genotype Bentanis, Bima Brebes and Tajuk were confirmed as flower-sensitive genotypes, with a lower content of proline and nitrogen compounds (ammonium carbamate and nitrogen

oxide), but with higher phytol compounds. Genotype Ilokos and Sumenep are flowering insensitive genotypes, which contain higher levels of organosulfur compounds (thiophene and trisulfide). Vernalization treatment caused the Sumenep genotype produces the highest proline compound among other genotypes.

ACKNOWLEDGEMENT

This researchs is supported by grants from National Innovation System Research Insentive 2016, Ministry of Research, Technology, and Higher Education, Indonesia.

REFERENCES

1. F.V. Golubev, N.A. Golubkina, and Y.N. Gorbunov. *Applied Biochemistry and Microbiology.* 39(5): 532–535 (2003)
2. G. Griffiths, L. Trueman, T. Crowther, B. Thomas, and B. Smith. *Phytotherapy research: PTR.* 16(7): 603–615 (2002)
3. T. Ritsema , and S. Smeekens. *Current Opinion in Plant Biology*. 6(3): 223–230 (2003)
4. H. Mohammadi-Motlagh , A. Mostafaie, and K. Mansouri *Archives of Medicaal Science.* 1: 38–44 (2011)
5. Marlin, A. Maharijaya, A. Purwito, and Sobir. SABRAO Journal of Breeding and Genetics. 50 (3) 313-328 (2018)
6. E.J. Finnegan, D. Bagnall, C. Helliwell, D. Rouse, C. Sheldon, M. Tadege, W.J. Peacock, and E. Dennis. *Vernalization*. Encyclopedia

of Life Sciences Macmillan Publishers Ltd, Nature Publishing Group. www.els.net (2001)

7. J. Song, A. Angel, M. Howard, and C. Dea. *Journal of Cell Science*. 125 (16): 3723–3731 (2012).
8. Y. Iijima. *Metabolite*. 4: 699–721 (2014).
9. D. Mnayer, A. Fabiano-Tixier, E. Petitcolas, T. Hamieh, N. Nehme, C. Ferrant, X. Fernandez, and F. Chemat. *Molecules*. 19: 20034–20053 (2014).
10. H. Xiao, and K.L. Parkin. *Phytochemistry*. 68(7): 1059–1067 (2007).
11. M.A. Vazquez-Prieto, and R.M. Miatello. *Molecular Aspects of Medicine*. 31(6): 540–5 (2010).
12. A.A. Dehpour, M. Yousefian, S.A. Jafary Kelarijani, M. Koshmoo, S. Mirzanegad, V. Mahdavi, S.E. Mousavi, E. Shirzad, M. Afzali, M.J. Javad Bayani, J.E. Olyaei, and M.K. Yahyapor. *Advances in Environmental Biology*. 6(3): 1020–1025 (2012).
13. V. Lanzotti, F. Scala, and G. Bonanomi. *Phytochemistry Reviews*. 13: 769–791 (2014).
14. N. Martins, S. Petropoulos, and I.C.F. Ferreira. *Food Chemistry*. 15(211): 41–50 (2016).
15. O. Fiehn. *Plant Molecular Biology*. 48: 155–171 (2002).
16. H. Wu, O.J. Gu, S. Chen, X. Liu, Y. Zhou, X. Zhang, and X. Xu. *Journal of pharmaceutical and biomedical analysis*. 72: 267–291 (2013).
17. T. Soininen. Ph.D. Thesis. University of Eastern Finland. Finland (2014).

18. S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, and J. Nielsen. *Mass Spectrometry Reviews.* 24: 613– 646 (2005).
19. N.C.J.P. Lekshmi, S. Viveka, M.B. Viswanathan, G. Manivannan, and T.M. Shobi. *Nanobio Pharmaceutical Technology.* doi: 10.13140/2.1.3278.7523. (2014).
20. S.M. Huang, W. Toh, P.I. Benke, C.S. Tan, and C.N. Ong. *Metabolomics.* 10:1084–1093 (2014).
21. Y.S. Wong, and P.A. Castelfranco *Plant Physiology*. 79(3): 730–733 (1985).
22. N. Baker, J. Harbinson, and D.M. Kramer. *Plant Cell Environment.* 30 1107–1125 (2007)
23. P. Rattanachaikunsopon, and P. Phumkhachorn P. *African Journal of Microbiology Research.* 3: 747–750 (2009).
24. A. Chehregani, F. Azimishad, and A.H. Haj. *International Journal of Agriculture Biology*. 9(6): 873–876 (2007)
25. C.L. Ye, D.H. Dai, and W.L. Hu. *Food Control.* 30: 48–53 (2013).
26. J.W. Kim, J.E. Huh, S.H. Kyung, and K.H. Kyung. *Food Science and Biotechnology.* 13: 235–239 (2004).
27. M.M. Lokke, M. Edelenbos, E. Larsen, and A. Feilberg. *Sensor*s. 12: 16060–16076 (2012).
28. M.G. Traber, J. Atkinson, and Atkinson. *Free Radical Biology and Medicine.* 43 (1): 4–15 (2007).
29. N. Verbruggen, and C. Hermans. *Amino Acids.* 35: 753–759 (2008).
30. E.A. El-Wakil, M.M. El-Sayed, and E.E. Abdel-Lateef. *International Journal of Pharma Sciences and Research IJPSR.* 6 (3) (2015).

31. S.A.B.E. Van Acker, D.J. van den Berg, N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, and W.J. van der Vijgh. *Free Radical Biology and Medicine* 20 (3): 331– 342 (1996).
32. M.A. Alvarez, N.B. Debattista, and N.B. Pappano. *Folia Microbiologica.* 53(1): 23–8 (2008).
33. A.P. Mukne, V. Viswanathan, and A.G. Phadatare. *Pharmacognosy Reviews.* 5(9): 13–18 (2011)
34. X. Lu, J. Wang, H.M. Al-Qadiri, C.F. Ross, J.R. Powers, J. Tang, and B.A. Rasco. *Food Chemistry.* 129: 637–644 (2011).
35. J. Yang, K.J. Meyers, J. van der Heide, and R.H. Liu. *Journal of Agriculture Food Chemistry*. 52: 6787–6793 (2004).

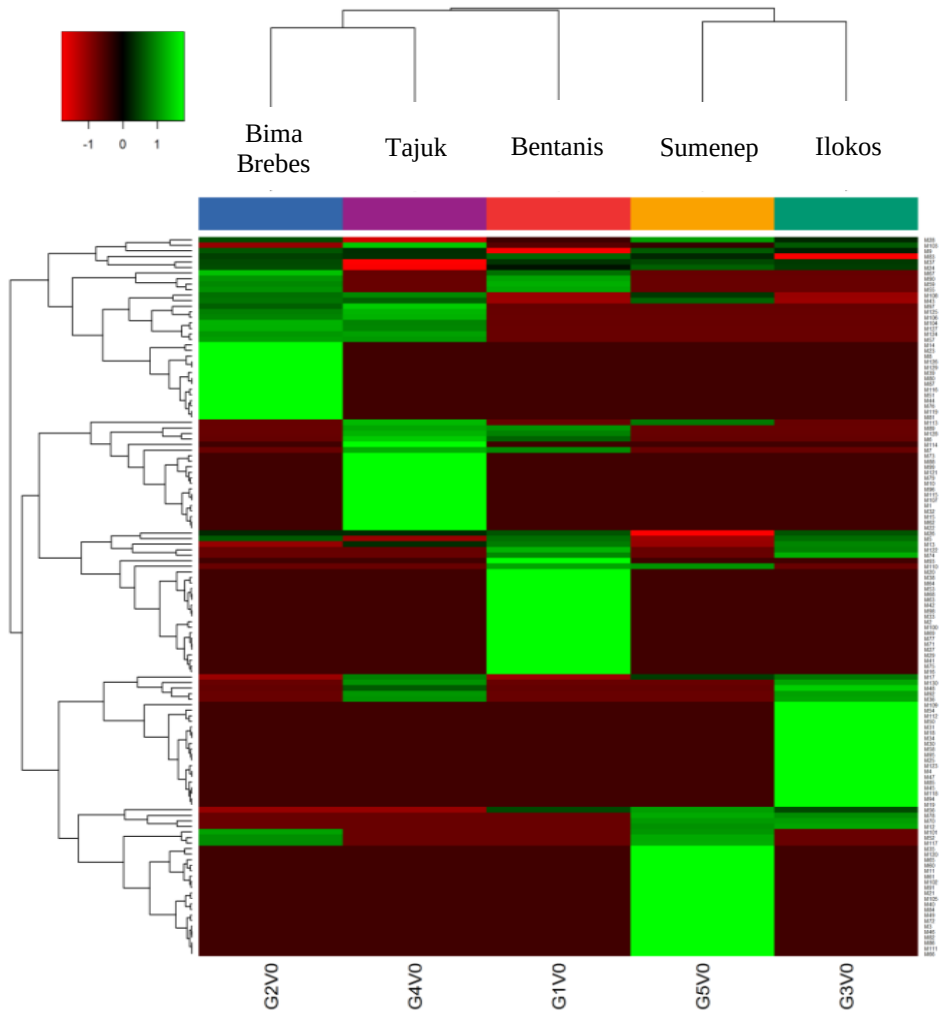


Figure 1 Heatmap of hierarchical clustering analysis based on non-targeted metabolomic in 5 shallot genotypes of 130 metabolites in non-vernalized bulbs.

Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered.

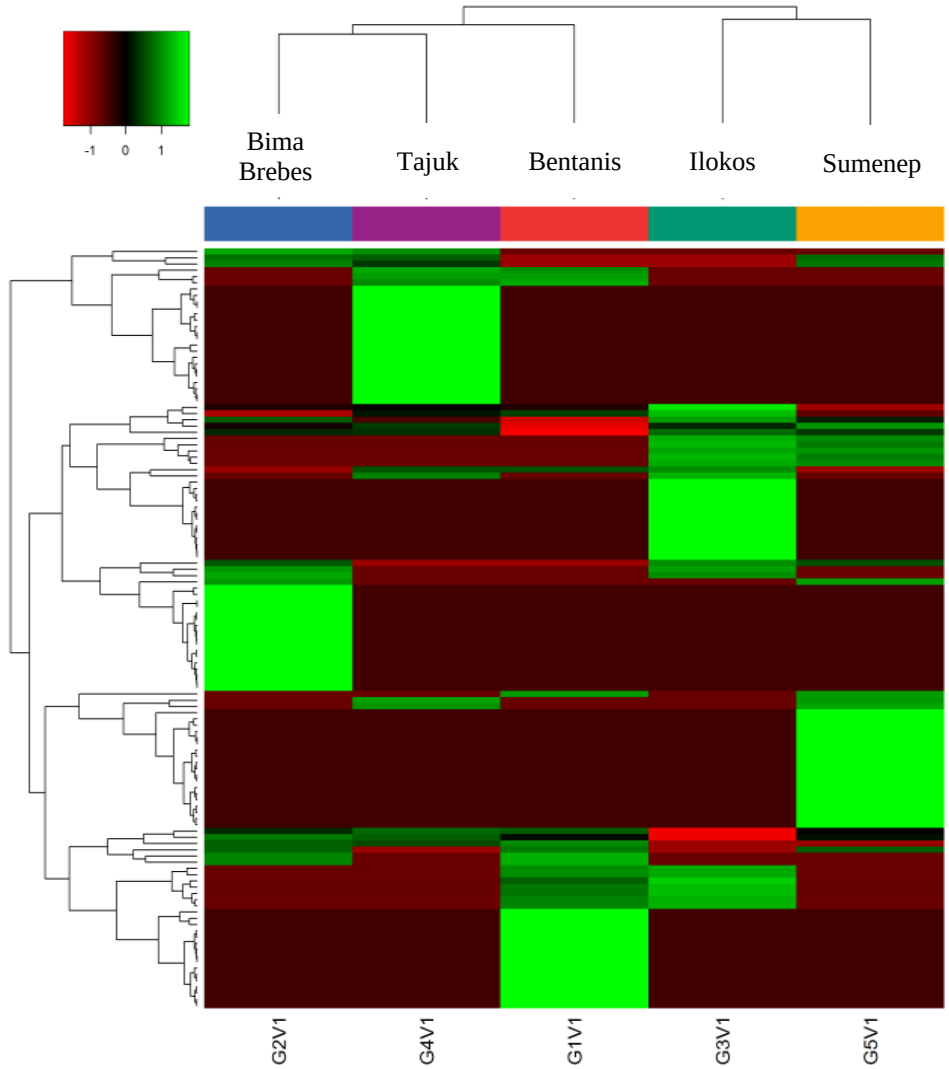


Figure 2 Heatmap of hierarchical group analysis based on non-targeted metabolomic in 5 shallot local genotypes of 122 metabolites in vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered.

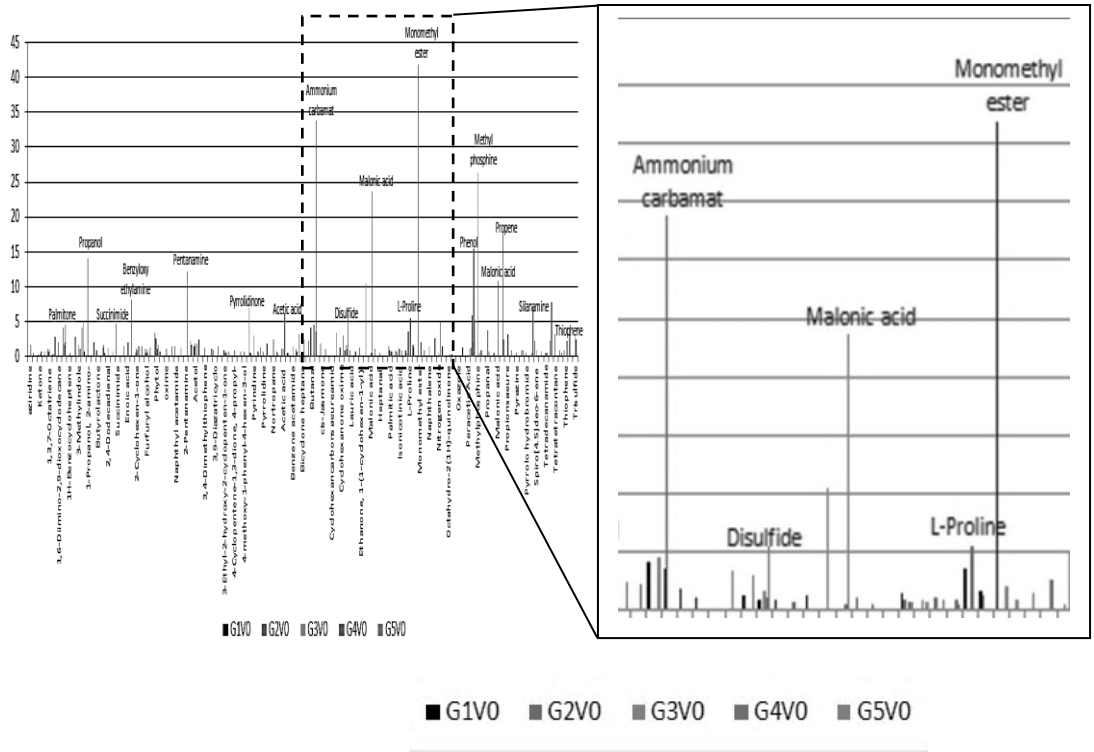


Figure 3 Metabolite composition in non-vernalized shallot genotypes. Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5)

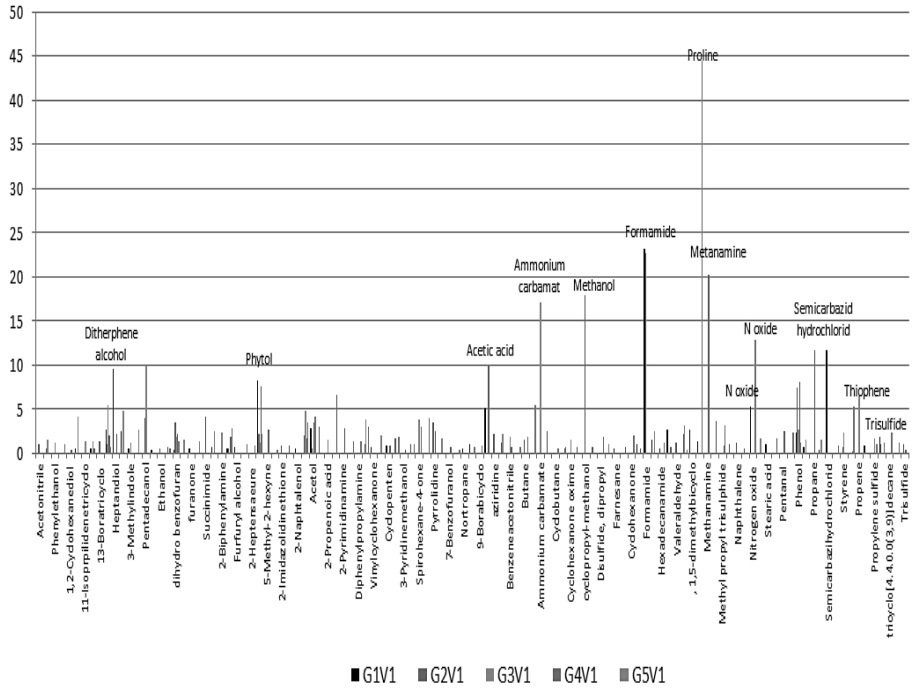


Figure 4 Metabolite compounds in 5 onion genotypes with vernalization treatment (5 mst). Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5)

Marlin UNIB <marlin@unib.ac.id>

Copyright of Transfer Form

marlin <marlin@unib.ac.id> 24 Mei 2019 pukul 23.32
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Dear Editors
Thank you for sending me the acceptance letter and invoice of my manuscript.
Herewith, I attached the Copyright of Transfer Form of my manuscript. Thank you.

Waiting for your next information

Best regards
Dr. Marlin
Fac. of Agriculture University of Bengkulu, Indonesia

Sent from my Samsung Galaxy smartphone.

Copyright (Marlin et al. 2019).pdf
15559K

Marlin UNIB <marlin@unib.ac.id>

Re: Manuscript Submission - Rasayan Journal

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 25 April 2019 pukul 18.40

Kepada: marlin@unib.ac.id

Dear Author,

Thanks for submitting your valuable manuscript for the review and subsequent publication in RASĀYAN Journal of Chemistry, which is A SCOPUS (Elsevier) indexed, Since 2008* and UGC (India) approved International Research Journal of Chemical Sciences.

Your Manuscript No. is: RJC- 5356/2019. Please use this number always in any of your future correspondence with us.

Revision-1

1. Please refer the attached **SAMPLE PAPER** send your manuscript again, as **Revision-1** mentioning the assigned Manuscript No. in the subject line. **References in the paper must be STRICTLY formatted as per the SAMPLE PAPER.**
2. Also, please provide the Names with Complete Affiliation and Contact Details of **03 Potential Reviewers** (who can review your manuscript promptly.), If not sent with the submission, Then only, it would be considered for the review purpose. Remember, no reviewer should be from your own institution and at least one of them must be from out of your Country.

Best regards,

Dr. Sanjay K. Sharma, FRSC

Editor, **RASĀYAN Journal of Chemistry**

On Tue, Apr 23, 2019 at 6:30 PM <marlin@unib.ac.id> wrote:

> Name: Marlin
> Address: Department of Crop science, Faculty of Agriculture, University of Bengkulu
> Jl. WR. Supratman Kandang Limun
> City: Bengkulu
> State: Indonesia
> Phone: +6285368227265
> Email: marlin@unib.ac.id
> Comment: Bengkulu, April 22nd 2019
>
> Prof.(Dr.) Sanjay K. Sharma

Editor-in-Chief
RASĀYAN Journal of Chemistry

Dear Editors,
I am pleased to submit an original research article entitled “The Secondary Metabolites and Its Relation to Flowering Competency in Shalot (Allium cepa var. aggregatum)” by Marlin, Awang Maharijaya, Agus Purwito, and Sobir for consideration for publication in RASĀYAN Journal of Chemistry. This manuscript builds on our study to determine the metabolites composition in shallot and its relation to flowering competency.
The non-targetted metabolites were identified using GC-MS and assigned by matching their mass spectra with those available in the WILEY7 LIB. In this manuscript, we show a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The metabolites composition was visualized in heatmap. Metabolites composition from 5 genotypes were difference and suggested correlated to flowering competency.
The submission has not been previously published, and is not under consideration for publication elsewhere. I certify that this article is new, and very appropriate to be published in RASĀYAN Journal of Chemistry. All authors have made substantial contributions to the concept and design of the research, acquisition of the data and its analysis, and drafting of the manuscript. We have no conflicts of interest to disclose.
Thank you for your consideration. We are looking forward to hearing from you soon.

Yours Sincerely,

Dr. Marlin
Department of Crop Production, Faculty of Agriculture, University of Bengkulu
Jalan WR. Supratman Kandang Limun Bengkulu 38121, Indonesia
Email : marlin@unib.ac.id

Attachment: Manuscript_Marlin et al. 2019.docx

--
Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)
www.rasayanjournal.com | www.rasayanjournal.co.in
Contact: +91 9001699997, +91 9414202678

_SAMPLE PAPER.doc
1024K

Marlin UNIB <marlin@unib.ac.id>

Revision-1 (RJC- 5356/2019)

Marlin UNIB <marlin@unib.ac.id> 26 April 2019 pukul 22.00
Kepada: rasayanjournal@gmail.com

Prof.(Dr.) Sanjay K. Sharma

Editor-in-Chief

RASĀYAN Journal of Chemistry

Dear Editors,

Thank you for your fast response to my submission entitled "The Secondary Metabolites and Its Relation to Flowering Competency in Shalot (*Allium cepa* var. *aggregatum*)" by Marlin, Awang Maharijaya, Agus Purwito, and Sobir. Here is I attach the document of Revision-1 for consideration for publication in RASĀYAN Journal of Chemistry. I also provide 3 potential reviewers for my manuscript as follow

1. Dr. Wahyuni, M.BioMed
Email : wahyuni@lipi.go.id
Indonesian Institute of Sciences (LIPI)
Jalan Jenderal Gatot Subroto No.10 Jakarta 12710, INDONESIA
2. Dr. Waras Nurcholis
Email : wnurcholis@gmail.com
Tropical Biopharmaca Research Center
Kampus IPB Taman Kencana
Jl. Taman Kencana No. 3, Bogor 16128, INDONESIA
3. Prof. Masayoshi SHIGYO
E-mail : shigyo@yamaguchi-u.ac.jp
Department. of Biological and Environmental Sciences
Faculty of Agriculture Yamaguchi University.
Yoshida 1677-1 Yamaguchi, 753-8515, JAPAN

Thank you for your consideration.

Yours Sincerely,

Dr. Marlin
Department of Crop Production, Faculty of Agriculture, University of Bengkulu
Jalan WR. Supratman Kandang Limun Bengkulu 38121, Indonesia

REVISION-1 (RJC- 53562019).docx
275K

Marlin UNIB <marlin@unib.ac.id>

Revision-1 (RJC- 5356/2019)

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 30 April 2019 pukul 13.24
Kepada: Marlin UNIB <marlin@unib.ac.id>

Well received with thanks.
[Kutipan teks disembunyikan]
--
Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)
www.rasayanjournal.com | www.rasayanjournal.co.in
Contact: +91 9001699997, +91 9414202678

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Date: 13/ 5/ 2019

Acceptance Letter

Dear **Dr. Marlin,**

We are glad to inform you that your manuscript, entitled-

MS No. RJC- 5356/2019: THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (*Allium cepa* var. *aggregatum*)

Marlin, A. Maharijaya, A. Purwito, and Sobir

has been reviewed and subsequently accepted for publication in **RASĀYAN Journal of Chemistry**. The paper will be published in **RJC, Vol.12, No.3, 2019** issue of the journal.

Article(s) will be published online on the website **www.rasayanjournal.com** prior to the printed version of **RASĀYAN Journal of Chemistry**.

Thanks for your interest in our journal.

Sincerely Yours,
S/d
Prof. Sanjay K. Sharma, FRSC
Editor-in-Chief

Abstracted/Indexed in :

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Date: 13/ 5/ 2019

Invoice

MS No. RJC- 5356/2019: THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (*Allium cepa* var. *aggregatum*)

Marlin, A. Maharijaya, A. Purwito, and Sobir

Herewith we are mentioning the charges towards publication and processing for the above said manuscript.

S. No.	Items	Net Payable Charges
1.	Publication and Procession Charges	**USD 400** (USD Four Hundred Only), Net Or Equivalent in Euro
2.	Bank Transfer Fee **(extra)**	Whatever applicable on the day of transfer

Note:

1. You can send the payment either by Draft in favor of **RASAYAN Journal of Chemistry**, payable at Jaipur (Rajasthan) or cash deposit in our account at Bank of Baroda or NEFT from any Bank or Net banking. Our **Bank Details** are given below.
2. To expedite the process of publication please clear the dues ASAP.Kindly send payment or confirm within10 days, otherwise, we shall presume, you are not interested in publishing this paper with us and we shall take up other pending papers for publication in this issue.

Bank Details:

A/C Holder: **Rasayan Journal of Chemistry, Jaipur**

A/C no.: **29720200000312**, Current Account

Bank: **Bank of Baroda, DCM Branch, Ajmer Road, Jaipur (Rajasthan) India**

SWIFT Code: **BARBINBBJAI**; IFS Code: **BARB0DCMAJM**

Abstracted/Indexed in :

Marlin UNIB <marlin@unib.ac.id>

Revision-1 (RJC- 5356/2019)

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 13 Mei 2019 pukul 15.23

Kepada: Marlin UNIB <marlin@unib.ac.id>

Dear Author,

Greetings from RASĀYAN Journal of Chemistry.

Your paper RJC-5356/2019 has been evaluated and can be published. We may inform you that we have a policy of Article Procession Charges, the cost of your paper shall be USD 400 **(USD Four Hundred only).**

The paper shall be published in Vol.12, No.3, 2019 issue of RASĀYAN Journal of Chemistry.

We are very happy to share with you that **SJR** powered by **SCOPUS (Elsevier)** announced the **Journal Ranking**# of Indian Journals abstracted in SCOPUS (Elsevier) and its matter of proud for us that **RASĀYAN J. Chem.** is on 2nd rank in this list and having significantly high H-index value = 17; which is quite encouraging and a proved evidence of the international quality publications in this journal. See the Link- http://www.scimagojr.com/journalrank.php?category=1601&area=1600&country=IN&year=2017

We assign **DOI** to every manuscript published in RASAYAN J. Chem. , which is assigned by **CrossRef**, an international organization known for quality control of scientific content. A manuscript linked with a DOI will be visible and searchable from all platforms and devices just on a click.

Kindly send payment within 10 days from the date of this mail, otherwise, we shall presume that you are not interested in publishing this paper with us and we shall take up other pending papers for publication in this issue.

Note:

1. *Formal acceptance letters and Comments of Reviewers for Revision shall be sent as soon as the payment is received.
2. You can send the payment either by Draft in favor of RASAYAN Journal of Chemistry, payable at Jaipur or cash deposit in Bank of Baroda in our account or NEFT from any Bank or Net banking.

Bank details:

A/C Holder: RASĀYAN Journal of Chemistry, Jaipur

A/C no.: **29720200000312**, Current Account

Bank: **Bank of Baroda, DCM Branch, Ajmer Road, Jaipur (Rajasthan) India**

SWIFT Code: **BARBINBBJAI**

IFS Code: **BARB0DCMAJM**

With Regards, your's-

Dr. Pratima Sharma

Managing Editor, RASĀYAN Journal of Chemistry

On Fri, Apr 26, 2019 at 8:31 PM Marlin UNIB <marlin@unib.ac.id> wrote:

[Kutipan teks disembunyikan]

[Kutipan teks disembunyikan]

Marlin UNIB <marlin@unib.ac.id>

Revision-1 (RJC- 5356/2019)

marlin <marlin@unib.ac.id> 13 Mei 2019 pukul 15.47
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Dear editors,
Thank you for your information about my payment responsibility. I'll send the publication payment within a week.

Kind regards
Dr. Marlin

Sent from my Samsung Galaxy smartphone.
[Kutipan teks disembunyikan]

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Reviewer's Report

SECTION-I : Details of Manuscript

Reviewer's Name:	
Complete Affiliation:	
E-Mail:	
Manuscript Number:	
Title:	THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (Allium cepa var. aggregatum)
Authors:	**Marlin[1*], A. Maharijaya[2], A. Purwito[2], and Sobir[2]** [1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia [2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia
Date of receiving by Reviewer:	May 12, 2019
Date of submission From Reviewer:	May 13, 2019

SECTION-II : Comments per Section of Manuscript

General comment:	**Please see in the text**
Introduction and Literature Review:	**Please see in the text**
Research Methodology:	**Please see in the text**
Results and Discussion:	**Please see in the text**
Bibliography/References:	**Please see in the text**
Others:	**Please see in the text**

UGC Recognized **and Abstracted/Indexed in:**

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

SECTION-III : Please rate the following

(1 = Excellent) (2 = Good) (3 = Fair) (4 = Poor)

Originality:	**3**
Contribution to the Field:	**2**
Technical Quality:	**3**
Clarity of Presentation :	**3**
Depth of Research:	**3**

SECTION-IV : Recommandation

(Kindly mark with an X)

Accept As it is:	
Requires Minor Revision:	
Requires Moderate Revision:	
Requires Major Revision:	**x**
Rejected for publication in RJC: (Please give reason)	

SECTION-V : Additional Comments (You may use additional sheet)

Signature of the Reviewer

Date: 13 May 2019

UGC Recognized **and Abstracted/Indexed in:**

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

UGC Recognized **and Abstracted/Indexed in:**

RJC

RASĀYAN *J. Chem.*
Vol. 12 | No. 2 |402 - 408| April - June | 2019
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (*Allium cepa* var. *aggregatum*)

Marlin[1*], A. Maharijaya[2], A. Purwito[2], and Sobir[2]
[1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia
[2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia
*E-mail : marlin@unib.ac.id

ABSTRACT

Shallot extract contains a great quantity of essential oil, volatiles, and other compounds. The composition of secondary metabolite can be regarded as the ultimate reactions of biological and physiological to genetic and environmental conditions. This study was objected to identify metabolomic composition and its relation to flowering competency in 5 genotypes of shallot. The non-targetted metabolites were identified using GC-MS and assigned by matching their mass spectra with those available in the WILEY7 LIB. Data matrix was analyzed by using metabolomic package of the R software. Heatmap was visualized for a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The composition metabolites from 5 genotypes were difference and suggested correlated to flowering competency. The 3 genotypes of flowering type of shallot produced highest concentration of phytol (ditherphene alcohol) and low concentration of nitrogen compound. Whereas, 2 genotypes of non-flowering types contained high concentration of organosulfur and nitrogen sources. Metabolite profile of 5 genotypes contained of volatile and non-volatile phytonutrients, vitamin, saturated fatty acid and organosulfur compounds.

Keywords: dissimilarity matrix, GC-MS, non-targetted metabolite, organosulfur, phytol

Commented [A1]: Please give "Vernalization" as your technic in flowering competency of shalot

Commented [A2]: Data not found in text!

Commented [A3]: What compounds that correlated with flowering competency?

INTRODUCTION

Shallot (*Allium cepa* var. *aggregatum*) is an important *Allium* plant that been used for food, ornamental, natural alternatives to food synthetic preservatives and medicinal for curing various diseases. *Allium* plants contain different sugars, amino acids, vitamins, sulfurous compounds, enzymes, flavonoids, saponins and minerals[1]. *Allium* are also valued for their therapeutic properties; they are active as diuretics and laxatives and have been used to treat headaches and parasitic worms[2]. Onion has beneficial effect as their prebiotic, and their ability to decrease levels of insulin, cholesterol, triacylglycerol and phospholipids for human health[3], and considered an important vegetable and medicinal plant prescribed as an effective therapy for several complaints in healing treatment such as natural cancer treatment[4]. These beneficial effects will become an important target in shallot breeding and management practices.

Shallot genotypes may vary in flowering pattern, naturally flowering, inducible flowering, and non-inducible flowering shallot[5]. Flowering in shallot can be induce by an exogenous treatment such as vernalization. Vernalization is an important adaptation of plants to initiate flowering in response to a prolonged exposure to low temperatures[6,7]. The principles derived from vernalization are likely to be widely relevant to epigenetic reprogramming in many organisms[7]. External condition plays important regulation in plant bioactivity and influences the metabolomic composition. Metabolomic composition in plant is various and specific, that depends on genetic and plant growth environment. Metabolomic describes plant phenotype differentiation.

CrossMark

Metabolomic technologies have revealed a new insights in biological systems through metabolic dynamics[8]. The metabolomic composition determines biological and physiological function of the plants. Metabolite compounds are observed in the 6 *Alliaceae* family[9], in *Allium cepa* (onion) extracts[10,11], in *Allium rotundum* L[12], and in *Allium sativum* (garlic) formulations[13,14]. Metabolomic defined as a comprehensive analysis in which all the metabolites of a biological system are identified and quantified[15]. Metabolites are the end products of cellular regulatory processes. Metabolites profiling in plant can be directly extracted from a part of plant to find new molecules contents. Profiling of the metabolite composition is an important way to evaluate the contents of the medical plants, as well as their safety and efficacy[16].

Mass spectrometry is an analytical method, which allows the determination of the mass formula and structure. With mass spectrometry, individual molecules can be measured based on their molecular peak (m/z) and characteristics spectra of fragment ions[17]. Mass spectrometry technology is high sensitivity to identify the unknown and unexpected of the components present in the complex biological samples[18]. These fragments often provide a clue to the molecule's structure, but without a reference compound, the difficulty of making an identification increases markedly[17]. The analytical strategy *gase chromatography mass spectrometry* (GC-MS) used to analyze the volatile compounds, and selected compounds were structurally measured by mass spectrometry transposing the method to GC-MS[19]. The mass analyzer separates the molecules or/and fragments according to their masses and the detector detects and quantifies the separated ions[17]. GC-MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal-mass information. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories databases[18]. This study was objected to identify secondary metabolite composition and its relation to flowering competency in 5 genotypes of shallot.

EXPERIMENTAL

Plant materials

Local genotypes of shallot with different pattern of flowering were selected as plant material. Shallot genotype 'Bentanis' is used as the naturally flowering, while genotypes 'Bima Brebes Ilokos, Tajuk and Sumenep' are selected as the induced flowering of shallot (Marlin *et al.*, 2018). Each genotypes were treated with vernalization in 8 °C temperature for 5 weeks, and no vernalization treatment as control. The bulbs were then planted in 45 cm diameter polythene bags containing 8 kg of growing media (soil: manure: husk = 2: 1: 1). Each polybag was planted with three bulbs. Before planting, shallots bulbs were soaked in a fungicide benomil 50% at a concentration of 2 $g.L^{-1}$ for 15 minutes. The bulbs were then soaked into solution of plant growth promoting rhizobacteria of 5 $g.L^{-1}$ for 15 minutes. NPK fertilizers with a ratio of 15:15:15 were applied in a dose of 600 $kg.ha^{-1}$ or 2.4 g per polybag.

Commented [A4]: Are you sure, this material has been identified? Please give information such as identified or voucher specimen in your work.

Commented [A5]: Where location? Please give information for coordinate geography your research location.

Metabolomic analysis

The samples were the leaves of shallot plants at 4 weeks after planting. GC-MS unit was carried out on an GCMS-QP2010 system (Shimadzu Corporation, Japan) coupled to mass spectrometer detector. The samples were inserted into the quartz chamber in the GC-MS unit. Helium was used as a carrier gas in a constant flow mode at 0.85 ml/min. The chamber was heated in an oxygen-free environment at a temperature of 400 °C for 0.2 minutes. The reaction will produce heat-mediated cleavage of chemical bonds in the macromolecular structure and produce low molecular weight with a chemical composition that identify specific compound of metabolite. Compound mixtures were then passed through the column GC-MS analysis. The column is Rt x 5 MS, with length 60.0 m, thickness 0.25 µm, and diameter 0.25 mm. The initial temperature of the column was 50 °C, which was gradually increased by 10 °C up to 280 °C. At the end of this period, the column oven temperature was 50 °C raised up to 280 °C. Injection port temperature was ensured as 280 °C and helium flow rate as 0.85 ml/min. Mass spectrometer detector was employed to detect compounds when they were vented from the column. Temperature of the detector was 200 °C. The volatile compounds of the plant samples were then identified for each treatments.

The identification of the components of non-~~targetted~~targeted metabolite was assigned by matching their mass spectra with those available in the WILEY7 LIB. The volatile compounds of the plant samples were then identified for each genotypes. Spectra were compared with National Institute of Standard and

Commented [A6]: Please give information type of your sample that injected in GC-MS? Extract or another types of your sample, please give explain more?

Technology (NIST, 2005 v2.1) library to identify the unknown compounds. The data were confirmed using PubChem online database in National Center for Biotechnology Information (https://pubchem.ncbi. nlm.nih.gov/), chemicalbook database (http://www.chemicalbook.com), and NIST webbook database http://webbook.nist.gov/. The datasets were then log-transformed in order to acquire the normalized data. Data matrix based on non-targetted metabolomic of shallot in each vernalization treatments were analyzed using Metabolomic package of the R software (R version 3.2.2 http://www.r-project.org/). Metabolomics package was operated to construct HCA (*hierarchical cluster analysis*) and visulized heatmap with dendrogram. HCA was performed using Euclidean distance method and complete linkage agglomerative method.

RESULTS AND DISCUSSION

Metabolite compounds in 5 shallot local genotypes were affected significantly by vernalization treatments. A total of 130 of metabolomic compound of non-vernalized bulbs, and 122 of metabolites compound of vernalized bulbs of 5 shallot local genotypes were identified by GC-MS. Metabolite compound obtained where then visually described by metabolite heatmap (Figure-1). Heatmap is widely used in metabolomic research because it is able to visualize large amounts of metabolite data and its differences in sample groups[20]. Heatmap of both non vernalized and vernalized bulbs clustered 5 shallot local genotypes into three groups (Figure-1). First group was G2 (Bima Brebes genotype) and G4 (Tajuk genotype), second group was G1 (Bentanis genotype), and the third group was G3 (Ilokos genotype) and G5 (Sumenep genotype).

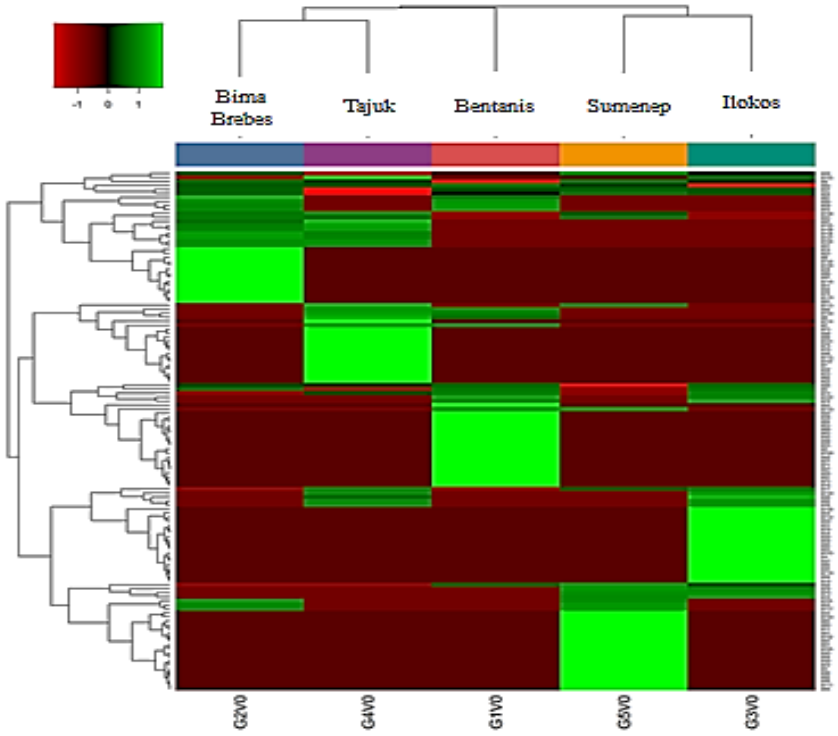


Fig.-1: Heatmap of hierarchical clustering analysis based on non-targeted metabolomic in 5 shallot genotypes of 130 metabolites in non-vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered.

Commented [A7]: In this section, I not found correlation between metabolite with flowering competency in shallot? Can you added more explain, because your novelty in this research was metabolite in flowering competency in shallot. If you not found correlation with Heatmap analysis, may be analysis data can be use with simple correlation between major metabolite and flowering competency data.

Commented [A8]: Please give data in table for these metabolites?

Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by different colour of each heatmap coloumn. Specific metabolite compounds were visualized on each genotype column with a bright green color. Hierarchical clustering analysis showed that the genotype had a significant effect on the metabolites composition of the shallot. Each genotype of shallot produces different metabolites that associated to the flowering competency. Hierarchical clustering analysis of shallot metabolites grouped shallot genotypes into three distinct genotypes, in both vernalization treatments (Figure-1 and 2). The result indicated that the presence of specific compounds produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability.

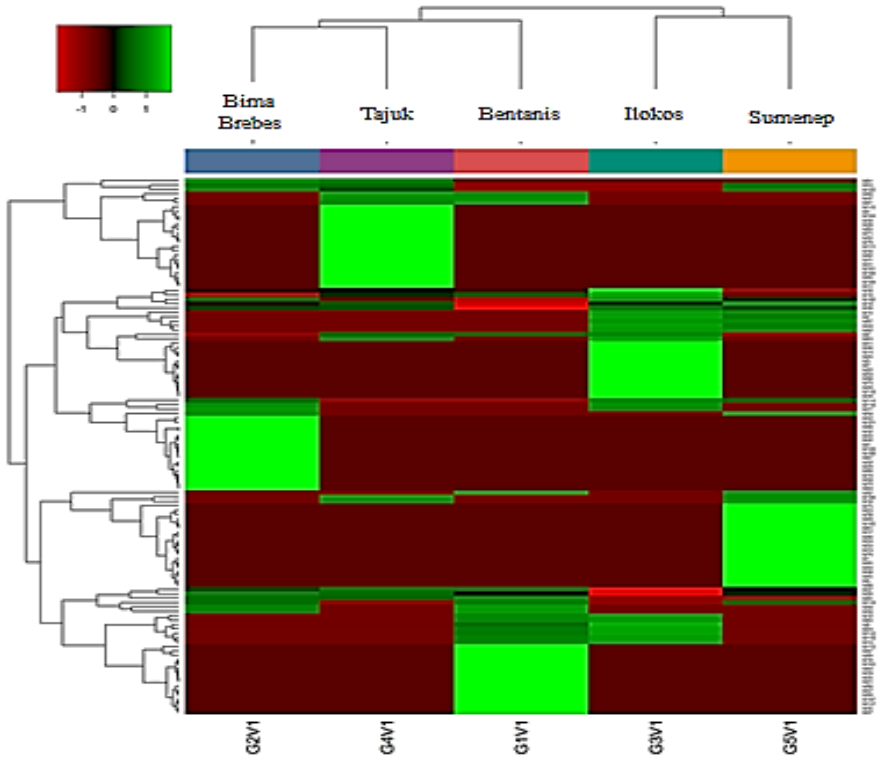


Fig.-2: Heatmap of hierarchical group analysis based on non-targeted metabolomic in 5 shallot local genotypes of 122 metabolites in vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered

Shallot genotypes located in the same group showed that the genotype had the same metabolite profile, and showed significant metabolite diversity with other groups. Visualization with heatmap also confirmed in the presence of different colors from each of the heatmap columns. The genotypes included in the first group were the Bentanis genotype (G1). In the second group there was the Bima Brebes (G2) and Tajuk genotype (G4), while in the third group there was Ilokos (G3) and Sumenep genotype (G5). The Bentanis

genotype separates on its own group, and has close proximity to the Bima Brebes and Tajuk groups. These three genotypes are genotypes that have the ability to generate interest (sensitive to flowering).

Visualization with heatmap is widely used in metabolomic research because it can visualize large amounts of metabolomic data and classify it in sample groups[20]. Specific metabolite compounds of each sample of onion are visualized on each genotype column in bright green.

Mass spectrometry methods can accurately detect compounds including identification of unknown and unexpected compounds[18]. Identification of the molecular structure is often given without reference compounds, making it difficult to make a real identification[18]. The genotypes of shallot that have the ability to produce flowers will produce the specific metabolite compounds. Metabolite compounds produced in the transition process can be an indicator to determine the ability of flowering in shallot. The distribution of metabolomic compounds produced in non-vernalized shallot genotypes is presented in Figure-3.

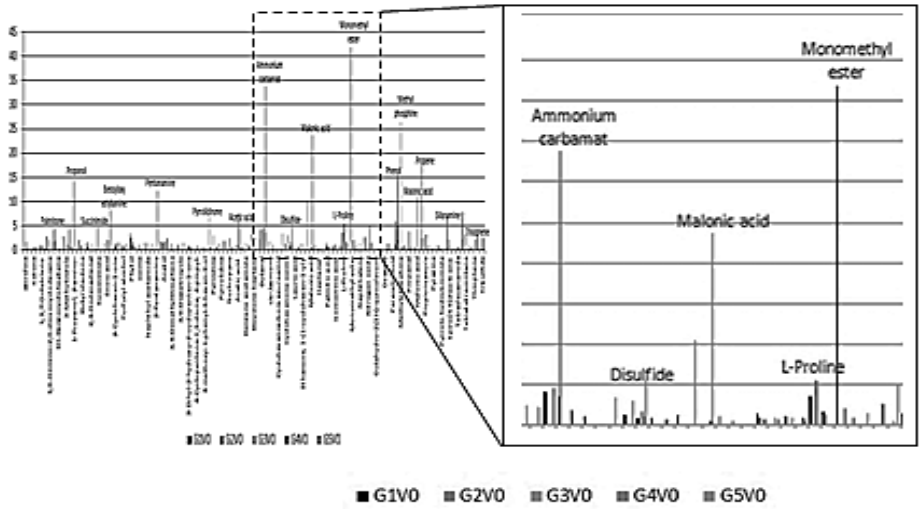


Fig.-3: Metabolite composition in non-vernalized shallot genotypes. Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5)

The results showed each genotype produced different metabolite compound. In Bentanis genotype showed the presence of monomethyl ester (41.82%) higher than Sumenep genotype (2.12%). The monomethyl ester compound, in the form of Mg-protoporphyrin IX mono-methyl ester cyclase is the catalyst required for the oxidation process of chlorophyll formation in plants[21]. The Bentanis genotype grows faster than Sumenep genotype. Bentanis genotype has robust maximum vegetative growth compared to Sumenep genotype at 5 weeks after planting. The results in Table 1 show that Bentanis genotype has higher plant height (36.96 cm) than of Sumenep (29.82 cm). The presence of monomethyl ester (Mg-protoporphyrin IX mono-methyl ester cyclase) compounds can increase the formation of chlorophyll more optimally, thus increasing the photosynthesis process for the Bentanis genotype growth.

Low concentration of proline (3.55% and 5.56%) was produced in Bentanis and Tajuk genotypes (Figure-3). Proline compounds are generally produced by plants as their response to stress condition. Bentanis genotype was also produced succinimide compounds at about 4.78%, those can not be found in other genotypes. Succinimide compounds are used as intermediate compounds in the process of forming organic compounds that stimulate plant growth.

The presence of high concentrate of ammonium carbamate compound is found in Bima Brebes genotype. Ammonium carbamate is an important compounds in the formation of carbamoyl phosphate and the formation of ATP into ADP[22]. While the presence of disulfide (dipropyl disulfide) compounds was found

higher in non-sensitive flowering genotypes, such as Ilokos and Sumenep (1.69% and 5.43%), compared with other genotypes (0.8–0.14%). The content of dipropyl disulfide is an important component of activity as an antimicrobial and antioxidant and gives a distinctive aroma to shallot plant. The sulfur compounds in dipropyl disulfide is important the components of these metabolite compounds[23]. The sulfur compounds are known to have important activities as antimicrobials and antioxidants[24,25]. Derivative products of allicin compounds such as diallyl disulfide, diallyl trisulfide found in garlic essential oil indicate activity as a good antimicrobial[26].

Onion bulbs had the highest concentrations of propanethiol compounds having an aroma 20 times higher than dipropyl disulfide compounds[27]. It also found in non-flowering shallot plant, Ilokos and Sumenep genotypes, they produce higher acetic acid compounds (1.04% and 5.98%) compared to the flowering type, the Bentanis genotype (1.02%).

Utilization of sensitive instruments in metabolomic analysis is an important way to detect the content of plant essential compounds.Plant metabolites consist of highly diverse chemical compounds ranging from inorganic compounds to hydrophilic, volatile alcohol and ketone compounds, amino and non-amino acids, hydrophobic lipids, and complex natural products[18].

Utilization of mass spectrometry technology is a great way to solve research and analysis problems in the metabolomic field[18]. Metabolomic analysis was succesfully to identify potential compounds extracted directly from shallot genotypes. The results showed higher concentrations of acyclic ditherphene alcohol (phytol) compounds in flowering type shallot, Bentanis genotype (8.23%), Bima Brebes (2.2%) and Tajuk (7.52%) than the Ilokos genotype (1.15%) and Sumenep (2.19). The phytol compounds is an indication that vernalization treatment has a positive effect in inducing flowering of shallot genotypes, explaining that these three genotypes are sensitive to vernalization treatment to induce flower formation. Phytol compound is a compound that serves as a precursor of vitamin E and K. The content of these vitamins plays an important role in the plant body as an enzymatic cofactor in the process of metabolism that stimulates reproductive response. The content of vitamin E in the form of tocopherol is an important compound as one of the factors in the context of a cellular antioxidant system[28]. The differences of metabolite profile might play a key role in flowering initiation in shallot.

In flowering type of shallot genotypes, Bentanis, Bima Brebes and Tajuk also showed the formation of nitrogen oxide and semicarbazid hydrochlorid which are relatively lower than the non-flowering type, Ilokos and Sumenep. Nitrogen compound is a compound that plays an important role in the vegetative phase of the plant. Once the plant enters the reproductive phase, the nitrogen compounds in the plant body will decrease. In contrast, the Ilokos and Sumenep genotypes show the formation of high nitrogen compounds ie ammonium compounds ammonium carbamat, and nitrogen oxide. High content of nitrogen compounds in Ilokos and Sumenep genotypes explains that these two genotypes are not sensitive to vernalization treatment, thus not encouraging the flowering formation.

The results also showed that the Sumenep genotype contained high organosulfur compounds compared with other genotypes, namely thiophene (2.31%) and trisulfide (4.25%). The organosulfur is an important compound in giving aroma and spiciness upsurge in shallot, and play an important role as an antioxidant. Essential compounds in *Allium* have strong antioxidant ability. Tests on antimicrobial activity showed that all essential oils on *Allium* were able to inhibit the activity of gram-positive and gram-negative bacteria[9].

Vernalization treatment triggered in the formation of proline compounds in the Sumenep genotype. Vernalization treatment provides a stress condition to the plants that increase the plant mechanism of adaptation by producing proline compounds. Proline accumulation in the plant body is a physiological response to stress, and as a generative tissue development[29]. Secondary metabolite is the cellular response in plant that receives external pressure.

The metabolite profile of 5 local onion genotypes is also rich in non-volatile phytonutrients, especially the content of phenolic compounds (phenol, 2-propenamide, 3-pyridinamine, N-furfurylidene 2-phenylethyl amine, pyrrolidinedione), amide compounds aziridine-2-carbothioic acid amide) and protein compound (pyroglutamic acid). The essential components, mono and sesquiterpenes, carbohydrates, phenols, alcohols, ether, aldehydes and ketones, are responsible for biological activity and also for their fragrance[30]. Phenols and polyphenol compounds play important roles as antioxidants in plants[31,32,33].

Research on *Allium rotundum* showed the presence of antioxidant activity of phenol compounds with high concentrations of up to 4%[12]. Onion extract contained the highest of phenol content, at 17.18 mg GAE per gram of fresh weight[34], and a phenol content of 114.70 mg GAE per 100 g of different onion samples[35].

Metabolite compound is difference among shallot genotypes. Each plant genotype performs metabolic activities that depend on their genetic conditions and the ability to adapt to its environment. The plant biological activity may imply to proportion of metabolite containing in a plant.

CONCLUSION

Secondary metabolite is associated to the flowering competency in shallot genotypes. GC-MS analysis detected 130 metabolites compounds in non-vernalized treatment, and detected 122 metabolites in vernalization treatment in 5 genotypes of shallot. Each genotypes produce different specific metabolites which is clustered according to the flowering competency. Genotype Bentanis, Bima Brebes and Tajuk were confirmed as flower-sensitive genotypes, with a lower content of proline and nitrogen (ammonium carbamate and nitrogen oxide), and higher phytol compounds. Genotype Ilokos and Sumenep are flowering insensitive genotypes, which contain higher level of organosulfur compounds (thiophene and trisulfide). Vernalization treatment caused the Sumenep genotype produces the highest proline compound among other genotypes.

Commented [A9]: Please answer your objective in this study

Commented [A10]: Data not found in text

ACKNOWLEDGMENT

This researchs is supported by grants from National Innovation System Research Insentive 2016, Ministry of Research, Technology, and Higher Education, Indonesia.

REFERENCES

1. F.V. Golubev, N.A. Golubkina, and Y.N. Gorbunov. *Applied Biochemistry and Microbiology.* 39(5): 532–535 (2003)
2. G. Griffiths, L. Trueman, T. Crowther, B. Thomas, and B. Smith. *Phytotherapy research: PTR.* 16(7): 603–615 (2002)
3. T. Ritsema , and S. Smeekens. *Current Opinion in Plant Biology.* 6(3): 223–230 (2003)
4. H. Mohammadi-Motlagh , A. Mostafaie, and K. Mansouri *Archives of Medicaal Science.* 1: 38–44 (2011)
5. Marlin, A. Maharijaya, A. Purwito, and Sobir. *SABRAO Journal of Breeding and Genetics*. 50 (3) 313-328 (2018)
6. E.J. Finnegan, D. Bagnall, C. Helliwell, D. Rouse, C. Sheldon, M. Tadege, W.J. Peacock, and E. Dennis. Vernalization. Encyclopedia of Life Sciences Macmillan Publishers Ltd, Nature Publishing Group. (2001). www.els.net.
7. J. Song, A. Angel, M. Howard, and C. Dea. *Journal of Cell Science.* 125 (16): 3723–3731 (2012).
8. Y. Iijima. *Metabolite.* 4: 699–721 (2014).
9. D. Mnayer, A. Fabiano-Tixier, E. Petitcolas, T. Hamieh, N. Nehme, C. Ferrant, X. Fernandez, and F. Chemat. *Molecules.* 19: 20034–20053 (2014).
10. H. Xiao, and K.L. Parkin. *Phytochemistry.* 68(7): 1059–1067 (2007).
11. M.A. Vazquez-Prieto, and R.M. Miatello. *Molecular Aspects of Medicine.* 31(6): 540–5 (2010).
12. A.A. Dehpour, M. Yousefian, S.A. Jafary Kelarijani, M. Koshmoo, S. Mirzanegad, V. Mahdavi, S.E. Mousavi, E. Shirzad, M. Afzali, M.J. Javad Bayani, J.E. Olyaei, and M.K. Yahyapor. *Advances in Environmental Biology.* 6(3): 1020–1025 (2012).
13. V. Lanzotti, F. Scala, and G. Bonanomi. *Phytochemistry Reviews.* 13: 769–791 (2014).
14. N. Martins, S. Petropoulos, and I.C.F. Ferreira. *Food Chemistry.* 15(211): 41–50 (2016).
15. O. Fiehn. *Plant Molecular Biology*. 48: 155–171 (2002).
16. H. Wu, O.J. Gu, S. Chen, X. Liu, Y. Zhou, X. Zhang, and X. Xu. *Journal of pharmaceutical and biomedical analysis.* 72: 267–291 (2013).

17. T. Soininen. Ph.D. Thesis. University of Eastern Finland. Finland (2014).
18. S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, and J. Nielsen. *Mass Spectrometry Reviews.* 24: 613– 646 (2005).
19. N.C.J.P. Lekshmi, S. Viveka, M.B. Viswanathan, G. Manivannan, and T.M. Shobi. *Nanobio Pharmaceutical Technology.* (2014). DOI : 10.13140/2.1.3278.7523.
20. S.M. Huang, W. Toh, P.I. Benke, C.S. Tan, and C.N. Ong. *Metabolomics.* 10:1084–1093 (2014).
21. Y.S. Wong, and P.A. Castelfranco *Plant Physiology.* 79(3): 730–733 (1985).
22. N. Baker, J. Harbinson, and D.M. Kramer. *Plant Cell Environment.* 30 1107–1125 (2007)
23. P. Rattanachaikunsopon, and P. Phumkhachorn P. *African Journal of Microbiology Research.* 3: 747–750 (2009).
24. A. Chehregani, F. Azimishad, and A.H. Haj. *International Journal of Agriculture Biology.* 9(6): 873–876 (2007)
25. C.L. Ye, D.H. Dai, and W.L. Hu. *Food Control.* 30: 48–53 (2013).
26. J.W. Kim, J.E. Huh, S.H. Kyung, and K.H. Kyung. *Food Science and Biotechnology.* 13: 235–239 (2004).
27. M.M. Lokke, M. Edelenbos, E. Larsen, and A. Feilberg. *Sensor*s. 12: 16060–16076 (2012).
28. *M.G. Traber, J. Atkinson, and Atkinson. Free Radical Biology and Medicine. 43 (1): 4–15 (2007).*
29. N. Verbruggen, and C. Hermans. *Amino Acids.* 35: 753–759 (2008).
30. E.A. El-Wakil, M.M. El-Sayed, and E.E. Abdel-Lateef. *International Journal of Pharma Sciences and Research IJPSR.* 6 (3) (2015).
31. S.A.B.E. Van Acker, D.J. van den Berg, N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, and W.J. van der Vijgh. *Free Radical Biology and Medicine* 20 (3): 331– 342 (1996).
32. M.A. Alvarez, N.B. Debattista, and N.B. Pappano. *Folia Microbiologica.* 53(1): 23–8 (2008).
33. A.P. Mukne, V. Viswanathan, and A.G. Phadatare. *Pharmacognosy Reviews.* 5(9): 13–18 (2011)
34. X. Lu, J. Wang, H.M. Al-Qadiri, C.F. Ross, J.R. Powers, J. Tang, and B.A. Rasco. *Food Chemistry.* 129: 637–644 (2011).
35. J. Yang, K.J. Meyers, J. van der Heide, and R.H. Liu. *Journal of Agriculture Food Chemistry.* 52: 6787–6793 (2004).

[RJC-4034/2018]

Marlin UNIB <marlin@unib.ac.id>

Proof of payment

Marlin UNIB <marlin@unib.ac.id> 18 Mei 2019 pukul 22.24
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Dear Editors

RASAYAN Journal of Chemistry.

I'd like to inform you that I've been transfer the article procession charges of my article (**RJC-5356/2019**), entittle : The Secondary Metabolites and Its Relation to Flowering Competency in Shalot (Allium cepa var. aggregatum, on May 17. 2019.

Here, I attach the proof of payment I have transferred to **RASĀYAN Journal of Chemistry, Jaipur**
A/C no.: **29720200000312**, Current Account
Bank of Baroda, DCM Branch, Ajmer Road, Jaipur (Rajasthan) India
SWIFT Code: **BARBINBBJAI**
IFS Code: **BARB0DCMAJM**, amounting to $ 400.

We are looking forward to hearing from you soon.

Sincerelly

Dr. Marlin

Department of Crop Production, Faculty of Agriculture, University of Bengkulu

3 lampiran

2.jpg
712K

3.jpg
666K

1.jpg
605K

SECTION-I : Details of Manuscript

Manuscript Number:	RJC_5356
Title:	THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (Allium cepa var. aggregatum)
Authors:	Marlin[1*], A. Maharijaya[2], A. Purwito[2], and Sobir[2] [1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia [2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia

SECTION-II : Comments per Section of Manuscript

Reviewer 1 Comments	Justification/Revision
General comment:	
A1. Please give "Vernalization" as your technic in flowering competency of shalot	Thank you for raising very interesting points. We have now strengthened the title with "vernalization" as follow : SECONDARY METABOLITES CHANGE UNDER VERNALIZATION AND ITS RELATION TO FLOWERING COMPETENCY IN SHALLOT (*Allium cepa* var. *aggregatum*)
A2. Data not found	Thank you for the comment. We have showed the data in results part (figure 1 and 2) that 130 0f metabolites were detected in non vernalized bulbs dan 122 of metabolites were found in vernalized bulbs.
A3. What compounds that correlated with flowering competency?	Thank you for the comment. In results and discussion, we showed that ditherphene alcohol (phytol) associated to flowering competency in shallot.
Introduction and Literature :	-
Research Methodology:	

A4. Are you sure, this material has been identified? Please give information such as identified or voucher specimen in your work	Thank you for the suggestion. Those material had been identified and published (Marlin et al. 2018), as describe in the text.
A5. Where location? Please give information for coordinate geography your research location.	Thank you for raising very interesting points. We have now stated that the experiments were located in Pasir Kuda research farm, Bogor Indonesia.
A6. Please give information type of your sample that injected in GC-MS? Extract or another types of your sample, please give explain more?	Thank you for the suggestion. We agree and have added more information on sample preparation, as follow : The samples were the leaves of shallot plants at 4 weeks after planting (bolting stage). Leaves were cutted for 10 g and were extracted with methanol (50 mL). The methanolic extract of shallot leaves were used as a sample in the GC-MS analysis.
Results and Discussion	
A7. In this section, I not found correlation between metabolite with flowering competency in shallot? Can you added more explain, because your novelty in this research was metabolite in flowering competency in shallot. If you not found correlation with Heatmap analysis, may be analysis data can be use with simple correlation between major metabolite and flowering competency data.	Thank you for the comment. We stated that metabolite had a correlation with flowering competency in shallot as describing in heatmap of hierarchical clustering analysis. The heatmap showed that each shallot genotype produces different metabolites associated to the flowering competency. The results indicated that there are a presence of acyclic ditherpene alcohol (phytol) which were produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability. The phytol presence in vernalized bulb is an indication that vernalization treatment has a positive effect in inducing flowering of shallot.
A8. Please give data in table for these metabolites?	Thank you for the comment. We have describe the data in figure 1 and 2. It will be redundant if the data showed in a table.
A9. Please answer your objective in this study	Thank you for the suggestion. We agree and change the conclusion.
A10. Data not found in text	Thank you for the comment. The data have clearly in Figure 1 and 2 in both vernalization treatments.

Bibliography/References	-
Others:	-

Reviewer 2 Comments	Justification/Revision
General comment: The manuscript describes the metabolite composition in leaves of 5 shallot genotypes whose bulbs were untreated and treated by vernalization. The results may have added values to the scientific community about metabolomic changes induced by vernalization that are important for breeding.	Thank you for the suggestions.
#1 However, the results were not delivered in a clear state, so it is difficult to follow and understand it. For example, the author describes a particular metabolite specifically present in one genotype, but the author does not describe from which treatments the metabolite is produced.	#1. What we want to say is that in each genotype produces specific metabolite in difference vernalization treatments. The data were showed in heatmap of hierarchical clustering analysis in Figure 1 and 2. The heatmap showed that each shallot genotype produces different metabolites associated to the flowering competency. The results indicated that there are a presence of acyclic ditherpene alcohol (phytol) which were produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability. The phytol presence in vernalized bulb is an indication that vernalization treatment has a positive effect in inducing flowering of shallot.
#2 In addition, significant data cited in the discussion is not present in the manuscript. The morphological data (Table 1) and data of prolin levels could not be found.	# 2 Thank you for the correction, we have re-checked and revised the statements in the manuscript.
#3 It is vital that the author must provide metabolite information to understand the hypothesis raised in the discussion.	#3 Thank you for the suggestions. Metabolomic composition of 5 genotypes shallot have been describe in heatmap of 5 genotypes.
#4 From the title, the reader expects to have an overview that there are metabolomic changes after bulb vernalization, which may induce the	#4 Thank you for the correction, we have revised the statements in the manuscript.

flowering time acceleration among 5 shallots. However, the discussion goes broadly to environmental stress and does not deeper to the flowering ability of 5 shallots author discuss his findings. #5 Somehow, the approach that done by the author in analyzing shallot leaf metabolites for understanding the response of vernalization is questionable, since the metabolites in leaves and generative organ could be different. It may also reconsider to analyze metabolites from the whole plant organs to get a big view of metabolites responsible for accelerating the flowering time in shallot.	#5 Thank you for the suggestions. We used shallot leaves as sample (4 weeks after planting) in generative (bolting stage) for metabolomic analysis in shallot. We do not use another generative organ due to non-flowering genotype is not produce the flower part.
Introduction and Literature a. The literature review has not included the current status of metabolomics research in *Allium*, major metabolites in leaves, bulbs and/or other organs, the importance of generative stage and logical backgrounds obstacles in producing seed from *Allium*. b. Authors mentioned the reason of using GC/MS approach is due to unavailable reference standards for identifying the observed metabolites. However, this is a weak reason. The author must explain the ultimate reasons related to the major issue that really want to unravel, i.e., metabolites related to flowering physiology. c. The stated research objective is to identify secondary metabolite composition in 5 shallots. However, this is not very clear and too broad, whereas the research scope focusses on volatile metabolites.	a. Thank you for the sugestion. Unfortunatelly, there is no information about current status of metabolomic composition in each plant organ of shallot. b. Thank you for the correction. We agree and have revised the statements in the manuscript. c. Thank you for the correction. We state clearly to identify the various components by GCMS.from their mass spectra. Hierarchical clustering analysis grouped shallot genotypes into three distinct genotypes, in both vernalization treatments.
Research Metodology a. It is important to put species name of samples along with information on the flowering pattern of	a. Thank you for the sugestion.

each genotype. b. The sample pretreatment and metabolite extraction steps from raw leaves into ready-injected samples should be described in the methodology. c. In methodology, it was mentioned that metabolites were normalized by log transformation. In contrast, the author stated that metabolite data were normalized by log2 transformation and mean centering. Which method is the correct one? d. When performing metabolomics data analysis, there are huge spectra data collected from the analysis. Filtering and clustering spectra data must be done before further analysis. These steps were crucial to perform prior to metabolite identification. However, these steps were not described in the methodology. Please include these steps in the method.	b. Thank you for raising very interesting points. We agree and have added more information on sample preparation. c. Thank you for the correction. We agree and re-check the consistency.
Results and Discussion a. The first sentence of Results and Discussion stated that "Metabolites in 5 shallot genotypes were significantly affected by vernalization treatment". This sentence is a conclusion sentence which should be stated after the findings have been described and discussed. b. Sentences: "Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by different colour of each heatmap coloumn. Specific metabolite compounds were visualized on each genotype column with a bright green color" This is contrary with Fig. 1 and Fig. 2 illustrations, which are stated that the color-coded matrix represents the intensities of metabolites, i.e., red is low values and green is high values	a. Thank you for the sugestion. We agree and have change the statement b. Thank you for the correction. We agree and have change the statement. The color-coded matrix represents the intensities of metabolites content.

metabolites. The author should redefine the statements to make it clear.	
c. Results were organized by displaying metabolites detected in non-vernalized samples and vernalized samples (Fig. 1 & Fig 2). However, it is difficult to understand which metabolites affected/expressed by vernalization. It is important that data processing should be performed in integrated analysis and resulted in only 1 figure metabolite heatmap, supplemented by Table of metabolite identity, to get a better understanding of which metabolites upregulated or downregulated or synthesized after vernalization.	c. Thank you for the suggestion. However we decided to use heatmap of HCA to display metabolites composition in each treatments.
d. Group names of G1, G2, G3, G4 and G5 in the text are not in line to any illustration in Fig 1 and Fig 2. Meanwhile, there are other different codes displayed in Fig. 1 and 2 which do not correspond to any information in the text. Authors should put the group codes/names which are annotated in the text.	d. Thank you for raising very interesting points. Actually we have addressed these points in the results and discussion. Genotype codes of G1, G2, G3, G4, and G5 represent as Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5). we used the codes in text clearly inline to the explained data. The genotypes showed in Fig 1 were clustered in different group compared to Fig 2. It is that because the code are located in different position, depend on clustering data.
e. Paragraph 2 of the Results & Discussion: Based on metabolites, 5 shallots were clustered into 3 groups and the author claims the clustering was based on genotypes. However, this hypothesis is not provided by any strong facts, such as genotype agronomical description (origin, flowering period, etc.). On the other hand, in the next sentence, the author stated contrary to the previous statement, which said that the clustering was due to the flowering competency. These statements are confusing for readers and the author should restructure the paragraph.	e. Thank you for he suggestion. In this manuscript, we clustered shallots genotype base on metabolite compositions by GCMS analysis. We have reported another data in molecular and morphological approach to clustering shallot genotypes related to flowering competency.

f. Fig. 3 is hardly readable, Table 1 is missing	f. Thank you for suggestion. We agree and revise
g. Paragraph 3 of Results & Discussion: There are many details in describing metabolites found in shallots. However, the explanation does not describe the metabolomic differences among groups and their correlation with the vernalization and the flowering competency. The author should reshape the discussion into the topic expressed in the title.	g. Thank you for suggestion.
h. Metabolites to flowering competency correlation analysis is strongly recommended to be performed in order to check and decide which whether there are correlation between leaves volatile metabolites to flowering competency.	h. Thank you for suggestion. As mentioned in previous response, in this experiment we used shallot leave in bolting stage (4 weeks of planting). Bolting stage of shallot indicated the transition stage of vegetative to generative. This transition periode may change metabolites compositions related to its flowering competency.

RJC

RASĀYAN *J. Chem.*
Vol. 12 | No. 2 |402 - 408| April - June | 2019
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

SECONDARY METABOLITES CHANGE UNDER VERNALIZATION AND ITS RELATION TO FLOWERING COMPETENCY IN SHALLOT (*Allium cepa* var. *aggregatum*)

Marlin[1*], A. Maharijaya[2], A. Purwito[2], and Sobir[2]
[1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia
[2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia
*E-mail : marlin@unib.ac.id

ABSTRACT

Shallot extract contains a great quantity of essential oil, volatiles, and other compounds. The composition of secondary metabolite can be regarded as the ultimate reactions of biological and physiological to genetic and environmental conditions. This study was objected to identify metabolomic composition and its relation to flowering competency in 5 genotypes of shallot. The non-targetted metabolites were identified using GC-MS and assigned by matching their mass spectra with those available in the WILEY7 LIB. Data matrix was analyzed by using metabolomic package of the R software. Heatmap was visualized for a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The composition metabolites from 5 genotypes were difference and suggested correlated to flowering competency. The 3 genotypes of flowering type of shallot produced highest concentration of phytol (ditherphene alcohol) and low concentration of nitrogen compound. Whereas, 2 genotypes of non-flowering types contained high concentration of organosulfur and nitrogen sources. Metabolite profile of 5 genotypes contained of volatile and non-volatile phytonutrients, vitamin, saturated fatty acid and organosulfur compounds.

Keywords: dissimilarity matrix, GC-MS, non-targetted metabolite, organosulfur, phytol

INTRODUCTION

Shallot (*Allium cepa* var. *aggregatum*) is an important *Allium* plant that been used for food, ornamental, natural alternatives to food synthetic preservatives and medicinal for curing various diseases. *Allium* plants contain different sugars, amino acids, vitamins, sulfurous compounds, enzymes, flavonoids, saponins and minerals[1]. *Allium* are also valued for their therapeutic properties; they are active as diuretics and laxatives and have been used to treat headaches and parasitic worms[2]. Onion has beneficial effect as their prebiotic, and their ability to decrease levels of insulin, cholesterol, triacylglycerol and phospholipids for human health[3], and considered an important vegetable and medicinal plant prescribed as an effective therapy for several complaints in healing treatment such as natural cancer treatment[4]. These beneficial effects will become an important target in shallot breeding and management practices.

Rasayan J. Chem., 12(2), 402-408(2019)
http://dx.doi.org/10.31788/RJC.2019.1224034

CrossMark

Formatted: Font color: Blue
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Default Paragraph Font, Font: 12 pt
Formatted: Font: 12 pt

Shallot genotypes may vary in flowering pattern, naturally flowering, inducible flowering, and non-inducible flowering shallot[5]. Flowering in shallot can be induce by an exogenous treatment such as vernalization. Vernalization is an important adaptation of plants to initiate flowering in response to a prolonged exposure to low temperatures[6,7]. The principles derived from vernalization are likely to be widely relevant to epigenetic reprogramming in many organisms[7]. External condition plays important regulation in plant bioactivity and influences the metabolomic composition. Metabolomic composition in plant is various and specific, that depends on genetic and plant growth environment. Metabolomic describes plant phenotype differentiation.

Metabolomic technologies have revealed a new insights in biological systems through metabolic dynamics[8]. The metabolomic composition determines biological and physiological function of the plants. Metabolite compounds are observed in the 6 *Alliaceae* family[9], in *Allium cepa* (onion) extracts[10,11], in *Allium rotundum* L[12], ~~and~~ in *Allium sativum* (garlic) formulations[13,14], and in shallot grown in tidal swampland[15]. Metabolomic defined as a comprehensive analysis in which all the metabolites of a biological system are identified and quantified[~~15~~16]. Metabolites are the end products of cellular regulatory processes. Metabolites profiling in plant can be directly extracted from a part of plant to find new molecules contents. Profiling of the metabolite composition is an important way to evaluate the contents of the medical plants, as well as their safety and efficacy[17~~16~~].

Mass spectrometry is an analytical method, which allows the determination of the mass formula and structure. With mass spectrometry, individual molecules can be measured based on their molecular peak (m/z) and characteristics spectra of fragment ions[18~~17~~]. Mass spectrometry technology is high sensitivity to identify the unknown and unexpected of the components present in the complex biological samples[19~~18~~]. These fragments often provide a clue to the molecule's structure, but without a reference compound, the difficulty of making an identification increases markedly[18~~17~~]. The analytical strategy *gase chromatography mass spectrometry* (GC-MS) used to analyze the volatile compounds, and selected compounds were structurally measured by mass spectrometry transposing the method to GC-MS[~~19~~,20, 21]. The mass analyzer separates the molecules or/and fragments according to their masses and the detector detects and quantifies the separated ions[18~~17~~]. GC-MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal-mass information. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories databases[19~~18~~]. This study was objected to identify secondary metabolite composition and its relation to flowering competency in 5 genotypes of shallot.

EXPERIMENTAL

Plant materials

Local genotypes of shallot with different pattern of flowering were selected as plant material. Shallot genotype 'Bentanis' is used as the naturally flowering, while genotypes 'Bima Brebes Ilokos, Tajuk and Sumenep' are selected as the induced flowering of shallot[5] ~~(Marlin *et al.*, 2018)~~. Experiments were planted in Pasir Kuda research farm, Bogor Indonesia. Each genotypes were treated with vernalization in 8 ^{o}C temperature for 5 weeks, and no vernalization treatment as control. The bulbs were then planted in 45 cm diameter polythene bags containing 8 kg of growing media (soil: manure: husk = 2: 1: 1). Each polybag was planted with three bulbs. Before planting, shallots bulbs were soaked in a fungicide benomil 50% at a concentration of 2 g.L^{-1} for 15 minutes. The bulbs were then soaked into solution of plant growth promoting rhizobacteria of 5 g.L^{-1} for 15 minutes. NPK fertilizers with a ratio of 15:15:15 were applied in a dose of 600 kg.ha^{-1} or 2.4 g per polybag.

Formatted: Font: 12 pt
Formatted: Font: 12 pt, Font color: Blue
Formatted: Font: 12 pt, Font color: Blue
Formatted: Font: 12 pt

Metabolomic analysis

The samples were the leaves of shallot plants at 4 weeks after planting (bolting stage ~~phase~~). Leaves were cutted for 10 g and were extracted with methanol (50 mL). The methanolic extract of shallot leaves were used as a sample in the GC-MS analysis. GC-MS unit was carried out on an GCMS-QP2010 system (Shimadzu Corporation, Japan) coupled to mass spectrometer detector. The samples were inserted into the quartz chamber in the GC-MS unit. Helium was used as a carrier gas in a constant flow mode at 0.85 ml/min. The chamber was heated in an oxygen-free environment at a temperature of 400 ºC for 0.2 minutes. The reaction will produce heat-mediated cleavage of chemical bonds in the macromolecular structure and produce low molecular weight with a chemical composition that identify specific compound of metabolite. Compound mixtures were then passed through the column GC-MS analysis. The column is Rt x 5 MS, with length 60.0 m, thickness 0.25 µm, and diameter 0.25 mm. The initial temperature of the column was 50 °C, which was gradually increased by 10 °C up to 280 °C. At the end of this period, the column oven temperature was 50 ºC raised up to 280 °C. Injection port temperature was ensured as 280 °C and helium flow rate as 0.85 ml/min. Mass spectrometer detector was employed to detect compounds when they were vented from the column. Temperature of the detector was 200 °C. ~~The volatile metabolite compounds~~Secondary metabolites of the plant samples were then identified for each treatments.

The identification of the components of non-targeted metabolite was assigned by matching their mass spectra with those available in the WILEY7 LIB. The ~~volatile~~ metabolite compounds of the plant samples were then identified for each genotypes. Spectra were compared with National Institute of Standard and Technology (NIST, 2005 v2.1) library to identify the unknown compounds. The data were confirmed using PubChem online database in National Center for Biotechnology Information (https://pubchem.ncbi. nlm.nih.gov/), chemicalbook database (http://www.chemicalbook.com), and NIST webbook database http://webbook.nist.gov/. The datasets had been transformed to log2 and mean-centered~~were then log-transformed~~ in order to acquire the normalized data. Data matrix based on non-targetted metabolomic of shallot in each vernalization treatments were analyzed using Metabolomic package of the R software (R version 3.2.2 http://www.r-project.org/). Metabolomics package was operated to construct HCA (*hierarchical cluster analysis*) and visulized heatmap with dendrogram. HCA was performed using Euclidean distance method and complete linkage agglomerative method.

RESULTS AND DISCUSSION

~~Metabolite compounds in 5 shallot local genotypes were affected significantly by vernalization treatments.~~ A total of 130 of metabolomic compound of non-vernalized bulbs, and 122 of metabolites ~~compound~~ of vernalized bulbs of 5 shallot local genotypes were identified by GC-MS. Metabolite compound obtained where then visually described by metabolite heatmap (Figure-1). Heatmap is widely used in metabolomic research because it is able to visualize large amounts of metabolite data and its differences in sample groups~~22120~~. Heatmap of both non vernalized and vernalized bulbs clustered 5 shallot local genotypes into three groups (Figure-1). First group was G2 (Bima Brebes genotype) and G4 (Tajuk genotype), second group was G1 (Bentanis genotype), and the third group was G3 (Ilokos genotype) and G5 (Sumenep genotype).

Formatted ...

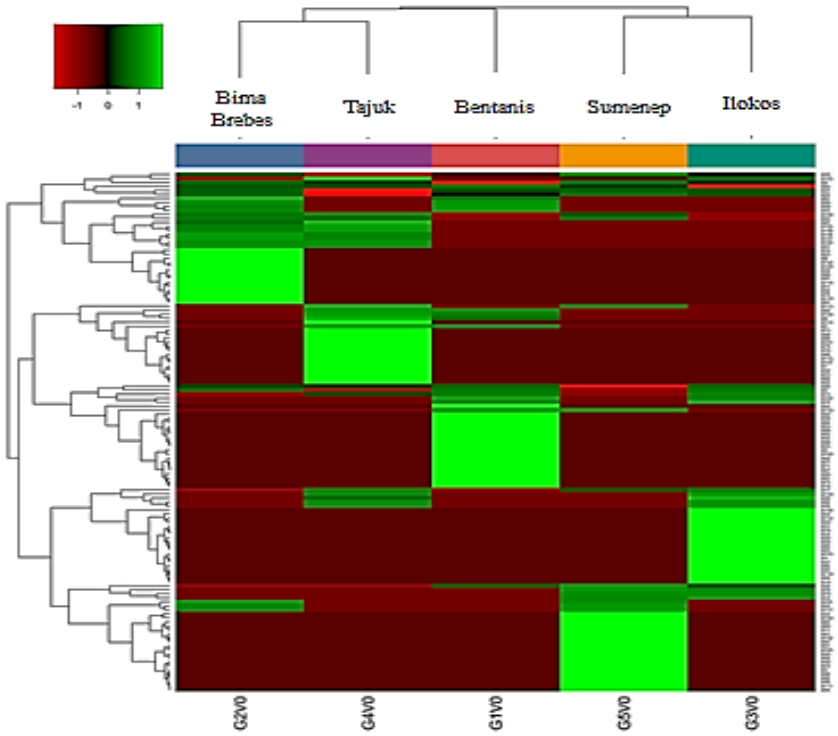


Fig.-1: Heatmap of hierarchical clustering analysis based on non-targeted metabolomic in 5 shallot genotypes of 130 metabolites in non-vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered.

Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by different colour of each heatmap coloumn. Intencity of s~~S~~pecific metabolite ~~compounds~~ was ~~were~~ visualized on each genotype column with a bright green color. Hierarchical clustering analysis showed that the genotype had a significant effect on the metabolites composition of the shallot. Each genotype of shallot produces different metabolites that associated to the flowering competency. Hierarchical clustering analysis of shallot metabolites grouped shallot genotypes into three distinct genotypes, in both vernalization treatments (Figure-1 and 2). The result indicated that the presence of specific compounds produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability.

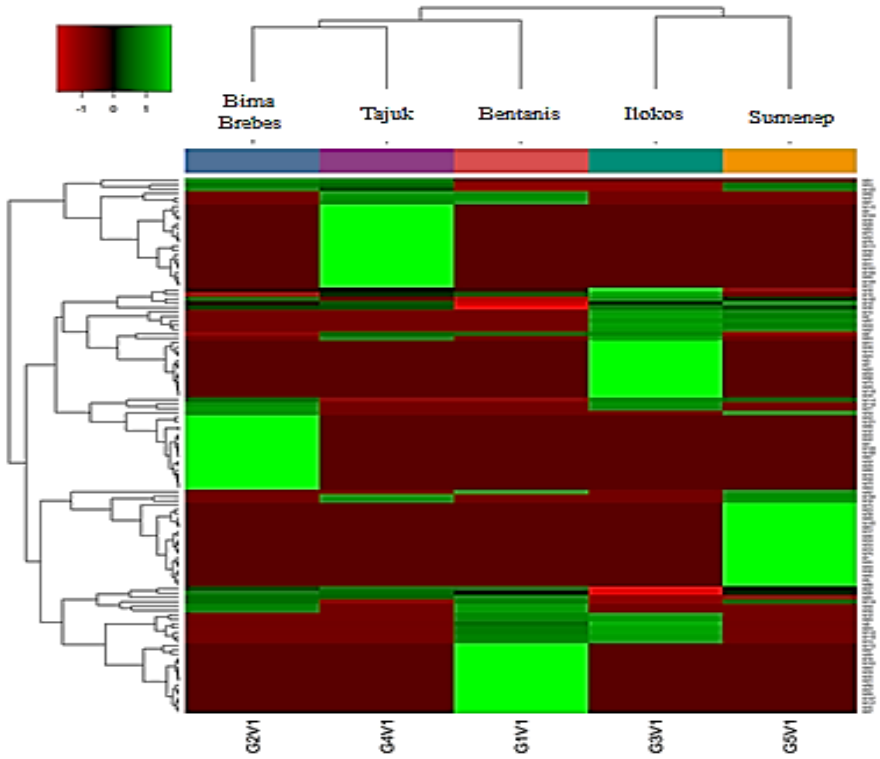


Fig.-2: Heatmap of hierarchical group analysis based on non-targeted metabolomic in 5 shallot local genotypes of 122 metabolites in vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered

Shallot genotypes located in the same group showed that the genotype had the same metabolite profile, and showed significant metabolite diversity with other groups. Visualization with heatmap also confirmed in the presence of different colors from each of the heatmap columns. The genotypes included in the first group were the Bentanis genotype (G1). In the second group there was the Bima Brebes (G2) and Tajuk genotype (G4), while in the third group there was Ilokos (G3) and Sumenep genotype (G5). The Bentanis genotype separates on its own group, and has close proximity to the Bima Brebes and Tajuk groups. These three genotypes are genotypes that have the ability to generate interest (sensitive to flowering).

Visualization with heatmap is widely used in metabolomic research because it can visualize large amounts of metabolomic data and classify it in sample groups[22,21,20]. ~~S~~intencitis of specific metabolite ~~compounds~~ of each sample of onion are visualized on each genotype column in bright green color.

Mass spectrometry methods can accurately detect compounds including identification of unknown and unexpected compounds[19,18]. Identification of the molecular structure is often given without reference compounds, making it difficult to make a real identification[19,18]. The genotypes of shallot that have the ability to produce flowers will produce the specific metabolite compounds. Metabolite compounds produced in the transition process of vegetative to generative

Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Font: (Default) Times New Roman, 12 pt

stage can be an indicator to determine the ability of flowering in shallot. The distribution of metabolomic compounds produced in non-vernalized shallot genotypes is presented in Figure-3.

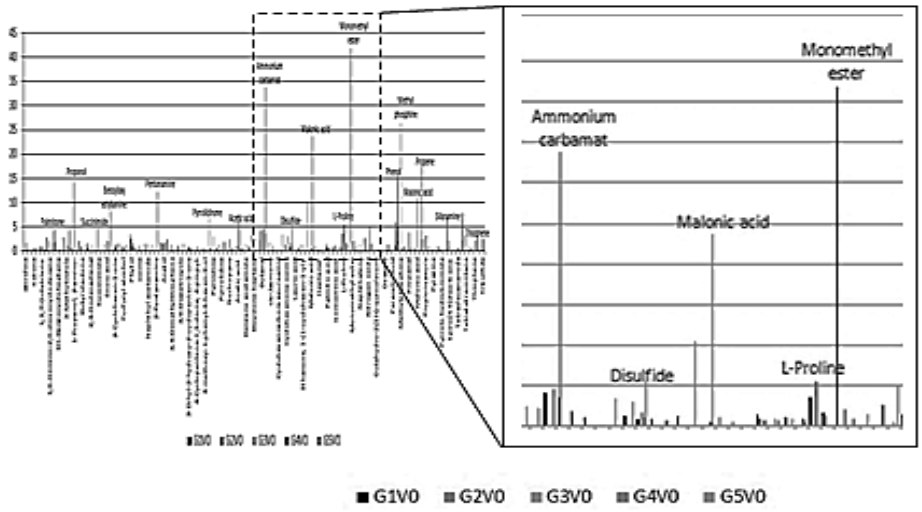


Fig.-3: Metabolite composition in non-vernalized shallot genotypes. Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5)

The results showed each genotype produced different metabolite compound. In Bentanis genotype showed the presence of monomethyl ester (41.82%) higher than Sumenep genotype (2.12%). The monomethyl ester compound, in the form of Mg-protoporphyrin IX mono-methyl ester cyclase is the catalyst required for the oxidation process of chlorophyll formation in plants[23,22,21]. The Bentanis genotype grows faster than Sumenep genotype. Bentanis genotype has robust maximum vegetative growth compared to Sumenep genotype at 5 weeks after planting. ~~The results in Table 1 show that~~ Morphological investigation showed that Bentanis genotype ~~h~~was higher ~~plant height~~ (36.96 cm) than of Sumenep genotype (29.82 cm). The presence of monomethyl ester (Mg-protoporphyrin IX mono-methyl ester cyclase) compounds can increase the formation of chlorophyll more optimally, thus increasing the photosynthesis process for the Bentanis genotype growth.

GCMS analysis showed ~~L~~low concentration of proline (3.55% and 5.56%) was produced in Bentanis and Tajuk genotypes (Figure-3). Proline compounds are generally produced by plants as their response to stress condition. Bentanis genotype was also produced succinimide compounds at about 4.78%, those can not be found in other genotypes. Succinimide compounds are used as intermediate compounds in the process of forming organic compounds that stimulate plant growth.

The presence of high concentrate of ammonium carbamate compound is found in Bima Brebes genotype. Ammonium carbamate is an important compounds in the formation of carbamoyl phosphate and the formation of ATP into ADP[24,~~22~~]. While the presence of disulfide (dipropyl disulfide) compounds was found higher in non-sensitive flowering genotypes, such as Ilokos and Sumenep (1.69% and 5.43%), compared with other genotypes (0.8–0.14%). The content of dipropyl disulfide is an important component of activity as an antimicrobial and antioxidant and gives a distinctive aroma to shallot plant. The sulfur compounds in dipropyl disulfide is

Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt, Font color: Blue
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt

important the components of these metabolite compounds[25,24,23]. The sulfur compounds are known to have important activities as antimicrobials and antioxidants[24,25,26,27]. Derivative products of allicin compounds such as diallyl disulfide, diallyl trisulfide found in garlic essential oil indicate activity as a good antimicrobial[28,27,26].

Onion bulbs had the highest concentrations of propanethiol compounds having an aroma 20 times higher than dipropyl disulfide compounds[29, 28,27]. It also found in non-flowering shallot plant, Ilokos and Sumenep genotypes, they produce higher acetic acid compounds (1.04% and 5.98%) compared to the flowering type, the Bentanis genotype (1.02%).

Utilization of sensitive instruments in metabolomic analysis is an important way to detect the content of plant essential compounds.Plant metabolites consist of highly diverse chemical compounds ranging from inorganic compounds to hydrophilic, volatile alcohol and ketone compounds, amino and non-amino acids, hydrophobic lipids, and complex natural products[19,18]. Utilization of mass spectrometry technology is a great way to solve research and analysis problems in the metabolomic field[19,18]. Metabolomic analysis was succesfully to identify potential compounds extracted directly from shallot genotypes. The results showed higher concentrations of acyclic ditherphene alcohol (phytol) compounds in flowering type shallot, Bentanis genotype (8.23%), Bima Brebes (2.2%) and Tajuk (7.52%) than the Ilokos genotype (1.15%) and Sumenep (2.19). The phytol compounds is an indication that vernalization treatment has a positive effect in inducing flowering of shallot genotypes, explaining that these three genotypes are sensitive to vernalization treatment to induce flower formation. Phytol compound is a compound that serves as a precursor of vitamin E and K. The content of these vitamins plays an important role in the plant body as an enzymatic cofactor in the process of metabolism that stimulates reproductive response. The content of vitamin E in the form of tocopherol is an important compound as one of the factors in the context of a cellular antioxidant system[30,28]. The differences of metabolite profile might play a key role in flowering initiation in shallot.

In flowering type of shallot genotypes, Bentanis, Bima Brebes and Tajuk also showed the formation of nitrogen oxide and semicarbazid hydrochlorid which are relatively lower than the non-flowering type, Ilokos and Sumenep. Nitrogen compound is a compound that plays an important role in the vegetative phase of the plant. Once the plant enters the reproductive phase, the nitrogen compounds in the plant body will decrease. In contrast, the Ilokos and Sumenep genotypes show the formation of high nitrogen compounds ie ammonium compounds ammonium carbamat, and nitrogen oxide. High content of nitrogen compounds in Ilokos and Sumenep genotypes explains that these two genotypes are not sensitive to vernalization treatment, thus not encouraging the flowering formation.

The results also showed that the Sumenep genotype contained high organosulfur compounds compared with other genotypes, namely thiophene (2.31%) and trisulfide (4.25%). The organosulfur is an important compound in giving aroma and spiciness upsurge in shallot, and play an important role as an antioxidant. Essential compounds in *Allium* have strong antioxidant ability. Tests on antimicrobial activity showed that all essential oils on *Allium* were able to inhibit the activity of gram-positive and gram-negative bacteria[9].

Vernalization treatment triggered in the formation of proline compounds in the Sumenep genotype. Vernalization treatment provides a stress condition to the plants that increase the plant mechanism of adaptation by producing proline compounds. Proline accumulation in the plant body is a physiological response to stress, and as a generative tissue development[31,29]. Secondary metabolite is the cellular response in plant that receives external pressure.

The metabolite profile of 5 local onion genotypes is also rich in non-volatile phytonutrients, especially the content of phenolic compounds (phenol, 2-propenamide, 3-pyridinamine, N-

Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Font: (Default) Times New Roman, 12 pt

Formatted: Font: 12 pt

Formatted: Font: 16 pt

Formatted: Font: 12 pt

furfurylidene 2-phenylethyl amine, pyrrolidinedione), amide compounds aziridine-2-carbothioic acid amide) and protein compound (pyroglutamic acid). The essential components, mono and sesquiterpenes, carbohydrates, phenols, alcohols, ether, aldehydes and ketones, are responsible for biological activity and also for their fragrance[32~~30~~]. Phenols and polyphenol compounds play important roles as antioxidants in plants[33,34,35 ~~31,32,33~~]. Research on *Allium rotundum* showed the presence of antioxidant activity of phenol compounds with high concentrations of up to 4%[12]. Onion extract contained the highest of phenol content, at 17.18 mg GAE per gram of fresh weight[36~~34~~], and a phenol content of 114.70 mg GAE per 100 g of different onion samples[37~~35~~]. Metabolite compound is difference among shallot genotypes. Each plant genotype performs metabolic activities that depend on their genetic conditions and the ability to adapt to its environment. The plant biological activity may imply to proportion of metabolite containing in a plant.

CONCLUSION

Secondary metabolite of 5 genotypes shallot were difference under vernalization treatment.~~Secondary metabolite is associated to the flowering competency in shallot genotypes.~~ Each genotypes produce different specific metabolites which is clustered according to the flowering competency. A high concentration ditherpene alcohol (phytol) found in flowering-sensitive genotypes (genotype of Bentanis, Bima Brebes and Tajuk), with a lower content of proline and nitrogen (ammonium carbamate and nitrogen oxide)~~,~~. While, in flowering-nonsensitive genotypes (genotype Ilokos and Sumenep) were found higher level of organosulfur compounds (thiophene and trisulfide). Vernalization treatment caused the Sumenep genotype produces the highest proline compound among other genotypes.

ACKNOWLEDGMENT

This researchs is supported by grants from National Innovation System Research Insentive 2016, Ministry of Research, Technology, and Higher Education, Indonesia.

REFERENCES

1. F.V. Golubev, N.A. Golubkina, and Y.N. Gorbunov. *Applied Biochemistry and Microbiology*. 39(5): 532–535 (2003). https://doi.org/10.1023/A:1025465106772
2. G. Griffiths, L. Trueman, T. Crowther, B. Thomas, and B. Smith. *Phytotherapy research: PTR*. 16(7): 603–615 (2002). DOI : 10.1002/ptr.1222
3. T. Ritsema , and S. Smeekens. *Current Opinion in Plant Biology*. 6(3): 223–230 (2003)
4. H. Mohammadi-Motlagh , A. Mostafaie, and K. Mansouri *Archives of Medicaal Science*. 1: 38–44 (2011)
5. Marlin, A. Maharijaya, A. Purwito, and Sobir. *SABRAO Journal of Breeding and Genetics*. 50 (3) 313-328 (2018). www.sabraojournal.org
6. E.J. Finnegan, D. Bagnall, C. Helliwell, D. Rouse, C. Sheldon, M. Tadege, W.J. Peacock, and E. Dennis. Vernalization. Encyclopedia of Life Sciences Macmillan Publishers Ltd, Nature Publishing Group. (2001). www.els.net.
7. J. Song, A. Angel, M. Howard, and C. Dea. *Journal of Cell Science*. 125 (16): 3723–3731 (2012).
8. Y. Iijima. *Metabolite*. 4: 699–721 (2014).
9. D. Mnayer, A. Fabiano-Tixier, E. Petitcolas, T. Hamieh, N. Nehme, C. Ferrant, X. Fernandez, and F. Chemat. *Molecules*. 19: 20034–20053 (2014).
10. H. Xiao, and K.L. Parkin. *Phytochemistry*. 68(7): 1059–1067 (2007).

Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue, English (United Kingdom)
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue, English (United Kingdom)
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue
Formatted
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt

11. M.A. Vazquez-Prieto, and R.M. Miatello. *Molecular Aspects of Medicine.* 31(6): 540–5 (2010).
12. A.A. Dehpour, M. Yousefian, S.A. Jafary Kelarijani, M. Koshmoo, S. Mirzanegad, V. Mahdavi, S.E. Mousavi, E. Shirzad, M. Afzali, M.J. Javad Bayani, J.E. Olyaei, and M.K. Yahyapor. *Advances in Environmental Biology.* 6(3): 1020–1025 (2012).
13. V. Lanzotti, F. Scala, and G. Bonanomi. *Phytochemistry Reviews.* 13: 769–791 (2014).
14. N. Martins, S. Petropoulos, and I.C.F. Ferreira. *Food Chemistry*. 15(211): 41–50 (2016).
15. R.Y. Galingging, Sobir, S.I. Aisyah, and A. Maharijaya. *Rasayan J. Chem*., 11(2), 575 - 581(2018). http://dx.doi.org/10.7324/RJC.2018.1123001
~~15.~~16. O. Fiehn. *Plant Molecular Biology*. 48: 155–171 (2002).
~~16.~~17. H. Wu, O.J. Gu, S. Chen, X. Liu, Y. Zhou, X. Zhang, and X. Xu. *Journal of pharmaceutical and biomedical analysis.* 72: 267–291 (2013).
~~17.~~18. T. Soininen. Ph.D. Thesis. University of Eastern Finland. Finland (2014).
~~18.~~19. S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, and J. Nielsen. *Mass Spectrometry Reviews.* 24: 613– 646 (2005).
20. P.P. Durgawale, S.A. Hendre, and R.S. Phatak. *Rasayan J. Chem*., 8(3), 271 – 278 (2015).
~~19.~~21. N.C.J.P. Lekshmi, S. Viveka, M.B. Viswanathan, G. Manivannan, and T.M. Shobi. *Nanobio Pharmaceutical Technology.* (2014). DOI : 10.13140/2.1.3278.7523.
~~20.~~22. S.M. Huang, W. Toh, P.I. Benke, C.S. Tan, and C.N. Ong. *Metabolomics.* 10:1084–1093 (2014).
~~21.~~23. Y.S. Wong, and P.A. Castelfranco *Plant Physiology.* 79(3): 730–733 (1985).
~~22.~~24. N. Baker, J. Harbinson, and D.M. Kramer. *Plant Cell Environment.* 30 1107–1125 (2007)
~~23.~~25. P. Rattanachaikunsopon, and P. Phumkhachorn P. *African Journal of Microbiology Research.* 3: 747–750 (2009).
~~24.~~26. A. Chehregani, F. Azimishad, and A.H. Haj. *International Journal of Agriculture Biology.* 9(6): 873–876 (2007)
~~25.~~27. C.L. Ye, D.H. Dai, and W.L. Hu. *Food Control.* 30: 48–53 (2013).
~~26.~~28. J.W. Kim, J.E. Huh, S.H. Kyung, and K.H. Kyung. *Food Science and Biotechnology.* 13: 235–239 (2004).
~~27.~~29. M.M. Lokke, M. Edelenbos, E. Larsen, and A. Feilberg. *Sensor*s. 12: 16060–16076 (2012).
~~28.~~30. *M.G. Traber, J. Atkinson, and Atkinson. Free Radical Biology and Medicine. 43 (1): 4–15 (2007).*
~~29.~~31. N. Verbruggen, and C. Hermans. *Amino Acids.* 35: 753–759 (2008).
~~30.~~32. E.A. El-Wakil, M.M. El-Sayed, and E.E. Abdel-Lateef. *International Journal of Pharma Sciences and Research IJPSR.* 6 (3) (2015).
~~31.~~33. S.A.B.E. Van Acker, D.J. van den Berg, N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, and W.J. van der Vijgh. *Free Radical Biology and Medicine* 20 (3): 331– 342 (1996).
~~32.~~34. M.A. Alvarez, N.B. Debattista, and N.B. Pappano. *Folia Microbiologica.* 53(1): 23–8 (2008).
~~33.~~35. A.P. Mukne, V. Viswanathan, and A.G. Phadatare. *Pharmacognosy Reviews.* 5(9): 13–18 (2011)
~~34.~~36. X. Lu, J. Wang, H.M. Al-Qadiri, C.F. Ross, J.R. Powers, J. Tang, and B.A. Rasco. *Food Chemistry.* 129: 637–644 (2011).
~~35.~~37. J. Yang, K.J. Meyers, J. van der Heide, and R.H. Liu. *Journal of Agriculture Food Chemistry.* 52: 6787–6793 (2004).

Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt

RASĀYAN ***J. Chem.***
Vol. 12 | No. 2 |402 - 408| April - June | 2019

[RJC-4034/2018]

Marlin UNIB <marlin@unib.ac.id>

Proof of payment

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 21 Mei 2019 pukul 12.47
Kepada: Marlin UNIB <marlin@unib.ac.id>

Acceptance Letter: RJC- 5356/2019

Dear Author,

Congratulations!
Your manuscript has been accepted for the publication in the latest issue of **RASĀYAN Journal of Chemistry,** i.e. **RJC, Vol.12, No.3, 2019.**

Herewith please find attached the **Acceptance Letter and Invoice** for your above-mentioned manuscript.

Also, find **Copyright Transfer Form** and **Letter of Original Work** attached. Please send these duly filled and signed docs as soon as possible.

Looking Forward.

Best regards,
Dr. Sanjay K. Sharma, FRSC
Editor, **RASĀYAN Journal of Chemistry**
[Kutipan teks disembunyikan]
--
Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)
www.rasayanjournal.com | www.rasayanjournal.co.in
Contact: +91 9001699997, +91 9414202678

3 lampiran

Acceptance Letter RJC-5356.pdf
177K

1_Copyright RJC.pdf
143K

2_Letter of Original work.doc
186K

Marlin UNIB <marlin@unib.ac.id>

Proof of payment

Marlin UNIB <marlin@unib.ac.id> 22 Mei 2019 pukul 10.28
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Received, thank you.
[Kutipan teks disembunyikan]

RASĀYAN

JOURNAL OF CHEMISTRY

(An International Quarterly Research Journal of Chemical Sciences)

ISSN:0974-1496 CODEN: RJCABP

Email: rasayanjournal@gmail.com **Website: www.rasayanjournal.com**

Copyright Form

FILL IN THE FORM IN CAPITALS AND RETURN TO:

RASĀYAN Journal of Chemistry
Prof. (Dr.) Sanjay K. Sharma
Editor-in-Chief
23, 'Anukampa', Janakpuri
Opp. Heerapura Power Station, Ajmer Road
Jaipur, Rajasthan. INDIA 302024
Email: drsanjay1973@gmail.com
Phone: 0091-141-2810628
Mobile: 09414202678, 09887050628

Principle/Corresponding Author:
Name: Dr. MARLIN
Add/Affiliation: DEPT. OF CROP PRODUCTION FACULTY OF AGRICULTURE, UNIVERSITY OF BENEKULU - INDONESIA 38121
Fax: +62736-21290
Tel: +62853 6822 7265
E-mail 1 marlin@unib.ac.id
E-mail 2: marlin_iin@yahoo.com

Title of Manuscript: THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALLOT (Allium cepa var. Aggregatum)

Author(s): Authors : MARLIN, A. MAHARIJAYA, A. PURWITO, SOBIR

LICENSE AGREEMENT

For the submission of an article (mention paper type here):
I hereby declare, on behalf of myself and my co-authors (if any), that:

[1] The article submitted is an original work of mine/ours and has neither been published in any other peer-reviewed journal nor is under consideration for publication by any other journal. Also, the article does not contravene any existing copyright or any other third party rights.

[2] I am/we are the sole author(s) of the article and maintain the authority to enter into this agreement and the granting of rights to The Publisher of **RASĀYAN Journal of Chemistry**, India (RJC in short) does not infringe any clause of this agreement.

[3] The article contains no such material that may be unlawful, defamatory, or which would, if published, in any way whatsoever, violate the terms and conditions as laid down in the agreement.

[4] I/we have taken due care that the scientific knowledge and all other statements contained in the article conform to true facts and authentic formulae and will not, if followed precisely, be detrimental to the user.

[5] I/we permit the adaptation, preparation of derivative works, oral presentation or distribution, along with the commercial application of the work.

[6] No responsibility is assumed by RJC and the Publisher of RJC, its staff or members of the editorial board for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products instruction, advertisements or ideas contained in a publication by RJC and the Publisher of RJC.

COPYRIGHT TRANSFER

Copyright to the above work (including without limitation, the right to publish the work in whole, or in part, in any and all forms) is hereby transferred to **RASĀYAN Journal of Chemistry** and the Publisher of **RASĀYAN Journal of Chemistry**, India, to ensure widest dissemination and protection against infringement. No proprietary right other than copyright is proclaimed by **RASĀYAN Journal of Chemistry** and the Publisher of **RASĀYAN Journal of Chemistry**.

Under the Following Conditions: Attribution

a. The services of the original author must be acknowledged;
b. In case of reuse or distribution, the license conditions must be clarified to the user of this work;
c. Any of these conditions can be ignored on the consent of the author.

SIGN HERE FOR COPYRIGHT TRANSFER: I hereby certify that I am authorized to sign this document either in my own right or as an agent of my employer, and have made no changes to the current valid document supplied by **RASĀYAN Journal of Chemistry** and the Publisher of **RASĀYAN Journal of Chemistry**

Print Authorized Name(s) and Title/Designation(s): Dr. MARLIN

Original Signature(s) (in ink):

Date: MAY. 22. 2019

RASĀYAN Journal of Chemistry

[An International Journal of Chemical Sciences]

ISSN: 0974-1496 (Print); ISSN: 0976-0083(Online) www.rasayanjournal.com

Abstracted in: SCOPUS (Elsevier), SCIRUS (USA), Chemical Abstracts (USA), CABI (UK), Global Health (UK), DOAJ

Editorial Office: 23, 'Anukampa', Janakpuri, Opp. Heerapura Power Stn.Ajmer Road, JAIPUR

E-mail: rasayanjournal@gmail.com ; Mobile: 094142102678, 07597925412

LETTER OF ORIGINAL WORK

[Mention your Manuscript No.:RJC. 5356/2019]

It is hereby declared that the manuscript entitled-

THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (*Allium cepa* var. *aggregatum*) authored by: Marlin*, A. Maharijaya, A. Purwito, and Sobir

is original work carried out by the authors and is not published in any form ever before.

Also, declared that, no part of this manuscript is plagiarized from published work of same or other authors without proper acknowledgement/written permission.

In case plagiarism is found the authors (specially corresponding author) will be responsible for the legal consequences.

Date: May, 22. 2019

Signature of Corresponding author

Address:

Department of Crop Production, Faculty of Agriculture,
University of Bengkulu, Indonesia
Jl. WR. Supratman Kandang Limun Bengkulu, INDONESIA 38121

E-mail: marlin@unib.ac.id **Mobile No.: +6285368227265**

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Reviewer's Report

SECTION-I : Details of Manuscript

Reviewer's Name:	Wahyuni
Complete Affiliation:	Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI)
E-Mail:	wahyuni@lipi.go.id; wahyu004@gmail.com
Manuscript Number:	**RJS 5356**
Title:	THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (Allium cepa var. aggregatum)
Authors:	Marlin1*, A. Maharijaya2, A. Purwito2, and Sobir2
Date of receiving by Reviewer:	May 12, 2019
Date of submission From Reviewer:	May 22, 2019

SECTION-II : Comments per Section of Manuscript

General comment:	The manuscript describes the metabolite composition in leaves of 5 shallot genotypes whose bulbs were untreated and treated by vernalization. The results may have added values to the scientific community about metabolomic changes induced by vernalization that are important for breeding. #1 However, the results were not delivered in a clear state, so it is difficult to follow and understand it. For example, the author describes a particular metabolite specifically present in one genotype, but the author does not describe from which treatments the metabolite is produced. #2 In addition, significant data cited in the discussion is not present in the manuscript. The morphological data (Table 1) and data of prolin levels could

UGC Recognized **and Abstracted/Indexed in:**

RASĀYAN Journal of Chemistry
(An SCOPUS Indexed International Journal of Chemical Sciences, Since 2008)
ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)
www.rasayanjournal.com | www.rasayanjournal.co.in
E-mail: rasayanjournal@gmail.com

	not be found. #3 It is vital that the author must provide metabolite information to understand the hypothesis raised in the discussion. #4 From the title, the reader expects to have an overview that there are metabolomic changes after bulb vernalization, which may induce the flowering time acceleration among 5 shallots. However, the discussion goes broadly to environmental stress and does not deeper to the flowering ability of 5 shallots author discuss his findings. #5 Somehow, the approach that done by the author in analyzing shallot leaf metabolites for understanding the response of vernalization is questionable, since the metabolites in leaves and generative organ could be different. It may also reconsider to analyze metabolites from the whole plant organs to get a big view of metabolites responsible for accelerating the flowering time in shallot.
Introduction and Literature Review:	a. The literature review has not included the current status of metabolomics research in *Allium*, major metabolites in leaves, bulbs and/or other organs, the importance of generative stage and logical backgrounds obstacles in producing seed from *Allium*. b. Authors mentioned the reason of using GC/MS approach is due to unavailable reference standards for identifying the observed metabolites. However, this is a weak reason. The author must explain the ultimate reasons related to the major issue that really want to unravel, i.e., metabolites related to flowering physiology. c. The stated research objective is to identify secondary metabolite composition in 5 shallots. However, this is not very clear and too broad, whereas the research scope focusses on volatile metabolites.
Research Methodology:	a. It is important to put species name of samples along with information on the flowering pattern of each genotype. b. The sample pretreatment and metabolite extraction steps from raw leaves into ready-injected samples should be described in the

UGC Recognized and Abstracted/Indexed in:

RASĀYAN Journal of Chemistry
(An SCOPUS Indexed International Journal of Chemical Sciences, Since 2008)
ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)
www.rasayanjournal.com | www.rasayanjournal.co.in
E-mail: rasayanjournal@gmail.com

	methodology. c. The sample set information, including the numbers of biological and technical replication, and the quality control samples should be provided in methods to make sure that the data set is statistically enough. d. In methodology, it was mentioned that metabolites were normalized by log transformation. In contrast, the author stated that metabolite data were normalized by log2 transformation and mean centering. Which method is the correct one? e. When performing metabolomics data analysis, there are huge spectra data collected from the analysis. Filtering and clustering spectra data must be done before further analysis. These steps were crucial to perform prior to metabolite identification. However, these steps were not described in the methodology. Please include these steps in the method.
Results and Discussion:	**a. The first sentence of Results and Discussion stated that "**Metabolites in 5 shallot genotypes were significantly affected by vernalization treatment**". This sentence is a conclusion sentence which should be stated after the findings have been described and discussed.** **b. Sentences:** "Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by different colour of each heatmap coloumn. Specific metabolite compounds were visualized on each genotype column with a bright green color" **This is contrary with Fig. 1 and Fig. 2 illustrations, which are stated that the color-coded matrix represents the intensities of metabolites, i.e., red is low values and green is high values metabolites. The author should redefine the statements to make it clear.** **c. Results were organized by displaying metabolites detected in non-vernalized samples and vernalized samples (Fig. 1 & Fig 2). However, it is difficult to understand which metabolites affected/expressed by**

UGC Recognized and Abstracted/Indexed in:

RASĀYAN Journal of Chemistry
(An SCOPUS Indexed International Journal of Chemical Sciences, Since 2008)
ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)
www.rasayanjournal.com | www.rasayanjournal.co.in
E-mail: rasayanjournal@gmail.com

vernalization. It is important that data processing should be performed in integrated analysis and resulted in only 1 figure metabolite heatmap, supplemented by Table of metabolite identity, to get a better understanding of which metabolites upregulated or downregulated or synthesized after vernalization.

d. Group names of G1, G2, G3, G4 and G5 in the text are not in line to any illustration in Fig 1 and Fig 2. Meanwhile, there are other different codes displayed in Fig. 1 and 2 which do not correspond to any information in the text. Authors should put the group codes/names which are annotated in the text.

e. Paragraph 2 of the Results & Discussion: Based on metabolites, 5 shallots were clustered into 3 groups and the author claims the clustering was based on genotypes. However, this hypothesis is not provided by any strong facts, such as genotype agronomical description (origin, flowering period, etc.).
On the other hand, in the next sentence, the author stated contrary to the previous statement, which said that the clustering was due to the flowering competency. These statements are confusing for readers and the author should restructure the paragraph.

f. Fig. 3 is hardly readable, Table 1 is missing

g. Paragraph 3 of Results & Discussion: There are many details in describing metabolites found in shallots. However, the explanation does not describe the metabolomic differences among groups and their correlation with the vernalization and the flowering competency. The author should reshape the discussion into the topic expressed in the title.

h. Metabolites to flowering competency correlation analysis is strongly recommended to be performed in order to check and decide which whether there are correlation between leaves volatile metabolites to flowering competency.

UGC Recognized and Abstracted/Indexed in:

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Bibliography/References:	**References suggested in Literature Review Section should be included**
Others:	# Other minor comments are the redundancy of word usage of metabolite and compound is applied together and all Figures were not supported in enough resolution, so it is difficult to understand the content. In addition, there are different font style used in the manuscript and some grammatical errors are detected.

SECTION-III : Please rate the following

(1 = Excellent) (2 = Good) (3 = Fair) (4 = Poor)

Originality:	**2**
Contribution to the Field:	**2**
Technical Quality:	**4**
Clarity of Presentation :	**4**
Depth of Research:	**3**

SECTION-IV : Recommandation

(Kindly mark with an X)

Accept As it is:	
Requires Minor Revision:	-
Requires Moderate Revision:	-
Requires Major Revision:	**X**
Rejected for publication in RJC: (Please give reason)	-

SECTION-V : Additional Comments (You may use additional sheet)

UGC Recognized **and Abstracted/Indexed in:**

RASĀYAN Journal of Chemistry

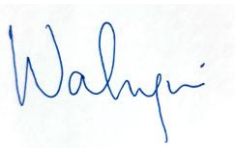
(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Signature of the Reviewer



Date: 22 May 2019

UGC Recognized **and Abstracted/Indexed in:**

Marlin UNIB <marlin@unib.ac.id>

Copyright of Transfer Form

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 29 Mei 2019 pukul 11.55
Kepada: marlin <marlin@unib.ac.id>

Well received with thanks.
[Kutipan teks disembunyikan]
--
Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)
www.rasayanjournal.com | www.rasayanjournal.co.in
Contact: +91 9001699997, +91 9414202678

Marlin UNIB <marlin@unib.ac.id>

Proof of payment

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 1 Juni 2019 pukul 14.16

Kepada: Marlin UNIB <marlin@unib.ac.id>

REVISION-2 : RJC-5356/2019

Plagiarism Check Status: (Please refer Point No. 9)

Dear Author,

Greetings from RASĀYAN Journal of Chemistry.

We have received your mail regarding clearance of printing charges, thanks.

You are requested to revise your above-mentioned manuscript (As per the Template attached) once again in the light of the REVIEWER'S REPORT(S) and PLAGIARISM CHECK REPORT (Attached) and following points given below [Note: Points No. 6, 7, 8 and 9 are very important, without addressing these points, your manuscript will not be considered for publication process. All your revisions must be visible in the Revised Manuscript, therefore you are requested to use Red/Blue ink for revisions.]:

1. Check the Title once again.
2. Check spelling and grammatical mistakes once again throughout the manuscript.
3. Check affiliations of all authors and corresponding author. Also, check the E-mail id of the corresponding author.
4. Check **Abstract** and **Keywords** once again.
5. References in the text should be cited as super-scripted and at the end of the sentence. Please rectify this mistake also, if there.
6. Important: If possible, cite (3-5) relevant papers from **RASAYAN Journal of Chemistry (RJC)** appropriately in your manuscript to show the relevance of your work to the journal.
7. MOST Important: References must be strictly as per the STYLE of the journal (Please refer Journal's Guidelines and published paper from the current issue). Also, Mention DOI with references, wherever possible. Which may otherwise cause unnecessary delay in publication of your paper. You are requested to re-check all your references with respect to its Volume No., Page No., Name of Journal / Name of Publisher, Year etc. and format according to the Guidelines of the Journal.
8. Go through the REVIEWER'S REPORT(S) and revise/ improve your manuscript accordingly. Give justification / revision of all comments in the Tabular form on a

separate word file pointwise. Name this file - 'ANSWERS to REVIEWER'S COMMENTS' Remember, without this sheet your REVISION will not be considered for publication process.

9. SIMILARITY INDEX **should not be more than 10-15%** in any case in your manuscript. Please take care of it. Otherwise, all the authors involved will be responsible, if any conflict arise.

After revising the manuscript, please send it as **Revised RJC-XXX,** where XXX stands for your manuscript Number mentioned above. Please mention your MS Number correctly in the subject line when you send the revised manuscript.

We value your contribution and association with **RASAYAN**. Kindly acknowledge this mail. It is necessary for follow-up.

Please note: Articles, data, figures, tables, scientific content and its interpretation and authenticity reported by author(s), published in **RASĀYAN J. Chem.** are the exclusive views of the author(s). The Editorial board, **RASĀYAN J. Chem.** is not responsible for any controversy arising out of them. In the case of any Plagiarism found, author (s) will be responsible and have to face the consequences.

Best regards,

Dr. Sanjay K. Sharma, FRSC
Editor, RASĀYAN Journal of Chemistry

On Wed, May 22, 2019 at 8:58 AM Marlin UNIB <marlin@unib.ac.id> wrote:
[Kutipan teks disembunyikan]
[Kutipan teks disembunyikan]

6 lampiran

Reviewer Report, RJC-5356_1.docx
159K

Reviewer Report, RJC-5356_2.docx
265K

Reviewer Report, RJC-5356_3.pdf
411K

RJC_5356.pdf
3544K

_RJC_Paper Submission_Template.docx
24K

_SAMPLE PAPER.doc
1025K

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Reviewer's Report

SECTION-I : Details of Manuscript

Reviewer's Name:	Wahyuni
Complete Affiliation:	Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI)
E-Mail:	wahyuni@lipi.go.id; wahyu004@gmail.com
Manuscript Number:	**RJS 5356**
Title:	THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (Allium cepa var. aggregatum)
Authors:	Marlin1*, A. Maharijaya2, A. Purwito2, and Sobir2
Date of receiving by Reviewer:	May 12, 2019
Date of submission From Reviewer:	May 22, 2019

SECTION-II : Comments per Section of Manuscript

General comment:	The manuscript describes the metabolite composition in leaves of 5 shallot genotypes whose bulbs were untreated and treated by vernalization. The results may have added values to the scientific community about metabolomic changes induced by vernalization that are important for breeding. #1 However, the results were not delivered in a clear state, so it is difficult to follow and understand it. For example, the author describes a particular metabolite specifically present in one genotype, but the author does not describe from which treatments the metabolite is produced. #2 In addition, significant data cited in the discussion is not present in the manuscript. The morphological data (Table 1) and data of prolin levels could

UGC Recognized **and Abstracted/Indexed in:**

RASĀYAN Journal of Chemistry
(An SCOPUS Indexed International Journal of Chemical Sciences, Since 2008)
ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)
www.rasayanjournal.com | www.rasayanjournal.co.in
E-mail: rasayanjournal@gmail.com

	not be found. #3 It is vital that the author must provide metabolite information to understand the hypothesis raised in the discussion. #4 From the title, the reader expects to have an overview that there are metabolomic changes after bulb vernalization, which may induce the flowering time acceleration among 5 shallots. However, the discussion goes broadly to environmental stress and does not deeper to the flowering ability of 5 shallots author discuss his findings. #5 Somehow, the approach that done by the author in analyzing shallot leaf metabolites for understanding the response of vernalization is questionable, since the metabolites in leaves and generative organ could be different. It may also reconsider to analyze metabolites from the whole plant organs to get a big view of metabolites responsible for accelerating the flowering time in shallot.
Introduction and Literature Review:	a. The literature review has not included the current status of metabolomics research in *Allium*, major metabolites in leaves, bulbs and/or other organs, the importance of generative stage and logical backgrounds obstacles in producing seed from *Allium*. b. Authors mentioned the reason of using GC/MS approach is due to unavailable reference standards for identifying the observed metabolites. However, this is a weak reason. The author must explain the ultimate reasons related to the major issue that really want to unravel, i.e., metabolites related to flowering physiology. c. The stated research objective is to identify secondary metabolite composition in 5 shallots. However, this is not very clear and too broad, whereas the research scope focusses on volatile metabolites.
Research Methodology:	a. It is important to put species name of samples along with information on the flowering pattern of each genotype. b. The sample pretreatment and metabolite extraction steps from raw leaves into ready-injected samples should be described in the

UGC Recognized and Abstracted/Indexed in:

RASĀYAN Journal of Chemistry
(An SCOPUS Indexed International Journal of Chemical Sciences, Since 2008)
ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)
www.rasayanjournal.com | www.rasayanjournal.co.in
E-mail: rasayanjournal@gmail.com

	methodology. c. The sample set information, including the numbers of biological and technical replication, and the quality control samples should be provided in methods to make sure that the data set is statistically enough. d. In methodology, it was mentioned that metabolites were normalized by log transformation. In contrast, the author stated that metabolite data were normalized by log2 transformation and mean centering. Which method is the correct one? e. When performing metabolomics data analysis, there are huge spectra data collected from the analysis. Filtering and clustering spectra data must be done before further analysis. These steps were crucial to perform prior to metabolite identification. However, these steps were not described in the methodology. Please include these steps in the method.
Results and Discussion:	**a. The first sentence of Results and Discussion stated that "**Metabolites in 5 shallot genotypes were significantly affected by vernalization treatment**". This sentence is a conclusion sentence which should be stated after the findings have been described and discussed.** **b. Sentences:** "Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by different colour of each heatmap coloumn. Specific metabolite compounds were visualized on each genotype column with a bright green color" **This is contrary with Fig. 1 and Fig. 2 illustrations, which are stated that the color-coded matrix represents the intensities of metabolites, i.e., red is low values and green is high values metabolites. The author should redefine the statements to make it clear.** **c. Results were organized by displaying metabolites detected in non-vernalized samples and vernalized samples (Fig. 1 & Fig 2). However, it is difficult to understand which metabolites affected/expressed by**

UGC Recognized and Abstracted/Indexed in:

RASĀYAN Journal of Chemistry
(An SCOPUS Indexed International Journal of Chemical Sciences, Since 2008)
ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)
www.rasayanjournal.com | www.rasayanjournal.co.in
E-mail: rasayanjournal@gmail.com

vernalization. It is important that data processing should be performed in integrated analysis and resulted in only 1 figure metabolite heatmap, supplemented by Table of metabolite identity, to get a better understanding of which metabolites upregulated or downregulated or synthesized after vernalization.

d. Group names of G1, G2, G3, G4 and G5 in the text are not in line to any illustration in Fig 1 and Fig 2. Meanwhile, there are other different codes displayed in Fig. 1 and 2 which do not correspond to any information in the text. Authors should put the group codes/names which are annotated in the text.

e. Paragraph 2 of the Results & Discussion: Based on metabolites, 5 shallots were clustered into 3 groups and the author claims the clustering was based on genotypes. However, this hypothesis is not provided by any strong facts, such as genotype agronomical description (origin, flowering period, etc.).
On the other hand, in the next sentence, the author stated contrary to the previous statement, which said that the clustering was due to the flowering competency. These statements are confusing for readers and the author should restructure the paragraph.

f. Fig. 3 is hardly readable, Table 1 is missing

g. Paragraph 3 of Results & Discussion: There are many details in describing metabolites found in shallots. However, the explanation does not describe the metabolomic differences among groups and their correlation with the vernalization and the flowering competency. The author should reshape the discussion into the topic expressed in the title.

h. Metabolites to flowering competency correlation analysis is strongly recommended to be performed in order to check and decide which whether there are correlation between leaves volatile metabolites to flowering competency.

UGC Recognized and Abstracted/Indexed in:

RASĀYAN Journal of Chemistry

(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Bibliography/References:	**References suggested in Literature Review Section should be included**
Others:	# Other minor comments are the redundancy of word usage of metabolite and compound is applied together and all Figures were not supported in enough resolution, so it is difficult to understand the content. In addition, there are different font style used in the manuscript and some grammatical errors are detected.

SECTION-III : Please rate the following

(1 = Excellent) (2 = Good) (3 = Fair) (4 = Poor)

Originality:	**2**
Contribution to the Field:	**2**
Technical Quality:	**4**
Clarity of Presentation :	**4**
Depth of Research:	**3**

SECTION-IV : Recommandation

(Kindly mark with an X)

Accept As it is:	
Requires Minor Revision:	-
Requires Moderate Revision:	-
Requires Major Revision:	**X**
Rejected for publication in RJC: (Please give reason)	-

SECTION-V : Additional Comments (You may use additional sheet)

UGC Recognized **and Abstracted/Indexed in:**

RASĀYAN Journal of Chemistry

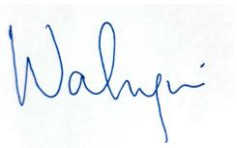
(An **SCOPUS Indexed** International Journal of Chemical Sciences, **Since 2008**)

ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)

www.rasayanjournal.com | www.rasayanjournal.co.in

E-mail: rasayanjournal@gmail.com

Signature of the Reviewer



Date: 22 May 2019

UGC Recognized **and Abstracted/Indexed in:**

RJC-5356

By Marlin Marlin

WORD COUNT	3604	TIME SUBMITTED	30-MAY-2019 09:55AM
		PAPER ID	47481590

RJC

RASĀYAN *J. Chem.*
Vol. 12 | No. 2 |402 - 408| April - June | 2019
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (*Allium cepa* var. *aggregatum*)

Marlin[1*], A. Maharijaya[2], A. Purwito[2], and Sobir[2]

[1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia
[2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia
*E-mail : marlin@unib.ac.id

ABSTRACT

Shallot extract contains a great quantity of essential oil, volatiles, and other compounds. The composition of secondary metabolite can be regarded as the ultimate reactions of biological and physiological to genetic and environmental conditions. This study was objected to identify metabolomic composition and its relation to flowering competency in 5 genotypes of shallot. The non-targetted metabolites were identified using GC-MS and assigned by matching their mass spectra with those available in the WILEY7 LIB. Data matrix was analyzed by using metabolomic package of the R software. Heatmap was visualized for a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The composition metabolites from 5 genotypes were difference and suggested correlated to flowering competency. The 3 genotypes of flowering type of shallot produced highest concentration of phytol (ditherphene alcohol) and low concentration of nitrogen compound. Whereas, 2 genotypes of non-flowering types contained high concentration of organosulfur and nitrogen sources. Metabolite profile of 5 genotypes contained of volatile and non-volatile phytonutrients, vitamin, saturated fatty acid and organosulfur compounds.

Keywords: dissimilarity matrix, GC-MS, non-targetted metabolite, organosulfur, phytol

INTRODUCTION

Shallot (*Allium cepa* var. *aggregatum*) is an important *Allium* plant that been used for food, ornamental, natural alternatives to food synthetic preservatives and medicinal for curing various diseases. *Allium* plants contain different sugar amino acids, vitamins, sulfurous compounds, enzymes, flavonoids, saponins and minerals[1]. *Allium* are also valued for their therapeutic properties; they are active as diuretic and laxatives and have been used to treat headaches and parasitic worms[2]. Onion has beneficial effect as their prebiotic, and their ability to decrease levels of insulin, cholesterol, triacylglycerol and phospholipids for human health[3], and considered an important vegetable and medicinal plant prescribed as an effective therapy for several complaints in healing treatment such as natural cancer treatment[4]. These beneficial effects will become an important target in shallot breeding and management practices.

Shallot genotypes may vary in flowering pattern, naturally flowering, inducible flowering, and non-inducible flowering shallot[5]. Flowering in shallot can be induce by an exogenous treatment such as vernalization. Vernalization is an important adaptation of plants to initiate flowering in response to a prolonged exposure to low temperatures[6,7]. The principles derived from vernalization are likely to be widely relevant to epigenetic reprogramming in many organisms[7]. External condition plays important regulation in plant bioactivity and influences the metabolomic composition. Metabolomic composition in plant is various and specific, that depends on genetic and plant growth environment. Metabolomic describes plant phenotype differentiation.

Metabolomic technologies have revealed a new insights in biological systems through metabolic dynamics[8]. The metabolomic composition determines biological and physiological function of the plants. Metabolite compounds are observed in the 6 *Alliaceae* family[9], in *Allium cepa* (onion) extracts[10,11], in

http://dx.doi.org/10.31788/RJC.2019.1224034

CrossMark

Allium rotundum L[12], and in *Allium sativum* (garlic) formulations[13,14]. Metabolomic defined as a comprehensive analysis in which all the metabolites of a biological system are identified and quantified[15]. Metabolites are the end products of cellular regulatory processes. Metabolites profiling in plant can be directly extracted from a part of plant to find new molecules contents. Profiling of the metabolite composition is an important way to evaluate the contents of the medical plants, as well as their safety and efficacy[16].

Mass spectrometry is an analytical method, which allows the determination of the mass formula and structure. With mass spectrometry, individual molecules can be measured based on their molecular peak (m/z) and characteristics spectra of fragment ions[17]. Mass spectrometry technology is high sensitivity to identify the unknown and unexpected of the components present in the complex biological samples[18]. These fragments often provide a clue to the molecule's structure, but without a reference compound, the difficulty of making identification increases markedly[17]. The analytical strategy *gase chromatography mass spectrometry* (GC-MS) used to analyze the volatile compounds, and selected compounds were structurally measured by mass spectrometry transposing the method to GC-MS[19]. The mass analyzer separates the molecules and fragments according to their masses and the detector detects and quantifies the separated ions[17]. GC-MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal-mass information. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories databases[18]. This study was objected to identify secondary metabolite composition and its relation to flowering competency in 5 genotypes of shallot.

EXPERIMENTAL

Plant materials

Local genotypes of shallot with different pattern of flowering were selected as plant material. Shallot genotype 'Bentanis' is used as the naturally flowering, while genotypes 'Bima Brebes Ilokos, Tajuk and Sumenep' are selected as the induced flowering of shallot (Marlin *et al.*, 2018). Each genotypes were treated with vernalization in 8 ºC temperature for 5 weeks, and no vernalization treatment as control. The bulbs were then planted in 45 cm diameter polythene bags containing 8 kg of growing media (soil: manure: husk = 2: 1: 1). Each polybag was planted with three bulbs. Before planting, shallots bulbs were soaked in a fungicide benomil 50% at a concentration of 2 $g.L^{-1}$ for 15 minutes. The bulbs were then soaked into solution of plant growth promoting rhizobacteria of 5 $g.L^{-1}$ for 15 minutes. NPK fertilizers with a ratio of 15:15:15 were applied in a dose of 600 $kg.ha^{-1}$ or 2.4 g per polybag.

Metabolomic analysis

The samples were the leaves of shallot plants at 4 weeks after planting. GC-MS unit was carried out on an GCMS-QP2010 system (Shimadzu Corporation, Japan) coupled to mass spectrometer detector. The samples were inserted into the quartz chamber in the GC-MS unit. Helium was used as a carrier gas in a constant flow mode at 0.85 ml/min. The chamber was heated in an oxygen-free environment at a temperature of 400 ºC for 0.2 minutes. The reaction will produce heat-mediated cleavage of chemical bonds in the macromolecular structure and produce low molecular weight with a chemical composition that identify specific compound of metabolite. Compound mixtures were then passed through the column GC-MS analysis. The column is Rt x 5 MS, with length 60.0 m, thickness 0.25 µm, and diameter 0.25 mm. The initial temperature of the column was 50 °C, which was gradually increased by 10 °C up to 280 °C. At the end of this period, the column oven temperature was 50 ºC raised up to 280 °C. Injection port temperature was ensured as 280 °C and helium flow rate as 0.85 ml/min. Mass spectrometer detector was employed to detect compounds when they were vented from the column. Temperature of the detector was 400 °C. The volatile compounds of the plant samples were then identified for each treatments.

The identification of the components of non-targeted metabolite was assigned by matching their mass spectra with those available in the WILEY7 LIB. The volatile compounds of the plant samples were then identified for each genotypes. Spectra were compared with National Institute of Standard and Technology (NIST, 2005 v2.1) library to identify the unknown compounds. The data were confirmed using PubChem online database in National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/), chemicalbook database (http://www.chemicalbook.com), and NIST webbook database

http://webbook.nist.gov/. The datasets were then log-transformed in order to acquire the normalized data. Data matrix based on non-targetted metabolomic of shallot in each vernalization treatments were analyzed using Metabolomic package of the R software (R version 3.2.2 http://www.r-project.org/). Metabolomics package was operated to construct HCA (*hierarchical cluster analysis*) and visulized heatmap with dendrogram. HCA was performed using Euclidean distance method and complete linkage agglomerative method.

RESULTS AND DISCUSSION

Metabolite compounds in 5 shallot local genotypes were affected significantly by vernalization treatments. A total of 130 of metabolomic compound of non-vernalized bulbs, and 122 of metabolites compound of vernalized bulbs of 5 shallot local genotypes were identified by GC-MS. Metabolite compound obtained where then visually described by metabolite heatmap (Figure-1). Heatmap is widely used in metabolomic research because it is able to visualize large amounts of metabolite data and its differences in sample groups[20]. Heatmap of both non vernalized and vernalized bulbs clustered 5 shallot local genotypes into three groups (Figure-1). First group was G2 (Bima Brebes genotype) and G4 (Tajuk genotype), second group was G1 (Bentanis genotype), and the third group was G3 (Ilokos genotype) and G5 (Sumenep genotype).

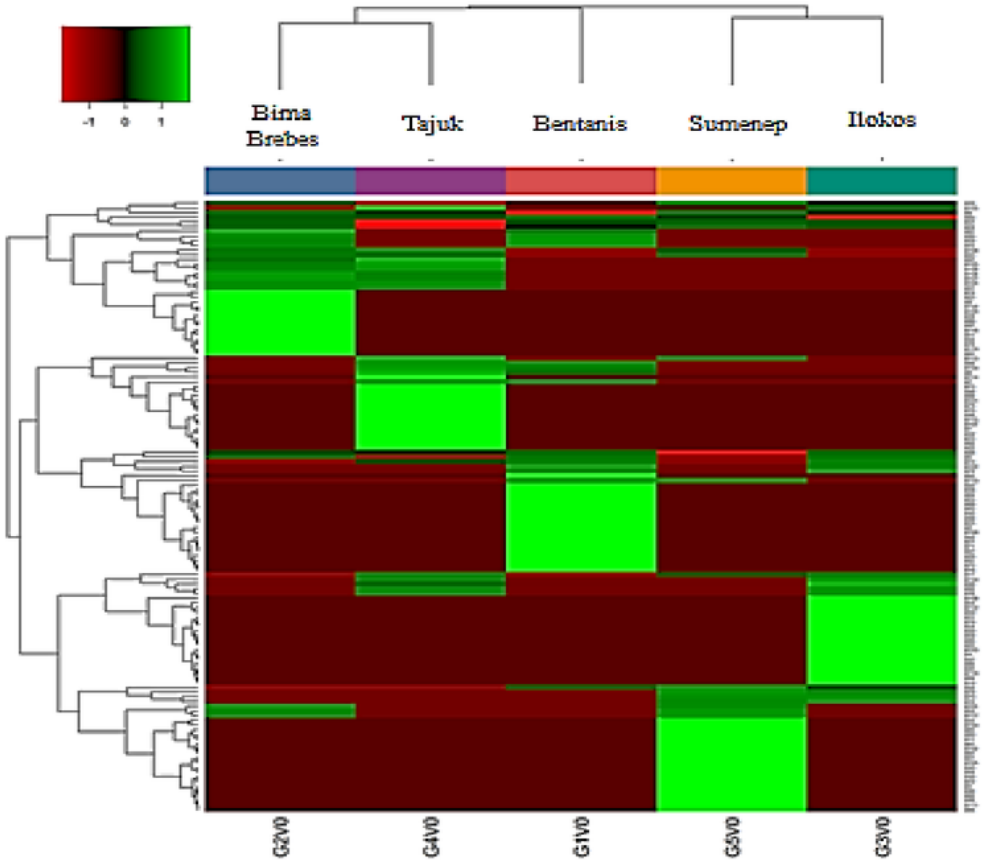


Fig.-1: Heatmap of hierarchical clustering analysis based on non-targeted metabolomic in 5 shallot genotypes of 130 metabolites in non-vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered.

Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a

relevant metabolite variability to another group. This was also confirmed by different colour of each heatmap coloumn. Specific metabolite compounds were visualized on each genotype column with a bright green color. Hierarchical clustering analysis showed that the genotype had a significant effect on the metabolites composition of the shallot. Each genotype of shallot produces different metabolites that associated to the flowering competency. Hierarchical clustering analysis of shallot metabolites grouped shallot genotypes into three distinct genotypes, in both vernalization treatments (Figure-1 and 2). The result indicated that the presence of specific compounds produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability.

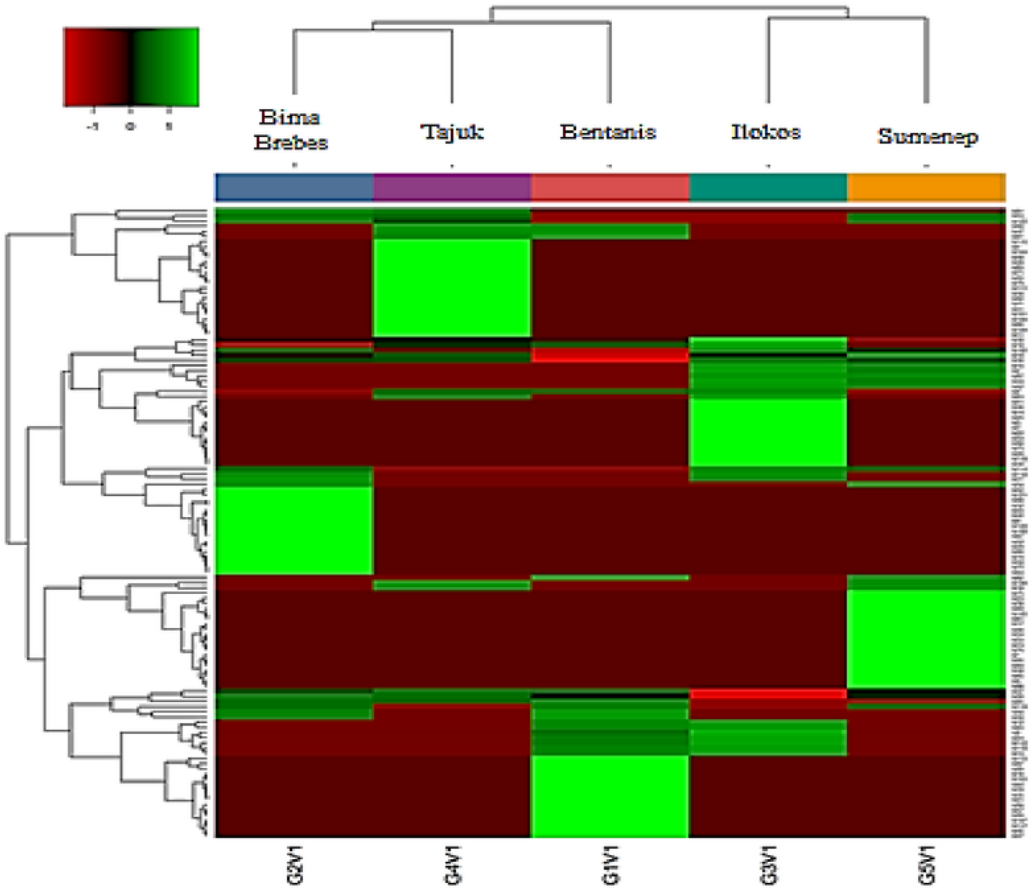


Fig.-2: Heatmap of hierarchical group analysis based on non-targeted metabolomic in 5 shallot local genotypes of 122 metabolites in vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered

Shallot genotypes located in the same group showed that the genotype had the same metabolite profile, and showed significant metabolite diversity with other groups. Visualization with heatmap also confirmed in the presence of different colors from each of the heatmap columns. The genotypes included in the first group were the Bentanis genotype (G1). In the second group there was the Bima Brebes (G2) and Tajuk genotype (G4), while in the third group there was Ilokos (G3) and Sumenep genotype (G5). The Bentanis genotype separates on its own group, and has close proximity to the Bima Brebes and Tajuk groups. These three genotypes are genotypes that have the ability to generate interest (sensitive to flowering).

Visualization with heatmap is widely used in metabolomic research because it can visualize large amounts of metabolomic data and classify it in sample groups[20]. Specific metabolite compounds of each sample of onion are visualized on each genotype column in bright green.

Mass spectrometry methods can accurately detect compounds including identification of unknown and unexpected compounds[18]. Identification of the molecular structure is often given without reference compounds, making it difficult to make a real identification[18]. The genotypes of shallot that have the ability to produce flowers will produce the specific metabolite compounds. Metabolite compounds produced in the transition process can be an indicator to determine the ability of flowering in shallot. The distribution of metabolomic compounds produced in non-vernalized shallot genotypes is presented in Figure-3.

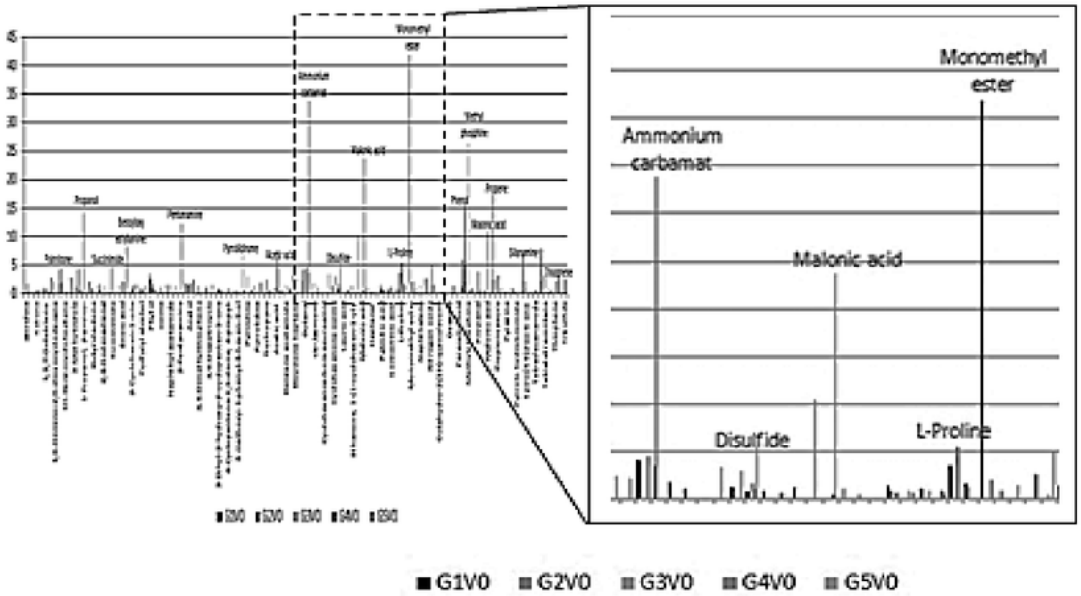


Fig.-3: Metabolite composition in non-vernalized shallot genotypes. Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5)

The results showed each genotype produced different metabolite compound. In Bentanis genotype showed the presence of monomethyl ester (21.82%) higher than Sumenep genotype (2.12%). The monomethyl ester compound, in the form of Mg-protoporphyrin IX mono-methyl ester cyclase is the catalyst required for the oxidation process of chlorophyll formation in plants[21]. The Bentanis genotype grows faster than Sumenep genotype. Bentanis genotype has robust maximum vegetative growth compared to Sumenep genotype at 5 weeks after planting. The results in Table 1 show that Bentanis genotype has higher plant height (36.96 cm) than of Sumenep (29.82 cm). The presence of monomethyl ester (Mg-protoporphyrin IX mono-methyl ester cyclase) compounds can increase the formation of chlorophyll more optimally, thus increasing the photosynthesis process for the Bentanis genotype growth.

Low concentration of proline (3.55% and 5.56%) was produced in Bentanis and Tajuk genotypes (Figure-3). Proline compounds are generally produced by plants as their response to stress condition. Bentanis genotype was also produced succinimide compounds at about 4.78%, those can not be found in other genotypes. Succinimide compounds are used as intermediate compounds in the process of forming organic compounds that stimulate plant growth.

The presence of high concentrate of ammonium carbamate compound is found in Bima Brebes genotype. Ammonium carbamate is an important compounds in the formation of carbamoyl phosphate and the formation of ATP into ADP[22]. While the presence of disulfide (dipropyl disulfide) compounds was found higher in non-sensitive flowering genotypes, such as Ilokos and Sumenep (1.69% and 5.43%), compared with other genotypes (0.8–0.14%). The content of dipropyl disulfide is an important component of

activity as an antimicrobial and antioxidant and gives a distinctive aroma to shallot plant. The sulfur compounds in dipropyl disulfide is important the components of these metabolite compounds[23]. The sulfur compounds are known to have important activities as antimicrobials and antioxidants[24,25]. Derivative products of allicin compounds such as diallyl disulfide, diallyl trisulfide found in garlic essential oil indicate activity as a good antimicrobial[26].

Onion bulbs had the highest concentrations of propanethiol compounds having an aroma 20 times higher than dipropyl disulfide compounds[27]. It also found in non-flowering shallot plant, Ilokos and Sumenep genotypes, they produce higher acetic acid compounds (1.04% and 5.98%) compared to the flowering type, the Bentanis genotype (1.02%).

Utilization of sensitive instruments in metabolomic analysis is an important way to detect the content of plant essential compounds.Plant metabolites consist of highly diverse chemical compounds ranging from inorganic compounds to hydrophilic, volatile alcohol and ketone compounds, amino and non-amino acids, hydrophobic lipids, and complex natural products[18].

Utilization of mass spectrometry technology is a great way to solve research and analysis problems in the metabolomic field[18]. Metabolomic analysis was succesfully to identify potential compounds extracted directly from shallot genotypes. The results showed higher concentrations of acyclic ditherphene alcohol (phytol) compounds in flowering type shallot, Bentanis genotype (8.23%), Bima Brebes (2.2%) and Tajuk (7.52%) than the Ilokos genotype (1.15%) and Sumenep (2.19). The phytol compounds is an indication that vernalization treatment has a positive effect in inducing flowering of shallot genotypes, explaining that these three genotypes are sensitive to vernalization treatment to induce flower formation. Phytol compound is a compound that serves as a precursor of vitamin E and K. The content of these vitamins plays an important role in the plant body as an enzymatic cofactor in the process of metabolism that stimulates reproductive response. The content of vitamin E in the form of tocopherol is an important compound as one of the factors in the context of a cellular antioxidant system[28]. The differences of metabolite profile might play a key role in flowering initiation in shallot.

In flowering type of shallot genotypes, Bentanis, Bima Brebes and Tajuk also showed the formation of nitrogen oxide and semicarbazid hydrochlorid which are relatively lower than the non-flowering type, Ilokos and Sumenep. Nitrogen compound is a compound that plays an important role in the vegetative phase of the plant. Once the plant enters the reproductive phase, the nitrogen compounds in the plant body will decrease. In contrast, the Ilokos and Sumenep genotypes show the formation of high nitrogen compounds ie ammonium compounds ammonium carbamat, and nitrogen oxide. High content of nitrogen compounds in Ilokos and Sumenep genotypes explains that these two genotypes are not sensitive to vernalization treatment, thus not encouraging the flowering formation.

The results also showed that the Sumenep genotype contained high organosulfur compounds compared with other genotypes, namely thiophene (2.31%) and trisulfide (4.25%). The organosulfur is an important compound in giving aroma and spiciness upsurge in shallot, and play an important role as an antioxidant. Essential compounds in *Allium* have strong antioxidant ability. Tests on antimicrobial activity showed that all essential oils on *Allium* were able to inhibit the activity of gram-positive and gram-negative bacteria[9].

Vernalization treatment triggered in the formation of proline compounds in the Sumenep genotype. Vernalization treatment provides a stress condition to the plants that increase the plant mechanism of adaptation by producing proline compounds. Proline accumulation in the plant body is a physiological response to stress, and as a generative tissue development[29]. Secondary metabolite is the cellular response in plant that receives external pressure.

The metabolite profile of 5 local onion genotypes is also rich in non-volatile phytonutrients, especially the content of phenolic compounds (phenol, 2-propenamide, 3-pyridinamine, N-furfurylidene 2-phenylethyl amine, pyrrolidinedione), amide compounds aziridine-2-carbothioic acid amide) and protein compound (pyroglutamic acid). The essential components, mono and sesquiterpenes, carbohydrates, phenols, alcohols, ether, aldehydes and ketones, are responsible for biological activity and also for their fragrance[30]. Phenols and polyphenol compounds play important roles as antioxidants in plants[31,32,33]. Research on *Allium rotundum* showed the presence of antioxidant activity of phenol compounds with high concentrations of up to 4%[12]. Onion extract contained the highest of phenol content, at 17.18 mg

GAE per gram of fresh weight[34], and a phenol content of 114.70 mg GAE per 100 g of different onion samples[35].

Metabolite compound is difference among shallot genotypes. Each plant genotype performs metabolic activities that depend on their genetic conditions and the ability to adapt to its environment. The plant biological activity may imply to proportion of metabolite containing in a plant.

CONCLUSION

Secondary metabolite is associated to the flowering competency in shallot genotypes. GC-MS analysis detected 130 metabolites compounds in non-vernalized treatment, and detected 122 metabolites in vernalization treatment in 5 genotypes of shallot. Each genotypes produce different specific metabolites which is clustered according to the flowering competency. Genotype Bentanis, Bima Brebes and Tajuk were confirmed as flower-sensitive genotypes, with a lower content of proline and nitrogen (ammonium carbamate and nitrogen oxide), and higher phytol compounds. Genotype Ilokos and Sumenep are flowering insensitive genotypes, which contain higher level of organosulfur compounds (thiophene and trisulfide). Vernalization treatment caused the Sumenep genotype produces the highest proline compound among other genotypes.

ACKNOWLEDGMENT

This researchs is supported by grants from National Innovation System Research Insentive 2016, Ministry of Research, Technology, and Higher Education, Indonesia.

REFERENCES

1. F.V. Golubev, N.A. Golubkina, and Y.N. Gorbunov. *Applied Biochemistry and Microbiology.* 39(5): 12–535 (2003)
2. G. Griffiths, L. Trueman, T. Crowther, B. Thomas, and B. Smith. *Phytotherapy research: PTR.* 16(7): 603–615 (2002)
3. T. Ritsema , and S. Smeekens. *Current Opinion in Plant Biology.* 6(3): 223–230 (2003)
4. H. Mohammadi-Motlagh , A. Mostafaie, and K. Mansouri *Archives of Medicaal Science.* 1: 38–44 (2011)
5. Marlin, A. Maharijaya, A. Purwito, and Sobir. *SABRAO Journal of Breeding and Genetics.* 50 (3) 313-328 (2018)
6. E.J. Finnegan, D. Bagn25 C. Helliwell, D. Rouse, C. Sheldon, M. Tadege, W.J. Peacock, and E. Dennis. Vernalization. Encyclopedia of Life Sciences Macmillan Publishers Ltd, Nature Publishing 26up. (2001). www.els.net.
7. J. Song, A. Angel, M. Howard, and C. Dea. *Journal of Cell Science.* 125 (16): 3723–3731 (2012).
8. 17ijima. *Metabolite.* 4: 699–721 (2014).
9. D. Mnayer, A. Fabiano-Tixier, E. Petitcolas, T. Hamieh, N. Nehme, C. Ferrant, X. Fernandez, and F. Chemat. *Molecules.* 19: 20034–20053 (2014).
10. 5 Xiao, and K.L. Parkin. *Phytochemistry.* 58(7): 1059–1067 (2007).
11. 8.A. Vazquez-Prieto, and R.M. Miatello. *Molecular Aspects of Medicine.* 31(6): 540–5 (2010).
12. A.A. Dehpour, M. Yousefian, S.A. Jafary Kelarijani, M. Koshmoo, S. Mirzanegad, V. Mahdavi, S.E. Mousavi, E. Shirzad, M. Afzali, M.J. Javad Bayani, J.E. Olyaei, and M.K. Yahyapor. *Advances in Environmental Biology.* 6(3): 1020–1025 (2012).
13. 33Lanzotti, F. Scala, and G. Bonanomi. *Phytochemistry Reviews.* 13: 769–791 (2014).
14. 36Martins, S. Petropoulos, and I.C.F. Ferreira. *Food Chemistry.* 15(211): 41–50 (2016).
15. 11Fiehn. *Plant Molecular Biology.* 48: 155–171 (2002).
16. H. Wu, O.J. Gu, S. Chen, X. Liu, Y. Zhou, X. Zhang, and X. Xu. *Journal of pharmaceutical and biomedical analysis.* 72: 267–291 (2013).
17. T. Soininen. Ph.D. Thesis. University of Eastern Finland. Finland (2014).

18. S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, and J. Nielsen. *Mass Spectrometry Reviews.* 24: 18–646 (2005).
19. N.C.J.P. Lekshmi, S. Viveka, M.B. Viswanathan, G. Manivannan, and T.M. Shobi. *Nanobio Pharmaceutical Technology.* (2014). DOI : 10.13140/2.1.3278.7523.
20. S.M. Huang, W. Toh, P.I. Benke, C.S. Tan, and C.N. Ong. *Metabolomics.* 10:1084–1093 (2014).
21. Y.S. Wong, and P.A. Castelfranco *Plant Physiology.* 79(3): 730–733 (1985).
22. N. Baker, J. Harbinson, and D.M. Kramer. *Plant Cell Environment.* 30 1107–1125 (2007)
23. P. Rattanachaikunsopon, and P. Phumkhachorn P. *African Journal of Microbiology Research.* 3: 747–750 (2009).
24. A. Chehregani, F. Azimishad, and A.H. Haj. *International Journal of Agriculture Biology.* 9(6): 873–(2007)
25. . Ye, D.H. Dai, and W.L. Hu. *Food Control.* 30: 48–53 (2013).
26. J.W. Kim, J.E. Huh, S.H. Kyung, and K.H. Kyung. *Food Science and Biotechnology.* 13: 235–239 04).
27. M. Lokke, M. Edelenbos, E. Larsen, and A. Feilberg. *Sensors.* 12: 16060–16076 (2012).
28. *M.G. Traber, J. Atkinson, and Atkinson. Free Radical Biology and Medicine. 43 (1): 4–15 (2007).*
29. N. Verbruggen, C. Hermans. *Amino Acids.* 35: 753–759 (2008).
30. E.A. El-Wakil, M.M. El-Sayed, and E.E. Abdel-Lateef. *International Journal of Pharma Sciences and Research IJPSR.* 6 (3) (2015).
31. S.A.B.E. Van Acker, D.J. van den Berg, N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, and W.J. der Vijgh. *Free Radical Biology and Medicine* 20 (3): 331– 342 (1996).
32. A. Alvarez, N.B. Debattista, and N.B. Pappano. *Folia Microbiologica.* 53(1): 23–8 (2008).
33. . Mukne, V. Viswanathan, and A.G. Phadatare. *Pharmacognosy Reviews.* 5(9): 13–18 (2011)
34. X. Lu, J. Wang, H.M. Al-Qadiri, C.F. Ross, J.R. Powers, J. Tang, and B.A. Rasco. *Food Chemistry.* : 637–644 (2011).
35. J. Yang, K.J. Meyers, J. van der Heide, and R.H. Liu. *Journal of Agriculture Food Chemistry.* 52: 6787–6793 (2004).

[RJC-4034/2018]

RJC-5356

ORIGINALITY REPORT

33%

SIMILARITY INDEX

PRIMARY SOURCES

1	sevgiligiyim.com Internet	372 words —	9%
2	epublications.uef.fi Internet	151 words —	4%
3	sabraojournal.org Internet	100 words —	2%
4	www.ijpsr.info Internet	44 words —	1%
5	Soininen, T.H.. "The combined use of constrained total-line-shape ^1H NMR and LC-MS/MS for quantitative analysis of bioactive components in yellow onion", Journal of Food Composition and Analysis, 201203 Crossref	43 words —	1%
6	Silas G. Villas-BÃ´as. "Mass spectrometry in metabolome analysis", Mass Spectrometry Reviews, 09/2005 Crossref	38 words —	1%
7	A Mantas, E Deretey, F.H Ferretti, M Estrada, I.G Csizmadia. "Ab initio conformational analysis of flavone and related compounds", Journal of Molecular Structure: THEOCHEM, 2000 Crossref	33 words —	1%
8	www.researchgate.net Internet	32 words —	1%
9	rasayanjournal.co.in		

Internet

32 words — 1%

10 Bamba, Takeshi, and Eiichiro Fukusaki. "Technical problems and practical operations in plant metabolomics", Journal of Pesticide Science, 2006.
Crossref

31 words — 1%

11 Borhane E.C. Ziani, Sandrina A. Heleno, Khaldoun Bachari, Maria Inês Dias et al. "Phenolic compounds characterization by LC-DAD- ESI/MSn and bioactive properties of Thymus algeriensis Boiss. & Reut. and Ephedra alata Decne", Food Research International, 2018
Crossref

27 words — 1%

12 "Optimization of Supercritical Extraction of Oil from Onion by Supercritical CO2", Asian Journal of Chemistry, 2014.
Crossref

26 words — 1%

13 edepot.wur.nl
Internet

24 words — 1%

14 pubs.rsc.org
Internet

21 words — 1%

15 Syed Rizwan Ahamad, Abdul Rahman Al-Ghadeer, Raisuddin Ali, Wajhul Qamar, Suliman Aljarboa. "Analysis of inorganic and organic constituents of myrrh resin by GC–MS and ICP-MS: An emphasis on medicinal assets", Saudi Pharmaceutical Journal, 2017
Crossref

21 words — 1%

16 www.mona.uwi.edu
Internet

20 words — < 1%

17 Mehmet Musa Özcan, Süleyman Doğu, Nurhan Uslu. "Effect of species on total phenol, antioxidant activity and phenolic compounds of different wild onion bulbs", Journal of Food Measurement and Characterization, 2017
Crossref

20 words — < 1%

18	electrochemsci.org Internet	19 words —	< 1%
19	Abu Hanifah, Awang Maharijaya, Sastia P. Putri, Walter A. Laviña, Sobir. "Untargeted Metabolomics Analysis of Eggplant (Solanum melongena L.) Fruit and Its Correlation to Fruit Morphologies", Metabolites, 2018 Crossref	18 words —	< 1%
20	Wang, Xin, Mingjie Jin, Venkatesh Balan, A. Daniel Jones, Xia Li, Bing-Zhi Li, Bruce E. Dale, and Ying-Jin Yuan. "Comparative metabolic profiling revealed limitations in xylose-fermenting yeast during co-fermentation of glucose and xylose in the presence of inhibitors : Metabolic Profiling Revealed Limitations in Xylose-Fermenting Yeast", Biotechnology and Bioengineering, 2013. Crossref	18 words —	< 1%
21	Advances in Photosynthesis and Respiration, 2013. Crossref	16 words —	< 1%
22	www.labome.org Internet	16 words —	< 1%
23	dspace-uniud.cineca.it Internet	15 words —	< 1%
24	Sharma, Kavita, Neelima Mahato, Shivraj Hariram Nile, Eul Tal Lee, and Yong Rok Lee. "Economical and environmentally-friendly approaches for usage of onion (Allium cepa L.) waste", Food & Function, 2016. Crossref	14 words —	< 1%
25	www.repositorio.ufop.br Internet	14 words —	< 1%
26	Amanda Malvessi Cattani, Tiago Sartor, Vítor da Silveira Falavigna, Diogo Denardi Porto et al. "The Control of Bud Break and Flowering Time in Plants: Contribution of Epigenetic Mechanisms and Consequences in Agriculture and Breeding", Elsevier BV, 2018 Crossref	13 words —	< 1%

27	gitisa.it Internet	13 words — < 1%
28	Lucian G. Bahrin, Mircea O. Apostu, Lucian M. Birsa, Marius Stefan. "The antibacterial properties of sulfur containing flavonoids", Bioorganic & Medicinal Chemistry Letters, 2014 Crossref	12 words — < 1%
29	Traber, M.G.. "Vitamin E, antioxidant and nothing more", Free Radical Biology and Medicine, 20070701 Crossref	12 words — < 1%
30	repository.ipb.ac.id Internet	12 words — < 1%
31	hal.archives-ouvertes.fr Internet	11 words — < 1%
32	Huang, Shao-Min, Weizhong Toh, Peter Imre Benke, Chuen Seng Tan, and Choon Nam Ong. "MetaboNexus: an interactive platform for integrated metabolomics analysis", Metabolomics, 2014. Crossref	10 words — < 1%
33	Tuty Arisuryanti, Zulfa Kurniawati, Johan Putra Koentjana. "Karyomorphological study on two local garlic cultivars (Allium sativum L.) from central Java, Indonesia", AIP Publishing, 2018 Crossref	10 words — < 1%
34	www.jmaterenvironsci.com Internet	9 words — < 1%
35	www.tandfonline.com Internet	9 words — < 1%
36	www.sunbio.com.cn Internet	9 words — < 1%

37	Chope, G.A.. "Use of canonical variate analysis to differentiate onion cultivars by mineral content as measured by ICP-AES", Food Chemistry, 20090801 Crossref	9 words — < 1%
38	Dima Mnayer, Anne-Sylvie Fabiano-Tixier, Emmanuel Petitcolas, Tayssir Hamieh et al. "Chemical Composition, Antibacterial and Antioxidant Activities of Six Essentials Oils from the Alliaceae Family", Molecules, 2014 Crossref	8 words — < 1%
39	E Jean Finnegan. "Vernalization", Encyclopedia of Life Sciences, 03/15/2010 Crossref	8 words — < 1%

EXCLUDE QUOTES	OFF	EXCLUDE MATCHES	OFF
EXCLUDE BIBLIOGRAPHY	OFF		

Marlin UNIB <marlin@unib.ac.id>

Proof of payment

Marlin UNIB <marlin@unib.ac.id> 2 Juni 2019 pukul 22.33
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Thank you for your mail.
[Kutipan teks disembunyikan]

Marlin UNIB <marlin@unib.ac.id>

Revised RJC_5356/2019

Marlin UNIB <marlin@unib.ac.id> 21 Juni 2019 pukul 08.02
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Dear Editors,

Thank you very much for sending me the Revision 2. In general, we really thank the editor and reviewers for the very constructive comments.
We have now revised the manuscript as suggested by reviewers.
I attach the more detail of the specific responses.

Thank you
Dr. Marlin

2 lampiran

ANSWERS to REVIEWER'S COMMENTS.docx
54K

REVISED MANUSCRIPT, RJC_5356.docx
291K

SECTION-I : Details of Manuscript

Manuscript Number:	RJC_5356
Title:	THE SECONDARY METABOLITES AND ITS RELATION TO FLOWERING COMPETENCY IN SHALOT (Allium cepa var. aggregatum)
Authors:	Marlin[1*], A. Maharijaya[2], A. Purwito[2], and Sobir[2] [1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia [2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia

SECTION-II : Comments per Section of Manuscript

Reviewer 1 Comments	Justification/Revision
General comment:	
A1. Please give "Vernalization" as your technic in flowering competency of shalot	Thank you for raising very interesting points. We have now strengthened the title with "vernalization" as follow : SECONDARY METABOLITES CHANGE UNDER VERNALIZATION AND ITS RELATION TO FLOWERING COMPETENCY IN SHALLOT (*Allium cepa* var. *aggregatum*)
A2. Data not found	Thank you for the comment. We have showed the data in results part (figure 1 and 2) that 130 0f metabolites were detected in non vernalized bulbs dan 122 of metabolites were found in vernalized bulbs.
A3. What compounds that correlated with flowering competency?	Thank you for the comment. In results and discussion, we showed that ditherphene alcohol (phytol) associated to flowering competency in shallot.
Introduction and Literature :	-
Research Methodology:	

A4. Are you sure, this material has been identified? Please give information such as identified or voucher specimen in your work	Thank you for the suggestion. Those material had been identified and published (Marlin et al. 2018), as describe in the text.
A5. Where location? Please give information for coordinate geography your research location.	Thank you for raising very interesting points. We have now stated that the experiments were located in Pasir Kuda research farm, Bogor Indonesia.
A6. Please give information type of your sample that injected in GC-MS? Extract or another types of your sample, please give explain more?	Thank you for the suggestion. We agree and have added more information on sample preparation, as follow : The samples were the leaves of shallot plants at 4 weeks after planting (bolting stage). Leaves were cutted for 10 g and were extracted with methanol (50 mL). The methanolic extract of shallot leaves were used as a sample in the GC-MS analysis.
Results and Discussion	
A7. In this section, I not found correlation between metabolite with flowering competency in shallot? Can you added more explain, because your novelty in this research was metabolite in flowering competency in shallot. If you not found correlation with Heatmap analysis, may be analysis data can be use with simple correlation between major metabolite and flowering competency data.	Thank you for the comment. We stated that metabolite had a correlation with flowering competency in shallot as describing in heatmap of hierarchical clustering analysis. The heatmap showed that each shallot genotype produces different metabolites associated to the flowering competency. The results indicated that there are a presence of acyclic ditherpene alcohol (phytol) which were produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability. The phytol presence in vernalized bulb is an indication that vernalization treatment has a positive effect in inducing flowering of shallot.
A8. Please give data in table for these metabolites?	Thank you for the comment. We have describe the data in figure 1 and 2. It will be redundant if the data showed in a table.
A9. Please answer your objective in this study	Thank you for the suggestion. We agree and change the conclusion.
A10. Data not found in text	Thank you for the comment. The data have clearly in Figure 1 and 2 in both vernalization treatments.

Bibliography/References	-
Others:	-

Reviewer 2 Comments	Justification/Revision
General comment: The manuscript describes the metabolite composition in leaves of 5 shallot genotypes whose bulbs were untreated and treated by vernalization. The results may have added values to the scientific community about metabolomic changes induced by vernalization that are important for breeding.	Thank you for the suggestions.
#1 However, the results were not delivered in a clear state, so it is difficult to follow and understand it. For example, the author describes a particular metabolite specifically present in one genotype, but the author does not describe from which treatments the metabolite is produced.	#1. What we want to say is that in each genotype produces specific metabolite in difference vernalization treatments. The data were showed in heatmap of hierarchical clustering analysis in Figure 1 and 2. The heatmap showed that each shallot genotype produces different metabolites associated to the flowering competency. The results indicated that there are a presence of acyclic ditherpene alcohol (phytol) which were produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability. The phytol presence in vernalized bulb is an indication that vernalization treatment has a positive effect in inducing flowering of shallot.
#2 In addition, significant data cited in the discussion is not present in the manuscript. The morphological data (Table 1) and data of prolin levels could not be found.	# 2 Thank you for the correction, we have re-checked and revised the statements in the manuscript.
#3 It is vital that the author must provide metabolite information to understand the hypothesis raised in the discussion.	#3 Thank you for the suggestions. Metabolomic composition of 5 genotypes shallot have been describe in heatmap of 5 genotypes.
#4 From the title, the reader expects to have an overview that there are metabolomic changes after bulb vernalization, which may induce the	#4 Thank you for the correction, we have revised the statements in the manuscript.

flowering time acceleration among 5 shallots. However, the discussion goes broadly to environmental stress and does not deeper to the flowering ability of 5 shallots author discuss his findings. #5 Somehow, the approach that done by the author in analyzing shallot leaf metabolites for understanding the response of vernalization is questionable, since the metabolites in leaves and generative organ could be different. It may also reconsider to analyze metabolites from the whole plant organs to get a big view of metabolites responsible for accelerating the flowering time in shallot.	#5 Thank you for the suggestions. We used shallot leaves as sample (4 weeks after planting) in generative (bolting stage) for metabolomic analysis in shallot. We do not use another generative organ due to non-flowering genotype is not produce the flower part.
Introduction and Literature a. The literature review has not included the current status of metabolomics research in *Allium*, major metabolites in leaves, bulbs and/or other organs, the importance of generative stage and logical backgrounds obstacles in producing seed from *Allium*. b. Authors mentioned the reason of using GC/MS approach is due to unavailable reference standards for identifying the observed metabolites. However, this is a weak reason. The author must explain the ultimate reasons related to the major issue that really want to unravel, i.e., metabolites related to flowering physiology. c. The stated research objective is to identify secondary metabolite composition in 5 shallots. However, this is not very clear and too broad, whereas the research scope focusses on volatile metabolites.	a. Thank you for the sugestion. Unfortunatelly, there is no information about current status of metabolomic composition in each plant organ of shallot. b. Thank you for the correction. We agree and have revised the statements in the manuscript. c. Thank you for the correction. We state clearly to identify the various components by GCMS.from their mass spectra. Hierarchical clustering analysis grouped shallot genotypes into three distinct genotypes, in both vernalization treatments.
Research Metodology a. It is important to put species name of samples along with information on the flowering pattern of	a. Thank you for the sugestion.

each genotype. b. The sample pretreatment and metabolite extraction steps from raw leaves into ready-injected samples should be described in the methodology. c. In methodology, it was mentioned that metabolites were normalized by log transformation. In contrast, the author stated that metabolite data were normalized by log2 transformation and mean centering. Which method is the correct one? d. When performing metabolomics data analysis, there are huge spectra data collected from the analysis. Filtering and clustering spectra data must be done before further analysis. These steps were crucial to perform prior to metabolite identification. However, these steps were not described in the methodology. Please include these steps in the method.	b. Thank you for raising very interesting points. We agree and have added more information on sample preparation. c. Thank you for the correction. We agree and re-check the consistency.
Results and Discussion a. The first sentence of Results and Discussion stated that "Metabolites in 5 shallot genotypes were significantly affected by vernalization treatment". This sentence is a conclusion sentence which should be stated after the findings have been described and discussed. b. Sentences: "Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by different colour of each heatmap coloumn. Specific metabolite compounds were visualized on each genotype column with a bright green color" This is contrary with Fig. 1 and Fig. 2 illustrations, which are stated that the color-coded matrix represents the intensities of metabolites, i.e., red is low values and green is high values	a. Thank you for the sugestion. We agree and have change the statement b. Thank you for the correction. We agree and have change the statement. The color-coded matrix represents the intensities of metabolites content.

metabolites. The author should redefine the statements to make it clear.	
c. Results were organized by displaying metabolites detected in non-vernalized samples and vernalized samples (Fig. 1 & Fig 2). However, it is difficult to understand which metabolites affected/expressed by vernalization. It is important that data processing should be performed in integrated analysis and resulted in only 1 figure metabolite heatmap, supplemented by Table of metabolite identity, to get a better understanding of which metabolites upregulated or downregulated or synthesized after vernalization.	c. Thank you for the suggestion. However we decided to use heatmap of HCA to display metabolites composition in each treatments.
d. Group names of G1, G2, G3, G4 and G5 in the text are not in line to any illustration in Fig 1 and Fig 2. Meanwhile, there are other different codes displayed in Fig. 1 and 2 which do not correspond to any information in the text. Authors should put the group codes/names which are annotated in the text.	d. Thank you for raising very interesting points. Actually we have addressed these points in the results and discussion. Genotype codes of G1, G2, G3, G4, and G5 represent as Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5). we used the codes in text clearly inline to the explained data. The genotypes showed in Fig 1 were clustered in different group compared to Fig 2. It is that because the code are located in different position, depend on clustering data.
e. Paragraph 2 of the Results & Discussion: Based on metabolites, 5 shallots were clustered into 3 groups and the author claims the clustering was based on genotypes. However, this hypothesis is not provided by any strong facts, such as genotype agronomical description (origin, flowering period, etc.). On the other hand, in the next sentence, the author stated contrary to the previous statement, which said that the clustering was due to the flowering competency. These statements are confusing for readers and the author should restructure the paragraph.	e. Thank you for he suggestion. In this manuscript, we clustered shallots genotype base on metabolite compositions by GCMS analysis. We have reported another data in molecular and morphological approach to clustering shallot genotypes related to flowering competency.

f. Fig. 3 is hardly readable, Table 1 is missing	f. Thank you for suggestion. We agree and revise
g. Paragraph 3 of Results & Discussion: There are many details in describing metabolites found in shallots. However, the explanation does not describe the metabolomic differences among groups and their correlation with the vernalization and the flowering competency. The author should reshape the discussion into the topic expressed in the title.	g. Thank you for suggestion.
h. Metabolites to flowering competency correlation analysis is strongly recommended to be performed in order to check and decide which whether there are correlation between leaves volatile metabolites to flowering competency.	h. Thank you for suggestion. As mentioned in previous response, in this experiment we used shallot leave in bolting stage (4 weeks of planting). Bolting stage of shallot indicated the transition stage of vegetative to generative. This transition periode may change metabolites compositions related to its flowering competency.

RJC

RASĀYAN *J. Chem.*
Vol. 12 | No. 2 |402 - 408| April - June | 2019
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

SECONDARY METABOLITES CHANGE UNDER VERNALIZATION AND ITS RELATION TO FLOWERING COMPETENCY IN SHALLOT (*Allium cepa* var. *aggregatum*)

Marlin[1*], A. Maharijaya[2], A. Purwito[2], and Sobir[2]
[1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia
[2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia
*E-mail : marlin@unib.ac.id

ABSTRACT

Shallot extract contains a great quantity of essential oil, volatiles, and other compounds. The composition of secondary metabolite can be regarded as the ultimate reactions of biological and physiological to genetic and environmental conditions. This study was objected to identify metabolomic composition and its relation to flowering competency in 5 genotypes of shallot. The non-targetted metabolites were identified using GC-MS and assigned by matching their mass spectra with those available in the WILEY7 LIB. Data matrix was analyzed by using metabolomic package of the R software. Heatmap was visualized for a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The composition metabolites from 5 genotypes were difference and suggested correlated to flowering competency. The 3 genotypes of flowering type of shallot produced highest concentration of phytol (ditherphene alcohol) and low concentration of nitrogen compound. Whereas, 2 genotypes of non-flowering types contained high concentration of organosulfur and nitrogen sources. Metabolite profile of 5 genotypes contained of volatile and non-volatile phytonutrients, vitamin, saturated fatty acid and organosulfur compounds.

Keywords: dissimilarity matrix, GC-MS, non-targetted metabolite, organosulfur, phytol

INTRODUCTION

Shallot (*Allium cepa* var. *aggregatum*) is an important *Allium* plant that been used for food, ornamental, natural alternatives to food synthetic preservatives and medicinal for curing various diseases. *Allium* plants contain different sugars, amino acids, vitamins, sulfurous compounds, enzymes, flavonoids, saponins and minerals[1]. *Allium* are also valued for their therapeutic properties; they are active as diuretics and laxatives and have been used to treat headaches and parasitic worms[2]. Onion has beneficial effect as their prebiotic, and their ability to decrease levels of insulin, cholesterol, triacylglycerol and phospholipids for human health[3], and considered an important vegetable and medicinal plant prescribed as an effective therapy for several complaints in healing treatment such as natural cancer treatment[4]. These beneficial effects will become an important target in shallot breeding and management practices.

Formatted: Font color: Blue
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Default Paragraph Font, Font: 12 pt
Formatted: Font: 12 pt

Shallot genotypes may vary in flowering pattern, naturally flowering, inducible flowering, and non-inducible flowering shallot[5]. Flowering in shallot can be induce by an exogenous treatment such as vernalization. Vernalization is an important adaptation of plants to initiate flowering in response to a prolonged exposure to low temperatures[6,7]. The principles derived from vernalization are likely to be widely relevant to epigenetic reprogramming in many organisms[7]. External condition plays important regulation in plant bioactivity and influences the metabolomic composition. Metabolomic composition in plant is various and specific, that depends on genetic and plant growth environment. Metabolomic describes plant phenotype differentiation.

Metabolomic technologies have revealed a new insights in biological systems through metabolic dynamics[8]. The metabolomic composition determines biological and physiological function of the plants. Metabolite compounds are observed in the 6 *Alliaceae* family[9], in *Allium cepa* (onion) extracts[10,11], in *Allium rotundum* L[12], ~~and~~ in *Allium sativum* (garlic) formulations[13,14], and in shallot grown in tidal swampland[15]. Metabolomic defined as a comprehensive analysis in which all the metabolites of a biological system are identified and quantified[15~~15~~16]. Metabolites are the end products of cellular regulatory processes. Metabolites profiling in plant can be directly extracted from a part of plant to find new molecules contents. Profiling of the metabolite composition is an important way to evaluate the contents of the medical plants, as well as their safety and efficacy[17~~16~~]. Mass spectrometry is an analytical method, which allows the determination of the mass formula and structure. With mass spectrometry, individual molecules can be measured based on their molecular peak (m/z) and characteristics spectra of fragment ions[18~~17~~]. Mass spectrometry technology is high sensitivity to identify the unknown and unexpected of the components present in the complex biological samples[19~~18~~]. These fragments often provide a clue to the molecule's structure, but without a reference compound, the difficulty of making an identification increases markedly[18~~17~~]. The analytical strategy *gase chromatography mass spectrometry* (GC-MS) used to analyze the volatile compounds, and selected compounds were structurally measured by mass spectrometry transposing the method to GC-MS[~~19~~,20, 21]. The mass analyzer separates the molecules or/and fragments according to their masses and the detector detects and quantifies the separated ions[18~~17~~]. GC-MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal-mass information. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories databases[19~~18~~]. This study was objected to identify secondary metabolite composition and its relation to flowering competency in 5 genotypes of shallot.

EXPERIMENTAL

Plant materials

Local genotypes of shallot with different pattern of flowering were selected as plant material. Shallot genotype 'Bentanis' is used as the naturally flowering, while genotypes 'Bima Brebes Ilokos, Tajuk and Sumenep' are selected as the induced flowering of shallot[5] ~~(Marlin *et al.*, 2018)~~. Experiments were planted in Pasir Kuda research farm, Bogor Indonesia. Each genotypes were treated with vernalization in 8 ^{o}C temperature for 5 weeks, and no vernalization treatment as control. The bulbs were then planted in 45 cm diameter polythene bags containing 8 kg of growing media (soil: manure: husk = 2: 1: 1). Each polybag was planted with three bulbs. Before planting, shallots bulbs were soaked in a fungicide benomil 50% at a concentration of 2 g.L^{-1} for 15 minutes. The bulbs were then soaked into solution of plant growth promoting rhizobacteria of 5 g.L^{-1} for 15 minutes. NPK fertilizers with a ratio of 15:15:15 were applied in a dose of 600 kg.ha^{-1} or 2.4 g per polybag.

Metabolomic analysis

Formatted: Font: 12 pt
Formatted: Font: 12 pt, Font color: Blue
Formatted: Font: 12 pt, Font color: Blue
Formatted: Font: 12 pt
Formatted: Font: 12 pt, Indonesian
Formatted: Font: 12 pt
Formatted: Font: 12 pt, Indonesian
Formatted: Font: 12 pt

The samples were the leaves of shallot plants at 4 weeks after planting (bolting stage phase). Leaves were cutted for 10 g and were extracted with methanol (50 mL). The methanolic extract of shallot leaves were used as a sample in the GC-MS analysis. GC-MS unit was carried out on an GCMS-QP2010 system (Shimadzu Corporation, Japan) coupled to mass spectrometer detector. The samples were inserted into the quartz chamber in the GC-MS unit. Helium was used as a carrier gas in a constant flow mode at 0.85 ml/min. The chamber was heated in an oxygen-free environment at a temperature of 400 ^{o}C for 0.2 minutes. The reaction will produce heat-mediated cleavage of chemical bonds in the macromolecular structure and produce low molecular weight with a chemical composition that identify specific compound of metabolite. Compound mixtures were then passed through the column GC-MS analysis. The column is Rt x 5 MS, with length 60.0 m, thickness 0.25 µm, and diameter 0.25 mm. The initial temperature of the column was 50 °C, which was gradually increased by 10 °C up to 280 °C. At the end of this period, the column oven temperature was 50 ^{o}C raised up to 280 °C. Injection port temperature was ensured as 280 °C and helium flow rate as 0.85 ml/min. Mass spectrometer detector was employed to detect compounds when they were vented from the column. Temperature of the detector was 200 °C. The volatile metabolite compoundsSecondary metabolites of the plant samples were then identified for each treatments.

The identification of the components of non-targeted metabolite was assigned by matching their mass spectra with those available in the WILEY7 LIB. The volatile metabolite compounds of the plant samples were then identified for each genotypes. Spectra were compared with National Institute of Standard and Technology (NIST, 2005 v2.1) library to identify the unknown compounds. The data were confirmed using PubChem online database in National Center for Biotechnology Information (https://pubchem.ncbi. nlm.nih.gov/), chemicalbook database (http://www.chemicalbook.com), and NIST webbook database http://webbook.nist.gov/. The datasets had been transformed to log2 and mean-centeredwere then log transformed in order to acquire the normalized data. Data matrix based on non-targetted metabolomic of shallot in each vernalization treatments were analyzed using Metabolomic package of the R software (R version 3.2.2 http://www.r-project.org/). Metabolomics package was operated to construct HCA (*hierarchical cluster analysis*) and visulized heatmap with dendrogram. HCA was performed using Euclidean distance method and complete linkage agglomerative method.

RESULTS AND DISCUSSION

Metabolite compounds in 5 shallot local genotypes were affected significantly by vernalization treatments. A total of 130 of metabolomic compound of non-vernalized bulbs, and 122 of metabolites compound of vernalized bulbs of 5 shallot local genotypes were identified by GC-MS. Metabolite compound obtained where then visually described by metabolite heatmap (Figure-1). Heatmap is widely used in metabolomic research because it is able to visualize large amounts of metabolite data and its differences in sample groups[222120]. Heatmap of both non vernalized and vernalized bulbs clustered 5 shallot local genotypes into three groups (Figure-1). First group was G2 (Bima Brebes genotype) and G4 (Tajuk genotype), second group was G1 (Bentanis genotype), and the third group was G3 (Ilokos genotype) and G5 (Sumenep genotype).

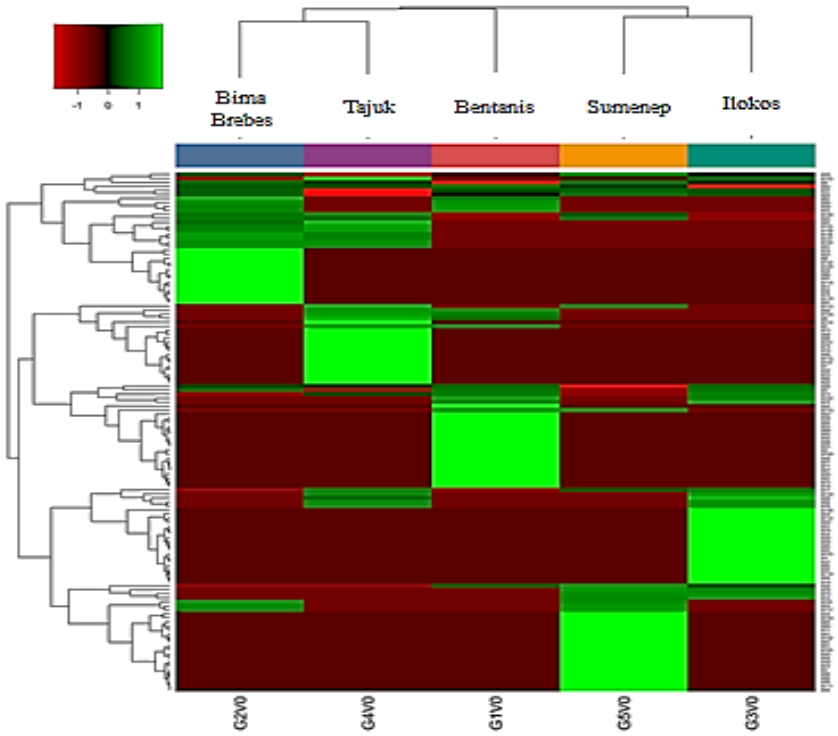


Fig.-1: Heatmap of hierarchical clustering analysis based on non-targeted metabolomic in 5 shallot genotypes of 130 metabolites in non-vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered.

Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by different colour of each heatmap coloumn. Intencity of s~~S~~pecific metabolite ~~compounds~~ was ~~were~~ visualized on each genotype column with a bright green color. Hierarchical clustering analysis showed that the genotype had a significant effect on the metabolites composition of the shallot. Each genotype of shallot produces different metabolites that associated to the flowering competency. Hierarchical clustering analysis of shallot metabolites grouped shallot genotypes into three distinct genotypes, in both vernalization treatments (Figure-1 and 2). The result indicated that the presence of specific compounds produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability.

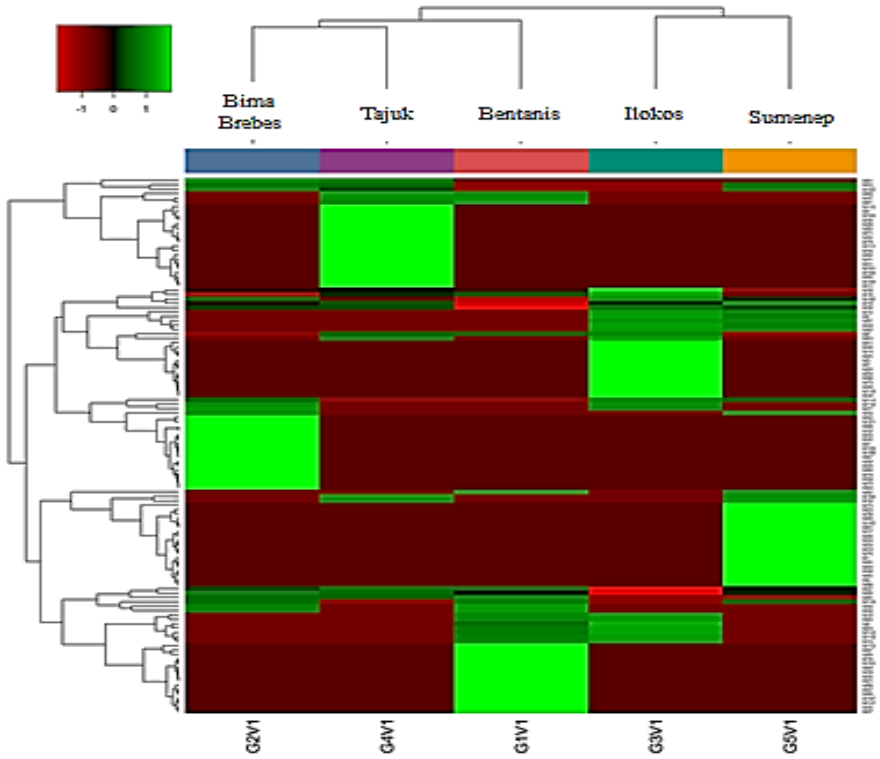


Fig.-2: Heatmap of hierarchical group analysis based on non-targeted metabolomic in 5 shallot local genotypes of 122 metabolites in vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered

Shallot genotypes located in the same group showed that the genotype had the same metabolite profile, and showed significant metabolite diversity with other groups. Visualization with heatmap also confirmed in the presence of different colors from each of the heatmap columns. The genotypes included in the first group were the Bentanis genotype (G1). In the second group there was the Bima Brebes (G2) and Tajuk genotype (G4), while in the third group there was Ilokos (G3) and Sumenep genotype (G5). The Bentanis genotype separates on its own group, and has close proximity to the Bima Brebes and Tajuk groups. These three genotypes are genotypes that have the ability to generate interest (sensitive to flowering).

Visualization with heatmap is widely used in metabolomic research because it can visualize large amounts of metabolomic data and classify it in sample groups[22,21,20]. ~~S~~intencitis of specific metabolite ~~compounds~~ of each sample of onion are visualized on each genotype column in bright green color.

Mass spectrometry methods can accurately detect compounds including identification of unknown and unexpected compounds[19,18]. Identification of the molecular structure is often given without reference compounds, making it difficult to make a real identification[19,18]. The genotypes of shallot that have the ability to produce flowers will produce the specific metabolite compounds. Metabolite compounds produced in the transition process of vegetative to generative stage can be

Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Font: (Default) Times New Roman, 12 pt

an indicator to determine the ability of flowering in shallot. The distribution of metabolomic compounds produced in non-vernalized shallot genotypes is presented in Figure-3.

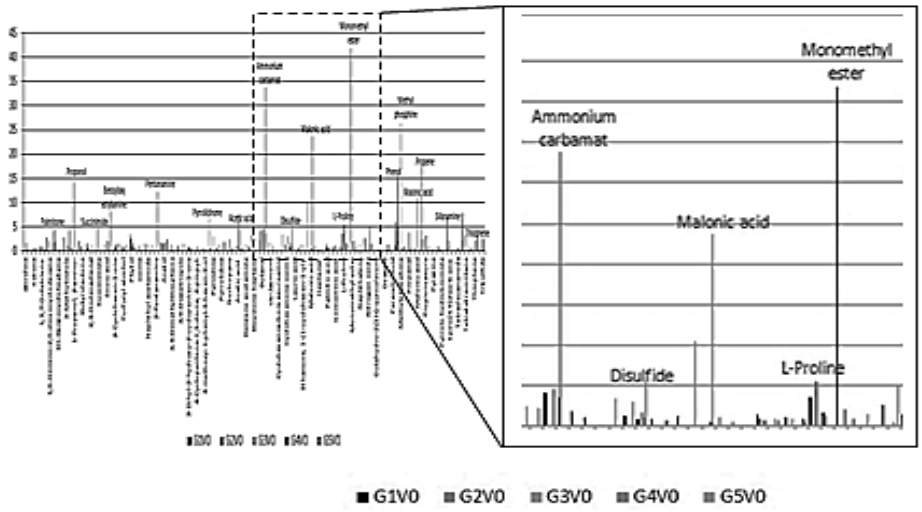


Fig.-3: Metabolite composition in non-vernalized shallot genotypes. Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5)

The results showed each genotype produced different metabolite compound. In Bentanis genotype showed the presence of monomethyl ester (41.82%) higher than Sumenep genotype (2.12%). The monomethyl ester compound, in the form of Mg-protoporphyrin IX mono-methyl ester cyclase is the catalyst required for the oxidation process of chlorophyll formation in plants[23 22 21]. The Bentanis genotype grows faster than Sumenep genotype. Bentanis genotype has robust maximum vegetative growth compared to Sumenep genotype at 5 weeks after planting. ~~The results in Table 1 show that~~ Morphological investigation showed that Bentanis genotype ~~h~~was higher ~~plant height~~ (36.96 cm) than of Sumenep genotype (29.82 cm). The presence of monomethyl ester (Mg-protoporphyrin IX mono-methyl ester cyclase) compounds can increase the formation of chlorophyll more optimally, thus increasing the photosynthesis process for the Bentanis genotype growth.

GCMS analysis showed ~~L~~low concentration of proline (3.55% and 5.56%) was produced in Bentanis and Tajuk genotypes (Figure-3). Proline compounds are generally produced by plants as their response to stress condition. Bentanis genotype was also produced succinimide compounds at about 4.78%, those can not be found in other genotypes. Succinimide compounds are used as intermediate compounds in the process of forming organic compounds that stimulate plant growth. The presence of high concentrate of ammonium carbamate compound is found in Bima Brebes genotype. Ammonium carbamate is an important compounds in the formation of carbamoyl phosphate and the formation of ATP into ADP[24 22]. While the presence of disulfide (dipropyl disulfide) compounds was found higher in non-sensitive flowering genotypes, such as Ilokos and Sumenep (1.69% and 5.43%), compared with other genotypes (0.8–0.14%). The content of dipropyl disulfide is an important component of activity as an antimicrobial and antioxidant and gives a distinctive aroma to shallot plant. The sulfur compounds in dipropyl disulfide is important the components of these metabolite compounds[25 24 23]. The sulfur compounds are known to have

Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt, Font color: Blue
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt

important activities as antimicrobials and antioxidants[24,25,26,27]. Derivative products of allicin compounds such as diallyl disulfide, diallyl trisulfide found in garlic essential oil indicate activity as a good antimicrobial[28 27 26].

Onion bulbs had the highest concentrations of propanethiol compounds having an aroma 20 times higher than dipropyl disulfide compounds[29 28 27]. It also found in non-flowering shallot plant, Ilokos and Sumenep genotypes, they produce higher acetic acid compounds (1.04% and 5.98%) compared to the flowering type, the Bentanis genotype (1.02%).

Utilization of sensitive instruments in metabolomic analysis is an important way to detect the content of plant essential compounds.Plant metabolites consist of highly diverse chemical compounds ranging from inorganic compounds to hydrophilic, volatile alcohol and ketone compounds, amino and non-amino acids, hydrophobic lipids, and complex natural products[19 18]. Utilization of mass spectrometry technology is a great way to solve research and analysis problems in the metabolomic field[19 18]. Metabolomic analysis was succesfully to identify potential compounds extracted directly from shallot genotypes. The results showed higher concentrations of acyclic ditherphene alcohol (phytol) compounds in flowering type shallot, Bentanis genotype (8.23%), Bima Brebes (2.2%) and Tajuk (7.52%) than the Ilokos genotype (1.15%) and Sumenep (2.19). The phytol compounds is an indication that vernalization treatment has a positive effect in inducing flowering of shallot genotypes, explaining that these three genotypes are sensitive to vernalization treatment to induce flower formation. Phytol compound is a compound that serves as a precursor of vitamin E and K. The content of these vitamins plays an important role in the plant body as an enzymatic cofactor in the process of metabolism that stimulates reproductive response. The content of vitamin E in the form of tocopherol is an important compound as one of the factors in the context of a cellular antioxidant system[30 28]. The differences of metabolite profile might play a key role in flowering initiation in shallot.

In flowering type of shallot genotypes, Bentanis, Bima Brebes and Tajuk also showed the formation of nitrogen oxide and semicarbazid hydrochlorid which are relatively lower than the non-flowering type, Ilokos and Sumenep. Nitrogen compound is a compound that plays an important role in the vegetative phase of the plant. Once the plant enters the reproductive phase, the nitrogen compounds in the plant body will decrease. In contrast, the Ilokos and Sumenep genotypes show the formation of high nitrogen compounds ie ammonium compounds ammonium carbamat, and nitrogen oxide. High content of nitrogen compounds in Ilokos and Sumenep genotypes explains that these two genotypes are not sensitive to vernalization treatment, thus not encouraging the flowering formation.

The results also showed that the Sumenep genotype contained high organosulfur compounds compared with other genotypes, namely thiophene (2.31%) and trisulfide (4.25%). The organosulfur is an important compound in giving aroma and spiciness upsurge in shallot, and play an important role as an antioxidant. Essential compounds in *Allium* have strong antioxidant ability. Tests on antimicrobial activity showed that all essential oils on *Allium* were able to inhibit the activity of gram-positive and gram-negative bacteria[9].

Vernalization treatment triggered in the formation of proline compounds in the Sumenep genotype. Vernalization treatment provides a stress condition to the plants that increase the plant mechanism of adaptation by producing proline compounds. Proline accumulation in the plant body is a physiological response to stress, and as a generative tissue development[31 29]. Secondary metabolite is the cellular response in plant that receives external pressure.

The metabolite profile of 5 local onion genotypes is also rich in non-volatile phytonutrients, especially the content of phenolic compounds (phenol, 2-propenamide, 3-pyridinamine, N-furfurylidene 2-phenylethyl amine, pyrrolidinedione), amide compounds aziridine-2-carbothioic

Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: 12 pt
Formatted: Font: 16 pt
Formatted: Font: 12 pt

acid amide) and protein compound (pyroglutamic acid). The essential components, mono and sesquiterpenes, carbohydrates, phenols, alcohols, ether, aldehydes and ketones, are responsible for biological activity and also for their fragrance[32,30]. Phenols and polyphenol compounds play important roles as antioxidants in plants[33,34,35,31,32,33]. Research on *Allium rotundum* showed the presence of antioxidant activity of phenol compounds with high concentrations of up to 4%[12]. Onion extract contained the highest of phenol content, at 17.18 mg GAE per gram of fresh weight[36,34], and a phenol content of 114.70 mg GAE per 100 g of different onion samples[37,35]. Metabolite compound is difference among shallot genotypes. Each plant genotype performs metabolic activities that depend on their genetic conditions and the ability to adapt to its environment. The plant biological activity may imply to proportion of metabolite containing in a plant.

CONCLUSION

Secondary metabolite of 5 genotypes shallot were difference under vernalization treatment. ~~Secondary metabolite is associated to the flowering competency in shallot genotypes.~~ Each genotypes produce different specific metabolites which is clustered according to the flowering competency. A high concentration ditherpene alcohol (phytol) found in flowering-sensitive genotypes (genotype of Bentanis, Bima Brebes and Tajuk), with a lower content of proline and nitrogen (ammonium carbamate and nitrogen oxide)~~,~~. While, in flowering-nonsensitive genotypes (genotype Ilokos and Sumenep) were found higher level of organosulfur compounds (thiophene and trisulfide). Vernalization treatment caused the Sumenep genotype produces the highest proline compound among other genotypes.

ACKNOWLEDGMENT

This researchs is supported by grants from National Innovation System Research Insentive 2016, Ministry of Research, Technology, and Higher Education, Indonesia.

REFERENCES

1. F.V. Golubev, N.A. Golubkina, and Y.N. Gorbunov. *Applied Biochemistry and Microbiology.* 39(5): 532–535 (2003). https://doi.org/10.1023/A:1025465106772
2. G. Griffiths, L. Trueman, T. Crowther, B. Thomas, and B. Smith. *Phytotherapy research: PTR.* 16(7): 603–615 (2002). DOI : 10.1002/ptr.1222
3. T. Ritsema , and S. Smeekens. *Current Opinion in Plant Biology*. 6(3): 223–230 (2003)
4. H. Mohammadi-Motlagh , A. Mostafaie, and K. Mansouri *Archives of Medicaal Science.* 1: 38–44 (2011)
5. Marlin, A. Maharijaya, A. Purwito, and Sobir. *SABRAO Journal of Breeding and Genetics*. 50 (3) 313-328 (2018). www.sabraojournal.org
6. E.J. Finnegan, D. Bagnall, C. Helliwell, D. Rouse, C. Sheldon, M. Tadege, W.J. Peacock, and E. Dennis. Vernalization. Encyclopedia of Life Sciences Macmillan Publishers Ltd, Nature Publishing Group. (2001). www.els.net.
7. J. Song, A. Angel, M. Howard, and C. Dea. *Journal of Cell Science.* 125 (16): 3723–3731 (2012).
8. Y. Iijima. *Metabolite*. 4: 699–721 (2014).
9. D. Mnayer, A. Fabiano-Tixier, E. Petitcolas, T. Hamieh, N. Nehme, C. Ferrant, X. Fernandez, and F. Chemat. *Molecules*. 19: 20034–20053 (2014).
10. H. Xiao, and K.L. Parkin. *Phytochemistry.* 68(7): 1059–1067 (2007).
11. M.A. Vazquez-Prieto, and R.M. Miatello. *Molecular Aspects of Medicine.* 31(6): 540–5 (2010).

Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue, English (United Kingdom)
Formatted: Font: (Default) Times New Roman, 12 pt, Font color: Blue
Formatted ...
Formatted ...
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted ...
Formatted ...
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted ...
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted ...
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted ...
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt

12. A.A. Dehpour, M. Yousefian, S.A. Jafary Kelarijani, M. Koshmoo, S. Mirzanegad, V. Mahdavi, S.E. Mousavi, E. Shirzad, M. Afzali, M.J. Javad Bayani, J.E. Olyaei, and M.K. Yahyapor. *Advances in Environmental Biology*. 6(3): 1020–1025 (2012).
13. V. Lanzotti, F. Scala, and G. Bonanomi. *Phytochemistry Reviews.* 13: 769–791 (2014).
14. N. Martins, S. Petropoulos, and I.C.F. Ferreira. *Food Chemistry*. 15(211): 41–50 (2016).
15. R.Y. Galingging, Sobir, S.I. Aisyah, and A. Maharijaya. *Rasayan J. Chem*., 11(2), 575 - 581(2018). http://dx.doi.org/10.7324/RJC.2018.1123001
~~15.~~16. O. Fiehn. *Plant Molecular Biology*. 48: 155–171 (2002).
~~16.~~17. H. Wu, O.J. Gu, S. Chen, X. Liu, Y. Zhou, X. Zhang, and X. Xu. *Journal of pharmaceutical and biomedical analysis.* 72: 267–291 (2013).
~~17.~~18. T. Soininen. Ph.D. Thesis. University of Eastern Finland. Finland (2014).
~~18.~~19. S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, and J. Nielsen. *Mass Spectrometry Reviews.* 24: 613– 646 (2005).
20. P.P. Durgawale, S.A. Hendre, and R.S. Phatak. *Rasayan J. Chem*., 8(3), 271 – 278 (2015).
~~19.~~21. N.C.J.P. Lekshmi, S. Viveka, M.B. Viswanathan, G. Manivannan, and T.M. Shobi. *Nanobio Pharmaceutical Technology*. (2014). DOI : 10.13140/2.1.3278.7523.
~~20.~~22. S.M. Huang, W. Toh, P.I. Benke, C.S. Tan, and C.N. Ong. *Metabolomics.* 10:1084–1093 (2014).
~~21.~~23. Y.S. Wong, and P.A. Castelfranco *Plant Physiology.* 79(3): 730–733 (1985).
~~22.~~24. N. Baker, J. Harbinson, and D.M. Kramer. *Plant Cell Environment.* 30 1107–1125 (2007)
~~23.~~25. P. Rattanachaikunsopon, and P. Phumkhachorn P. *African Journal of Microbiology Research.* 3: 747–750 (2009).
~~24.~~26. A. Chehregani, F. Azimishad, and A.H. Haj. *International Journal of Agriculture Biology.* 9(6): 873–876 (2007)
~~25.~~27. C.L. Ye, D.H. Dai, and W.L. Hu. *Food Control*. 30: 48–53 (2013).
~~26.~~28. J.W. Kim, J.E. Huh, S.H. Kyung, and K.H. Kyung. *Food Science and Biotechnology*. 13: 235–239 (2004).
~~27.~~29. M.M. Lokke, M. Edelenbos, E. Larsen, and A. Feilberg. *Sensor*s. 12: 16060–16076 (2012).
~~28.~~30. *M.G. Traber, J. Atkinson, and Atkinson. Free Radical Biology and Medicine. 43 (1): 4–15 (2007).*
~~29.~~31. N. Verbruggen, and C. Hermans. *Amino Acids.* 35: 753–759 (2008).
~~30.~~32. E.A. El-Wakil, M.M. El-Sayed, and E.E. Abdel-Lateef. *International Journal of Pharma Sciences and Research IJPSR.* 6 (3) (2015).
~~31.~~33. S.A.B.E. Van Acker, D.J. van den Berg, N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, and W.J. van der Vijgh. *Free Radical Biology and Medicine* 20 (3): 331– 342 (1996).
~~32.~~34. M.A. Alvarez, N.B. Debattista, and N.B. Pappano. *Folia Microbiologica.* 53(1): 23–8 (2008).
~~33.~~35. A.P. Mukne, V. Viswanathan, and A.G. Phadatare. *Pharmacognosy Reviews.* 5(9): 13–18 (2011)
~~34.~~36. X. Lu, J. Wang, H.M. Al-Qadiri, C.F. Ross, J.R. Powers, J. Tang, and B.A. Rasco. *Food Chemistry.* 129: 637–644 (2011).
~~35.~~37. J. Yang, K.J. Meyers, J. van der Heide, and R.H. Liu. *Journal of Agriculture Food Chemistry*. 52: 6787–6793 (2004).

[RJC-4034/2018]

Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt

Marlin UNIB <marlin@unib.ac.id>

Revised RJC_5356/2019

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 21 Juni 2019 pukul 16.46
Kepada: Marlin UNIB <marlin@unib.ac.id>

Thank you very much.
[Kutipan teks disembunyikan]
--
Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)
www.rasayanjournal.com | www.rasayanjournal.co.in
Contact: +91 9001699997, +91 9414202678

Marlin UNIB <marlin@unib.ac.id>

Galley Proofs of your paper.

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 27 Juni 2019 pukul 18.25
Bcc: marlin@unib.ac.id

Dear Contributor,

Soon you'll receive the Galley Proofs of your accepted paper, which is likely to be published in **Vol.12, No.3, July- September, 2019.**

Thanks for your interest in Rasayan J. Chem.

--
Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)
www.rasayanjournal.com | www.rasayanjournal.co.in
Contact: +91 9001699997, +91 9414202678

Marlin UNIB <marlin@unib.ac.id>

Galley Proofs of your paper.

Marlin UNIB <marlin@unib.ac.id> 3 Juli 2019 pukul 11.54
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Thanks a lot.
[Kutipan teks disembunyikan]

Marlin UNIB <marlin@unib.ac.id>

Galley Proofs of your paper.

Marlin UNIB <marlin@unib.ac.id> 16 Juli 2019 pukul 06.47
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Dear Editors
Rasayan Journal of chemistry

As your annoucement in June 27. 2019, my article entitle " Secondary metabolies change under vernalization and its relation to flowering competency in shallot (*Allium cepa* var. *aggregatum*)" will be publish in **Vol.12, No.3, July- September, 2019.**
However, I could not find my article in that publicattion. Please let me know the problem.
Thank you.

Dr. Marlin
University of Bengkulu, Indonesia

Pada tanggal Kam, 27 Jun 2019 pukul 18.26 RASĀYAN J. Chem. <rasayanjournal@gmail.com> menulis:
[Kutipan teks disembunyikan]

Marlin UNIB <marlin@unib.ac.id>

Galley Proofs of your paper.

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 22 Juli 2019 pukul 12.27
Kepada: marlin <marlin@unib.ac.id>

Be patient. This issue is still open.
[Kutipan teks disembunyikan]

Marlin UNIB <marlin@unib.ac.id>

Galley Proofs of your paper.

marlin <marlin@unib.ac.id> 22 Juli 2019 pukul 12.46
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Thank you. Waiting for the article publication.
[Kutipan teks disembunyikan]

Marlin UNIB <marlin@unib.ac.id>

Galley Proofs: RJC-5356/2019

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 22 Juli 2019 pukul 19.00
Kepada: Marlin UNIB <marlin@unib.ac.id>

Subject: Please verify the **Galley Proofs** of your paper which is going to be published in the **RASĀYAN JOURNAL OF CHEMISTRY**.

Dear Author,

Greetings from **RASĀYAN**.

First of all, we thank you for contributing your valuable research work in **RASĀYAN JOURNAL OF CHEMISTRY**.

Please find herewith the **Proof Draft** of your paper (in editable **Word Format** with a **PDF e-Proof**), which is going to be published in the **RASĀYAN JOURNAL OF CHEMISTRY**. **Before start correcting your Galley Proofs, Please refer the any published PAPER from the Current issue.**

1. Please go through it once again as per the **Guidelines** of the journal provided you earlier. If anything highlighted in the Proof or mentioned in last as **Author's Query (A.Q.)**, please check and correct it STRICTLY as per the demand of the Query.
2. **Please don't do any change with pagination and font type & size. The actual pagination will be carried out after receiving the corrected proof from you.**
3. Check author's names, affiliation and E-mail id of Corresponding author. If not given properly, give the same.
4. If you make any textual change, please do it in **red** or **blue** **color** in **Word File** attached, so that the typesetter could understand and correct it appropriately.
5. Check spellings, grammar and units of different quantities throughout the manuscript.
6. Check all references once again for their correctness in terms of name of the journal, Volume and Number, Year, Page no etc.

Please send it back **within 48 hours** (to: rasayanjournal@gmail.com) from the time of receiving this mail **with the following verification text** (without which the manuscript will not be finally published online) that-

"I,…….. as corresponding author for the manuscript no……(mention your manuscript no. here correctly)on behalf of myself and all my co-authors confirm that we have gone

through the **Proof Draft** of my manuscript; which is going to be published in the coming issue of **RASAYAN JOURNAL OF CHEMISTRY**. I take complete responsibility about the correctness of matter and content presented in this paper."

Since **RASĀYAN J. Chem.** **(SCOPUS H Index= 15)** is a rapidly growing International journal, you are requested to suggest the journal to your colleagues and friends.
Thanks for your cooperation and support. Looking forward

--
Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)
www.rasayanjournal.com | www.rasayanjournal.co.in
Contact: +91 9001699997, +91 9414202678

2 lampiran

Galley Proofs_RJC-5356.pdf
293K

Galley Proofs_RJC-5356.docx
298K

RJC

RASĀYAN *J. Chem.*
GALLEY PROOFS
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

SECONDARY METABOLITES CHANGE UNDER VERNALIZATIONAND ITS RELATION TO FLOWERING COMPETENCY IN SHALLOT (*Allium cepa* var. *aggregatum*)

Marlin[1,*], A. Maharijaya[2], A. Purwito[2] and Sobir[2]
[1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia
[2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia
*E-mail: marlin@unib.ac.id

ABSTRACT

Shallot extract contains a great quantity of essential oil, volatiles, and other compounds. The composition of a secondary metabolite can be regarded as the ultimate reactions of biological and physiological to genetic and environmental conditions. This study was objected to identifying metabolomic composition and its relation to flowering competency in 5 genotypes of shallot. The non-targetted metabolites were identified using GC-MSand assigned by matching their mass spectra with those available in the WILEY7 LIB. Data matrix was analyzed by using a metabolomic package of the R software. Heatmap was visualized for a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The composition metabolites from 5 genotypes were the difference and suggested correlated to flowering competency. The 3 genotypes of flowering type of shallot produced the highest concentration of phytol(ditherphene alcohol) and low concentration of nitrogen compound. Whereas, 2 genotypes of non-flowering types contained a high concentration of organosulfur and nitrogen sources. Metabolite profile of 5 genotypes contained volatile and non-volatile phytonutrients, vitamin, saturated fatty acid and organosulfur compounds.

Keywords: Dissimilarity Matrix, GC-MS, Non-targetted Metabolite, Organosulfur, Phytol

INTRODUCTION

Shallot (*Allium cepa* var.*aggregatum*) is an important *Allium* plant that been used for food, ornamental, natural alternatives to food synthetic preservatives and medicinal for curing various diseases. *Allium* plants contain different sugars, amino acids, vitamins, sulfurous compounds, enzymes, flavonoids, saponins and minerals[1]. *Allium* is also valued for their therapeutic properties; they are active as diuretics and laxatives and have been used to treat headaches and parasitic worms[2]. Onion has a beneficial effect as their prebiotic, and their ability to decrease levels of insulin, cholesterol, triacylglycerol and phospholipids for human health[3], and considered an important vegetable and medicinal plant prescribed as an effective therapy for several complaints in healing treatment such as natural cancer treatment[4]**.** These beneficial effects will become an important target in shallot breeding and management practices.

Shallot genotypes may vary in flowering pattern, naturally flowering, inducible flowering, and non-inducible flowering shallot[5]. Flowering in shallot can be induced by an exogenous treatment such as vernalization. Vernalization is an important adaptation of plants to initiate flowering in response to prolonged exposure to low temperatures[6,7]. The principles derived from vernalization are likely to be widely relevant to epigenetic reprogramming in many organisms[7]. External condition plays an important regulation in plant bioactivity and influences the metabolomic composition. Metabolomic composition in the plant is various and specific, that depends on genetic and plant growth environment. Metabolomic describes plant phenotype differentiation. Metabolomic technologies have revealed new insights in biological systems through metabolic dynamics[8]. The metabolomic composition determines the biological

CrossMark

and physiological function of the plants. Metabolite compounds are observed in the 6 *Alliaceae* family[9], in *Allium cepa* (onion) extracts[10,11], in *Allium rotundum* L[12], in *Allium sativum* (garlic) formulations[13,14], and in shallot grown in tidal swampland[15]. Metabolomic defined as a comprehensive analysis in which all the metabolites of a biological system are identified and quantified[16]. Metabolites are the end products of cellular regulatory processes. Metabolites profiling in plants can be directly extracted from a part of plantthe to find new molecules contents. Profiling of the metabolite composition is an important way to evaluate the contents of the medical plants, as well as their safety and efficacy[17].

Mass spectrometry is an analytical method, which allows the determination of the mass formula and structure. With mass spectrometry, individual molecules can be measured based on their molecular peak (m/z) and characteristics spectra of fragment ions[18]. Mass spectrometry technology is high sensitivity to identify the unknown and unexpected of the components present in the complex biological samples[19]. These fragments often provide a clue to the molecule's structure, but without a reference compound, the difficulty of making an identification increases markedly[18]. The analytical strategy *gase chromatography mass spectrometry* (GC-MS) used to analyze the volatile compounds, and selectedcompounds were structurally measured by mass spectrometry transposing the method to GC-MS[,20, 21]. The mass analyzer separates the moleculesor/and fragments according to their masses and the detector detects and quantifies the separated ions[18]. GC-MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal-mass information. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories databases[19].This study w as objected to identify secondary metabolite composition and its relation to flowering competency in 5 genotypes of shallot.

EXPERIMENTAL

Plant materials

Local genotypes of shallot with a different pattern of flowering were selected as plant material. Shallot genotype 'Bentanis' is used as the naturally flowering, while genotypes 'Bima Brebes Ilokos, Tajuk and Sumenep' are selected as the induced flowering of shallot[5]. Experiments were planted in Pasir Kuda research farm, Bogor Indonesia. Each genotypes were treated with vernalization in 8 ^{o}C temperature for 5 weeks, and no vernalization treatment as control.The bulbs were then pl anted in 45 cm diameter polythene bags containing 8 kg of growing media (soil: manure: husk = 2: 1: 1). Each polybag was planted with three bulbs. Before planting, shallots bulbs were soaked in a fungicide benomil 50% at a concentration of 2 g.L^{-1} for 15 minutes. The bulbs were then soaked into solution of plant growth-promoting rhizobacteria of 5 g.L^{-1} for 15 minutes. NPK fertilizers with a ratio of 15:15:15 wereapplied in a dose of 600 kg.ha^{-1} or 2.4 g per polybag.

Metabolomic Analysis

The samples were the leaves of shallot plants at 4 weeks after planting(bolting stage). Leaves were cutted for 10 g and were extracted with methanol (50 mL). The methanolic extract of shallot leaves were used as a sample in the GC-MS analysis. GC-MS unit was carried out on an GCMS-QP2010 system (Shimadzu Corporation, Japan) coupled to mass a spectrometer detector. The sampleswere inserted into the quartz chamber in the GC-MS unit. Helium was used as a carrier gas in a constant flow mode at 0.85 ml/min. The chamber was heated in an oxygen-free environment at a temperature of 400^{o}C for 0.2 minutes. The reaction will produce heat-mediated cleavage of chemical bonds in the macromolecular structure and produce low molecular weight with a chemical composition that identify specific compound of metabolite. Compound mixtureswere then passed through the column GC-MS analysis. The column is Rt x 5 MS, with length 60.0 m, thickness 0.25 µm, and diameter 0.25 mm. The initial temperature of the column was 50 °C, which was gradually increased by 10 °C up to 280 °C. At the end of this period, the column oven temperature was 50 ^{o}C raised up to 280 °C. Injection port temperature was ensured as 280 °C and helium flow rate as 0.85 ml/min. Mass spectrometer detector was employed to detect compounds when they were vented from the column. Temperature of the detector was 200 °C. Secondary metabolites of the plant samples were then identified for each treatments.

The identification of the components of the non-targeted metabolite was assigned by matching their mass spectra with those available in the WILEY7 LIB. The metabolitecompounds of the plant samples were

then identified for each genotypes. Spectra were compared with National the Institute of Standard and Technology (NIST, 2005 v2.1) library to identify the unknown compounds. The data were confirmed using PubChem online database in National Center for Biotechnology Information (https://pubchem.ncbi. nlm.nih.gov/), chemicalbook database (http://www.chemicalbook.com), and NIST webbook database http://webbook.nist.gov/. The datasets had been transformed to log2 and mean-centered in order to acquire the normalized data. Data matrix based on non-targetted metabolomic of shallot in each vernalization treatments were analyzed using Metabolomic package of the R software (R version 3.2.2 http://www.r-project.org/). Metabolomics package was operated to construct HCA (*hierarchical cluster analysis*) and visulized heatmap with dendrogram. HCA was performed using Euclidean distance method and complete linkage agglomerative method.

RESULTS AND DISCUSSION

A total of 130 of metabolomic compound of non-vernalized bulbs, and 122 of metabolites of vernalized bulbs of 5 shallot local genotypes were identified by GC-MS. Metabolite compound obtained where then visually described by metabolite heatmap (Figure-1). Heatmap is widely used in metabolomic research because it is able to visualize large amounts of metabolite data and its differences in sample groups[22]. Heatmap of both non vernalized and vernalized bulbs clustered 5 shallot local genotypes into three groups (Figure-1). First group was G2 (Bima Brebes genotype) and G4 (Tajuk genotype), second group was G1 (Bentanis genotype), and the third group was G3 (Ilokos genotype) and G5 (Sumenep genotype).

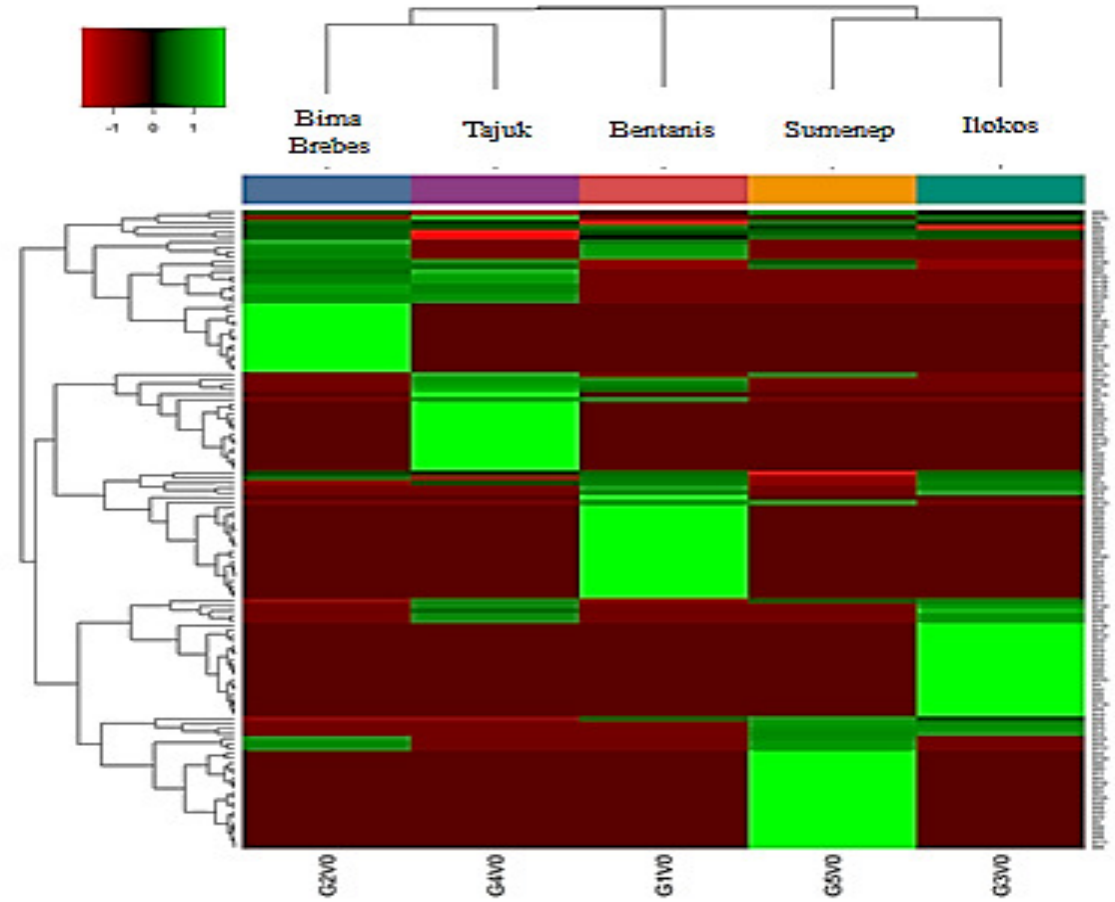


Fig.-1: Heatmap of hierarchical clustering analysis based on non-targeted metabolomic in 5 shallot genotypes of 130 metabolites in non-vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered.

Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by the different color of each heatmap

column. The intensity of specific metabolite was visualized on each genotype column with a bright green color. Hierarchical clustering analysis showed that the genotype had a significant effect on the metabolites composition of the shallot. Each genotype of shallot produces different metabolites that associated to the flowering competency.Hierarchical clustering analysisof shallot metabolites grouped shallot genotypes into three distinct genotypes, in both vernalization treatments (Figure-1 and 2). The result indicated that the presence of specific compounds produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability.

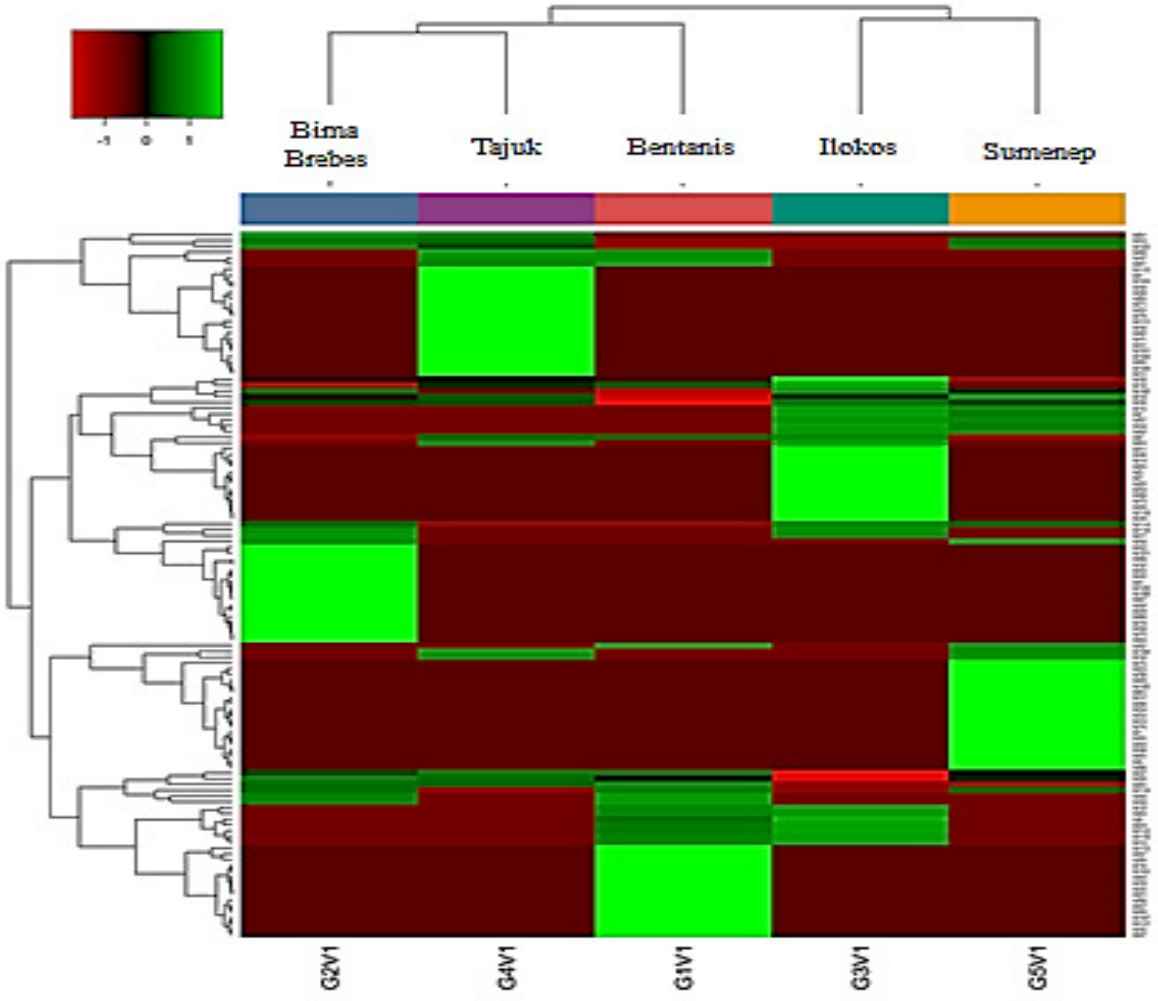


Fig.-2: Heatmap of hierarchical group analysis based on non-targeted metabolomic in 5 shallot local genotypes of 122 metabolites in vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered

Shallot genotypes located in the same group showed that the genotype had the same metabolite profile, and showed significant metabolite diversity with other groups. Visualization with heatmap also confirmed in the presence of different colors from each of the heatmap columns. The genotypes included in the first group were the Bentanis genotype (G1). In the second group, there was the Bima Brebes (G2) and Tajuk genotype (G4), while in the third group there was Ilokos (G3) and Sumenep genotype (G5). The Bentanis genotype separates on its own group, and has close proximity to the Bima Brebes and Tajuk groups. These three genotypes are genotypes that have the ability to generate interest (sensitive to flowering).

Visualization with heatmap is widely used in metabolomic research because it can visualize large amounts of metabolomic data and classify it in sample groups[22]. intensities of a specific metabolite of each sample of onion are visualized on each genotype column in bright green color.

Mass spectrometry methods can accurately detect compounds including identification of unknown and

unexpected compounds[19]. Identification of the molecular structure is often given without reference compounds, making it difficult to make a real identification[19]. The genotypes of shallot that have the ability to produce flowers will produce the specific metabolite compounds. Metabolite compounds produced in the transition process of vegetative to generative stage can be an indicator to determine the ability of flowering in shallot. The distribution of metabolomic compounds produced in non-vernalizedshallot genotypes is presented in Fig.-3.

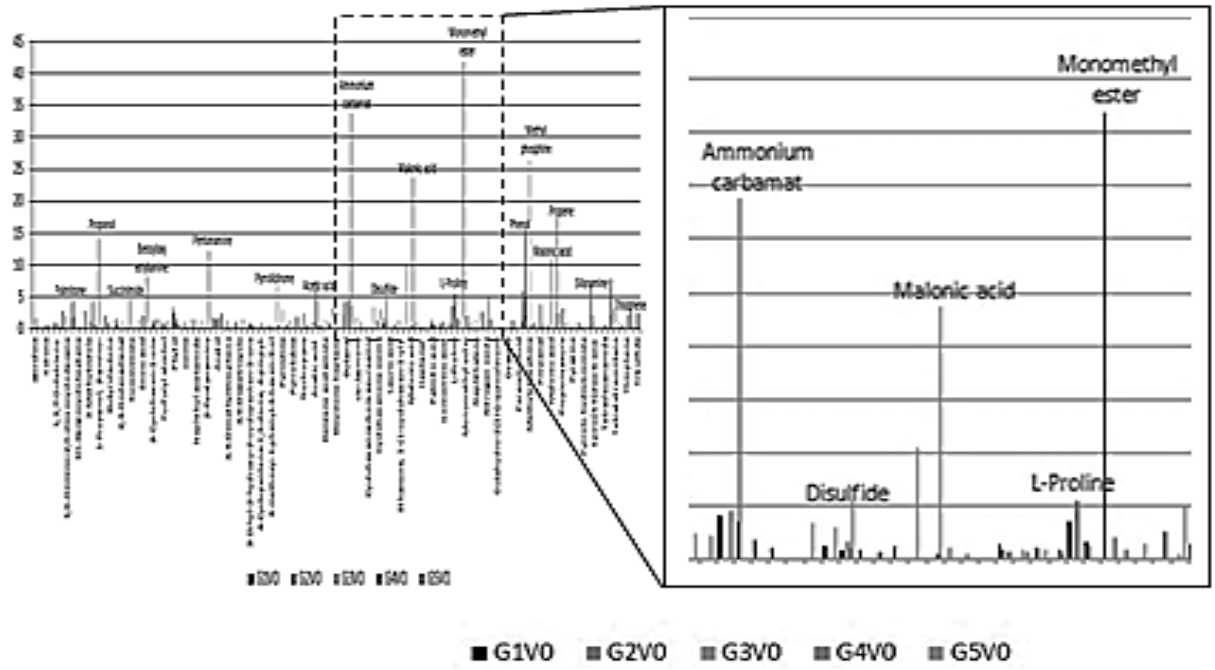


Fig.-3: Metabolite composition in non-vernalizedshallot genotypes. Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5)

The results showed each genotype produced different metabolite compound. In Bentanis genotype showed the presence of monomethyl ester (41.82%) higher than Sumenep genotype (2.12%). The monomethyl ester compound, in the form of Mg-protoporphyrin IX mono-methyl ester cyclase is the catalyst required for the oxidation process of chlorophyll formation in plants[23]. The Bentanis genotype growsfaster than Sumenep genotype. Bentanis genotype has robust maximum vegetative growth compared to Sumenep genotype at 5 weeks after planting.Morp hological investigation showed that Bentanis genotype was higher (36.96 cm) than of Sumenep genotype (29.82 cm). The presence of monomethyl ester (Mg-protoporphyrin IX mono-methyl ester cyclase) compounds can increase the formation of chlorophyll more optimally, thus increasing the photosynthesis process for the Bentanis genotype growth.

GCMS analysis showed a low concentration of proline (3.55% and 5.56%) was produced in Bentanis and Tajuk genotypes (Figure-3). Proline compounds are generally produced by plants as their responseto stress condition.Bentanis genotype was also produced succinimide compounds at about 4.78%, those can not be found in other genotypes. Succinimide compounds are used as intermediate compounds in the process of forming organic compounds that stimulate plant growth.

The presence of high concentrate of ammonium carbamate compound is found in Bima Brebes genotype. Ammonium carbamate is an important compound in the formation of carbamoyl phosphate and the formation of ATP into ADP[24] . While the presence of disulfide (dipropyl disulfide) compounds was found higher in non-sensitive flowering genotypes, such as Ilokos and Sumenep (1.69% and 5.43%), compared with other genotypes (0.8–0.14%).The content of dipropyl disulfide is an important component of activity as an antimicrobial and antioxidant and gives a distinctive aroma to shallot plant. The sulfur compounds indipropyl disulfide is important the components of these metabolite compounds[25].The su lfur compounds are known to have important activities as antimicrobials and antioxidants[26,27]. Derivative

products of allicin compounds such as diallyl disulfide, diallyl trisulfide found in garlic essential oil indicate activity as a good antimicrobial[28] .

Onion bulbs had the highest concentrations of propanethiol compounds having an aroma 20 times higher than dipropyl disulfide compounds[29] . It also found in non-flowering shallot plant, Ilokos and Sumenep genotypes, they produce higher acetic acid compounds (1.04% and 5.98%) compared to the flowering type, the Bentanis genotype (1.02%).

Utilization of sensitive instruments in the metabolomic analysis is an important way to detect the content of plant essential compounds. Plant metabolites consist of highly diverse chemical compounds ranging from inorganic compounds to hydrophilic, volatile alcohol and ketone compounds, amino and non-amino acids, hydrophobic lipids, and complex natural products[19].

Utilization of mass spectrometry technology is a great way to solve research and analysis problems in the metabolomic field[19]. Metabolomic analysis was succesfully to identify potential compounds extracted directly from shallot genotypes.Th e results showed higher concentrations of acyclic ditherphene alcohol (phytol) compounds in flowering type shallot, Bentanis genotype (8.23%), Bima Brebes (2.2%) and Tajuk (7.52%) than the Ilokos genotype (1.15%) and Sumenep (2.19). The phytol compounds is an indication that vernalization treatment has a positive effect in inducing flowering of shallot genotypes, explaining that these three genotypes are sensitive to vernalization treatment to induce flower formation.Phytol compound is a compound that serves as a precursor of vitamin E and K. The content of these vitamins plays an important role in the plant body as an enzymatic cofactor in the process of metabolism that stimulates rea productive response.The content of vitamin E in the form of tocopherol is an important compound as one of the factors in the context of a cellular antioxidant system[30]. The differences of metabolite profile might play a key role in flowering initiation in shallot.

In flowering type of shallot genotypes, Bentanis, Bima Brebes and Tajuk also showed the formation of nitrogen oxide and semicarbazid hydrochlorid which are relatively lower than the non-flowering type, Ilokos and Sumenep.Nitrogen compound is a compound that plays an important role in the vegetative phase of the plant. Once the plant enters the reproductive phase, the nitrogen compounds in the plant body will decrease.In contrast, the Ilokos and Sumenep genotypes show the formation of high nitrogen compounds ie ammonium compounds ammonium carbamat, and nitrogen oxide. The high content of nitrogen compounds in Ilokos and Sumenep genotypes explains that these two genotypes are not sensitive to vernalization treatment, thus not encouraging the flowering formation.

The results also showed that the Sumenep genotype contained high organosulfur compounds compared with other genotypes, namely thiophene (2.31%) and trisulfide (4.25%). The organosulfur is an important compound in giving aroma and spiciness upsurge in shallot, and play an important role as an antioxidant. Essential compounds in *Allium*have strong antioxidant ability. Tests on antimicrobial activity showed that all essential oils on *Allium* were able to inhibit the activity of gram-positive and gram-negative bacteria[9].

Vernalization treatment triggered in the formation of proline compounds in the Sumenep genotype. Vernalization treatment provides a stress condition to the plants that increase the plant mechanism of adaptation by producing proline compounds. Proline accumulation in the plant body is a physiological response to stress, and as a generative tissue development[31] . Secondary metabolite is the cellular response in plant that receives external pressure.

The metabolite profile of 5 local onion genotypes is also rich in non-volatile phytonutrients, especially the content of phenolic compounds (phenol, 2-propenamide, 3-pyridinamine, N-furfurylidene 2-phenylethyl amine, pyrrolidinedione), amide compounds aziridine-2-carbothioic acid amide) and protein compound (pyroglutamic acid).The essential components, mono and sesquiterpenes, carbohydrates, phenols, alcohols, ether, aldehydes and ketones, are responsible for biological activity and also for their fragrance[32]. Phenols and polyphenol compoundsplay important roles as antioxidants in plants[33,34,35] .Research on *Allium rotundum* showed the presence of antioxidant activity of phenol compounds with high concentrations of up to 4%[12]. Onion extract contained the highest of phenol content, at 17.18 mg GAE per gram of fresh weight[36], and a phenol content of 114.70 mg GAE per 100 g of different onion samples[37].

Metabolite compound is the difference among shallot genotypes. Each plant genotype performs metabolic activities that depend on their genetic conditions and the ability to adapt to its environment. The plant biological activity may imply to the proportion of metabolite containing a plant.

CONCLUSION

A secondary metabolite of 5 genotypes shallot was difference under vernalization treatment.Each genotypes produce different specific metabolites which is clustered according to the flowering competency.A high concentration ditherpene alcohol (phytol) found in flowering-sensitive genotypes (genotype of Bentanis, Bima Brebes and Tajuk), with a lower content of proline and nitrogen (ammonium carbamate and nitrogen oxide).While, in flowering-nonsensitive genotypes(genotype Ilokos and Sumenep) were found a higher level of organosulfur compounds (thiophene and trisulfide). Vernalization treatment caused the Sumenep genotype produces the highest proline compound among other genotypes.

ACKNOWLEDGMENT

This research is supported by grants from National Innovation System Research Incentive 2016, Ministry of Research, Technology, and Higher Education, Indonesia.

REFERENCES

1. F.V. Golubev, N.A. Golubkina, and Y.N. Gorbunov. *Applied Biochemistry and Microbiology*.39(5): 532–535 (2003). https://doi.org/10.1023/A:1025465106772
2. G. Griffiths, L. Trueman, T. Crowther, B. Thomas, and B. Smith. *Phytotherapy research: PTR.* 16(7): 603–615 (2002). DOI : 10.1002/ptr.1222
3. T. Ritsema , and S. Smeekens. *Current Opinion in Plant Biology*. 6(3): 223–230 (2003)
4. H. Mohammadi-Motlagh , A. Mostafaie, and K. Mansouri *Archives of Medicaal Science.* 1: 38–44 (2011)
5. Marlin, A. Maharijaya, A. Purwito, and Sobir. *SABRAO Journal of Breeding and Genetics*.50 (3) 313-328 (2018). www.sabraojournal.org
6. E.J. Finnegan, D. Bagnall, C. Helliwell, D. Rouse, C. Sheldon, M. Tadege, W.J. Peacock, and E. Dennis. Vernalization. Encyclopedia of Life Sciences Macmillan Publishers Ltd, Nature Publishing Group. (2001).www.els.net.
7. J. Song, A. Angel, M. Howard, and C. Dea. *Journal of Cell Science.* 125 (16): 3723–3731 (2012).
8. Y. Iijima. *Metabolite.*4: 699–721 (2014).
9. D. Mnayer, A. Fabiano-Tixier, E. Petitcolas, T. Hamieh, N. Nehme, C. Ferrant, X. Fernandez, and F. Chemat. *Molecules*. 19: 20034–20053 (2014).
10. H. Xiao, and K.L. Parkin. *Phytochemistry.* 68(7): 1059–1067 (2007).
11. M.A. Vazquez-Prieto, and R.M. Miatello. *Molecular Aspects of Medicine.*31(6): 540–5 (2010).
12. A.A. Dehpour, M. Yousefian, S.A. Jafary Kelarijani, M. Koshmoo, S. Mirzanegad, V. Mahdavi, S.E. Mousavi, E. Shirzad, M. Afzali, M.J. Javad Bayani, J.E. Olyaei, and M.K. Yahyapor.*Advances in Environmental Biology.* 6(3): 1020–1025 (2012).
13. V. Lanzotti, F. Scala, and G. Bonanomi. *Phytochemistry Reviews.* 13: 769–791 (2014).
14. N. Martins, S. Petropoulos, and I.C.F. Ferreira. *Food Chemistry.*15(211): 41–50 (2016).
15. R.Y. Galingging, Sobir, S.I. Aisyah, and A. Maharijaya. *Rasayan J. Chem*., 11(2), 575 - 581(2018). http://dx.doi.org/10.7324/RJC.2018.1123001
16. O. Fiehn. *Plant Molecular Biology*. 48: 155–171 (2002).
17. H. Wu, O.J. Gu, S. Chen, X. Liu, Y. Zhou, X. Zhang, and X. Xu. *Journal of pharmaceutical and biomedical analysis.* 72: 267–291 (2013).
18. T. Soininen. Ph.D. Thesis. University of Eastern Finland. Finland (2014).
19. S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, and J. Nielsen. *Mass Spectrometry Reviews.* 24: 613– 646 (2005).
20. P.P. Durgawale, S.A. Hendre, and R.S. Phatak.*Rasayan J. Chem*., 8(3), 271 – 278 (2015).

21. N.C.J.P. Lekshmi, S. Viveka, M.B. Viswanathan, G. Manivannan, and T.M. Shobi.*Nanobio Pharmaceutical Technology.* (2014). DOI : 10.13140/2.1.3278.7523.
22. S.M. Huang, W. Toh, P.I. Benke, C.S. Tan, and C.N. Ong. *Metabolomics.* 10:1084–1093 (2014).
23. Y.S. Wong, and P.A. Castelfranco *Plant Physiology.*79(3): 730–733 (1985).
24. N. Baker, J. Harbinson, and D.M. Kramer. *Plant Cell Environment.*30 1107–1125 (2007)
25. P. Rattanachaikunsopon, and P. Phumkhachorn P. *African Journal of Microbiology Research.*3: 747–750 (2009).
26. A. Chehregani, F. Azimishad, and A.H. Haj. *InternationalJournal of Agriculture Biology.* 9(6): 873–876 (2007)
27. C.L. Ye, D.H. Dai, and W.L. Hu. *Food Control.*30: 48–53 (2013).
28. J.W. Kim, J.E. Huh, S.H. Kyung, and K.H. Kyung. *Food Science and Biotechnology.* 13: 235–239 (2004).
29. M.M. Lokke, M. Edelenbos, E. Larsen, and A. Feilberg. *Sensor*s.12: 16060–16076 (2012).
30. *M.G. Traber, J. Atkinson, and Atkinson. Free Radical Biology and Medicine. 43 (1): 4–15 (2007).*
31. N. Verbruggen, and C. Hermans. *Amino Acids.* 35: 753–759 (2008).
32. E.A. El-Wakil, M.M. El-Sayed, and E.E. Abdel-Lateef.*International Journal of Pharma Sciences and Research IJPSR.* 6 (3) (2015).
33. S.A.B.E. Van Acker, D.J. van den Berg, N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, and W.J. van der Vijgh. *Free Radical Biology and Medicine* 20 (3): 331– 342 (1996).
34. M.A. Alvarez, N.B. Debattista, and N.B. Pappano. *Folia Microbiologica.* 53(1): 23–8 (2008).
35. A.P. Mukne, V. Viswanathan, and A.G. Phadatare. *Pharmacognosy Reviews.* 5(9): 13–18 (2011)
36. X. Lu, J. Wang, H.M. Al-Qadiri, C.F. Ross, J.R. Powers, J. Tang, and B.A. Rasco. *Food Chemistry.*129: 637–644 (2011).
37. J. Yang, K.J. Meyers, J. van der Heide, and R.H. Liu. *Journal of Agriculture Food Chemistry.*52: 6787–6793 (2004).

[RJC-4034/2018]

Author's Queries (To be sent with Galley Proofs)

1. Biological names must be italic trough out the manuscript(if any).
2. Check the Title, Names of Author(s), their affiliations and E-mail of Corresponding Author once again.
3. Check the complete Manuscript once again for Grammar Check.
4. MOST Important: References must be strict as per the STYLE of the journal (Please refer Guidelines and Sample paper), which may otherwise cause unnecessary delay in publication of your paper. Also,Mention DOI with references, wherever possible.
5. Check the terms highlighted in the Galley Proofs, if it is correct? If not, please correct the same throughout the manuscript in Red/Blue ink, so that it could be visible to us.
6. Check/ correct all Figure and Table numbers and their proper citation/position/alignment in the text.

Marlin UNIB <marlin@unib.ac.id>

Galley Proofs: RJC-5356/2019

Marlin UNIB <marlin@unib.ac.id> 28 Juli 2019 pukul 20.55
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Dear Editors

Herewith I resend the revision of galley proof of my article (RJC_53560).
I'd like to inform you that I change the authors name as **M. Marlin[1,*], A. Maharijaya[2], A. Purwito[2] and S. Sobir[2].**

Thank you for your consideration and help.

Sincerelly

Dr. Marlin
Department of Crop Production
Faculty of Agriculture, University of Bengkulu
[Kutipan teks disembunyikan]

2 lampiran

NewRevision_Galley Proofs_RJC-5356.docx
299K

NewRevision_Galley Proofs_RJC-5356.pdf
1006K

RJC

RASĀYAN *J. Chem.*
GALLEY PROOFS
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

SECONDARY METABOLITES CHANGE UNDER VERNALIZATION AND ITS RELATION TO FLOWERING COMPETENCY IN SHALLOT (*Allium cepa* var. *aggregatum*)

M. Marlin[1,*], A. Maharijaya[2], A. Purwito[2] and S. Sobir[2]
[1]Department of Crop Production, Faculty of Agriculture, University of Bengkulu, Indonesia
[2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Indonesia
*E-mail: marlin@unib.ac.id

ABSTRACT

Shallot extract contains a great quantity of essential oil, volatiles, and other compounds. The composition of a secondary metabolite can be regarded as the ultimate reactions of biological and physiological to genetic and environmental conditions. This study was objected to identifying metabolomic composition and its relation to flowering competency in 5 genotypes of shallot. The non-targetted metabolites were identified using GC-MS and assigned by matching their mass spectra with those available in the WILEY7 LIB. Data matrix was analyzed by using a metabolomic package of the R software. Heatmap was visualized for a total of 130 of metabolites in non vernalized bulbs and 122 of metabolites in vernalized bulbs of 5 shallot genotypes. The composition metabolites from 5 genotypes were the difference and suggested correlated to flowering competency. The 3 genotypes of flowering type of shallot produced the highest concentration of phytol (ditherphene alcohol) and low concentration of nitrogen compound. Whereas, 2 genotypes of non-flowering types contained a high concentration of organosulfur and nitrogen sources. Metabolite profile of 5 genotypes contained volatile and non-volatile phytonutrients, vitamin, saturated fatty acid and organosulfur compounds.

Keywords: Dissimilarity Matrix, GC-MS, Non-targetted Metabolite, Organosulfur, Phytol

INTRODUCTION

Shallot (*Allium cepa* var.*aggregatum*) is an important *Allium* plant that been used for food, ornamental, natural alternatives to food synthetic preservatives and medicinal for curing various diseases. *Allium* plants contain different sugars, amino acids, vitamins, sulfurous compounds, enzymes, flavonoids, saponins and minerals[1]. *Allium* is also valued for their therapeutic properties; they are active as diuretics and laxatives and have been used to treat headaches and parasitic worms[2]. Onion has a beneficial effect as their prebiotic, and their ability to decrease levels of insulin, cholesterol, triacylglycerol and phospholipids for human health[3], and considered an important vegetable and medicinal plant prescribed as an effective therapy for several complaints in healing treatment such as natural cancer treatment[4]. These beneficial effects will become an important target in shallot breeding and management practices.

Shallot genotypes may vary in flowering pattern, naturally flowering, inducible flowering, and non-inducible flowering shallot[5]. Flowering in shallot can be induced by an exogenous treatment such as vernalization. Vernalization is an important adaptation of plants to initiate flowering in response to prolonged exposure to low temperatures[6,7]. The principles derived from vernalization are likely to be widely relevant to epigenetic reprogramming in many organisms[7]. External condition plays an important regulation in plant bioactivity and influences the metabolomic composition. Metabolomic composition in the plant is various and specific, that depends on genetic and plant growth environment. Metabolomic describes plant phenotype differentiation. Metabolomic technologies have revealed new insights in

Formatted: Default Paragraph Font
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold

http://dx.doi.org/10.31788/RJC.2019.1235356

biological systems through metabolic dynamics[8]. The metabolomic composition determines the biological and physiological function of the plants. Metabolite compounds are observed in the 6 *Alliaceae* family[9], in *Allium cepa* (onion) extracts[10,11], in *Allium rotundum* L[12], ~~and~~ in *Allium sativum* (garlic) formulations[13,14], and in shallot grown in tidal swampland[15]. Metabolomic defined as a comprehensive analysis in which all the metabolites of a biological system are identified and quantified[~~15~~16]. Metabolites are the end products of cellular regulatory processes. Metabolites profiling in plants can be directly extracted from a part of plantthe to find new molecules contents. Profiling of the metabolite composition is an important way to evaluate the contents of the medical plants, as well as their safety and efficacy[17~~16~~]. Mass spectrometry is an analytical method, which allows the determination of the mass formula and structure. With mass spectrometry, individual molecules can be measured based on their molecular peak (m/z) and characteristics spectra of fragment ions[18~~17~~]. Mass spectrometry technology is high sensitivity to identify the unknown and unexpected of the components present in the complex biological samples[19~~18~~]. These fragments often provide a clue to the molecule's structure, but without a reference compound, the difficulty of making an identification increases markedly[18~~17~~]. The analytical strategy *gase chromatography mass spectrometry* (GC-MS) used to analyze the volatile compounds, and selected compounds were structurally measured by mass spectrometry transposing the method to GC-MS[~~19~~,20, 21]. The mass analyzer separates the molecules or/and fragments according to their masses and the detector detects and quantifies the separated ions[18~~17~~]. GC-MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal-mass information. Quadrupole MS spectra can be easily compared with commercial or inter-laboratories databases[19~~18~~].This study w as objected to identify secondary metabolite composition and its relation to flowering competency in 5 genotypes of shallot.

EXPERIMENTAL

Plant materials

Local genotypes of shallot with a different pattern of flowering were selected as plant material. Shallot genotype 'Bentanis' is used as the naturally flowering, while genotypes 'Bima Brebes Ilokos, Tajuk and Sumenep' are selected as the induced flowering of shallot[5] ~~(Marlin *et al.*, 2018)~~. Experiments were planted in Pasir Kuda research farm, Bogor Indonesia. Each genotypes were treated with vernalization in 8^{o}C temperature for 5 weeks, and no vernalization treatment as control.The bulbs were then planted in 45 cm diameter polythene bags containing 8 kg of growing media (soil: manure: husk = 2: 1: 1). Each polybag was planted with three bulbs. Before planting, shallots bulbs were soaked in a fungicide benomil 50% at a concentration of 2 g.L^{-1} for 15 minutes. The bulbs were then soaked into solution of plant growth-promoting rhizobacteria of 5 g.L^{-1} for 15 minutes. NPK fertilizers with a ratio of 15:15:15 were applied in a dose of 600 kg.ha^{-1} or 2.4 g per polybag.

Metabolomic Analysis

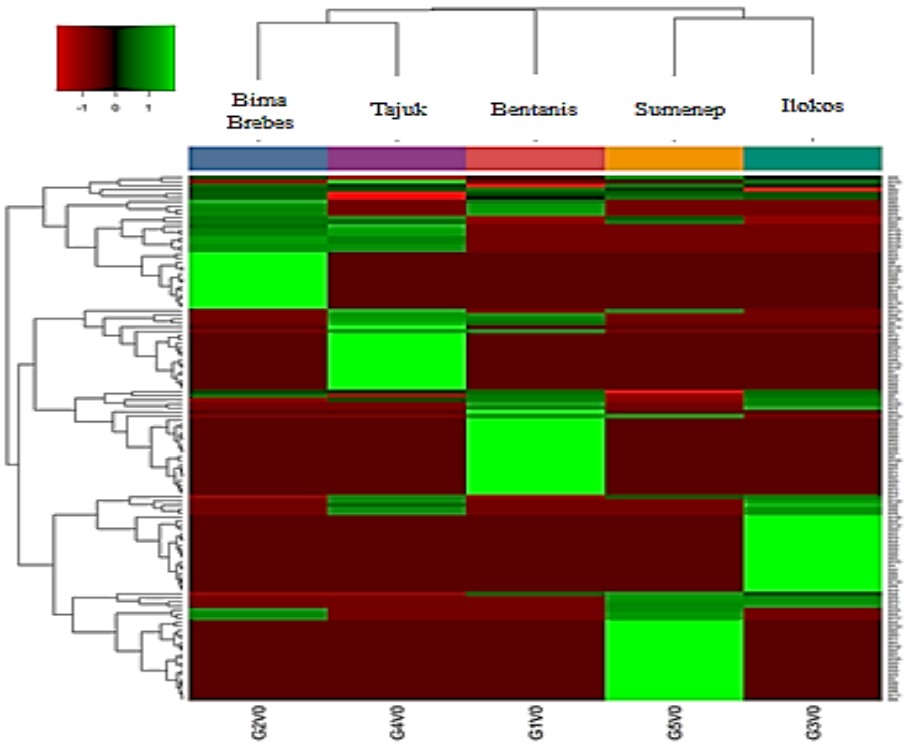
The samples were the leaves of shallot plants at 4 weeks after planting (bolting stage~~phase~~). Leaves were cutted for 10 g and were extracted with methanol (50 mL). The methanolic extract of shallot leaves were used as a sample in the GC-MS analysis. GC-MS unit was carried out on an GCMS-QP2010 system (Shimadzu Corporation, Japan) coupled to mass a spectrometer detector. The samples were inserted into the quartz chamber in the GC-MS unit. Helium was used as a carrier gas in a constant flow mode at 0.85 ml/min. The chamber was heated in an oxygen-free environment at a temperature of 400^{o}C for 0.2 minutes. The reaction will produce heat-mediated cleavage of chemical bonds in the macromolecular structure and produce low molecular weight with a chemical composition that identify specific compound of metabolite. Compound mixtures were then passed through the column GC-MS analysis. The column is Rt x 5 MS, with length 60.0 m, thickness 0.25 µm, and diameter 0.25 mm. The initial temperature of the column was 50 °C, which was gradually increased by 10 °C up to 280 °C. At the end of this period, the column oven temperature was 50 ^{o}C raised up to 280 °C. Injection port temperature was ensured as 280 °C and helium flow rate as 0.85 ml/min. Mass spectrometer detector was employed to detect compounds when they were vented from the column. Temperature of the detector was 200 °C. ~~The volatile metabolite compounds~~Secondary metabolites of the plant samples were then identified for each treatments.

Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Italic, Indonesian
Formatted: Font: Not Italic, Indonesian
Formatted: Font: Not Bold, Indonesian
Formatted: Font: 11 pt, Not Bold, Indonesian
Formatted: Font: 11 pt, Not Bold, Indonesian
Formatted: Font: Not Bold, Indonesian
Formatted: Font: 11 pt, Not Bold, Font color: Auto, Indonesian
Formatted: Font: Not Bold, Indonesian
Formatted: Font: Not Bold, Indonesian
Formatted: Font: Not Bold, Indonesian
Formatted: Font: Not Bold, Font color: Auto, Indonesian
Formatted: Font: Not Bold, Font color: Auto, Indonesian
Formatted: Font: Not Bold, Font color: Auto, Indonesian

The identification of the components of the non-targeted metabolite was assigned by matching their mass spectra with those available in the WILEY7 LIB. The ~~volatile~~ metabolitecompounds of the plant samples were then identified for each genotypes. Spectra were compared with National the Institute of Standard and Technology (NIST, 2005 v2.1) library to identify the unknown compounds. The data were confirmed using PubChem online database in National Center for Biotechnology Information (https://pubchem.ncbi. nlm.nih.gov/), chemicalbook database (http://www.chemicalbook.com), and NIST webbook database http://webbook.nist.gov/. The datasets had been transformed to log2 and mean-centered ~~were then log transformed~~in order to acquire the normalized data. Data matrix based on non-targetted metabolomic of shallot in each vernalization treatments were analyzed using Metabolomic package of the R software (R version 3.2.2 http://www.r-project.org/). Metabolomics package was operated to construct HCA (*hierarchical cluster analysis*) and visulized heatmap with dendrogram. HCA was performed using Euclidean distance method and complete linkage agglomerative method.

RESULTS AND DISCUSSION

~~Metabolite compounds in 5 shallot local genotypes were affected significantly by vernalization treatments.~~ A total of 130 of metabolomic of non-vernalized bulbs, and 122 of metabolites ~~compound~~ of vernalized bulbs of 5 shallot local genotypes were identified by GC-MS. Metabolite compound obtained where then visually described by metabolite heatmap (Figure-1). Heatmap is widely used in metabolomic research because it is able to visualize large amounts of metabolite data and its differences in sample groups[~~22120~~]. Heatmap of both non vernalized and vernalized bulbs clustered 5 shallot local genotypes into three groups (Figure-1). First group was G2 (Bima Brebes genotype) and G4 (Tajuk genotype), second group was G1 (Bentanis genotype), and the third group was G3 (Ilokos genotype) and G5 (Sumenep genotype).



Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Font: Not Bold
Formatted: Default Paragraph Font, Font: (Default) Calibri
Formatted: No underline, Font color: Auto
Formatted: Default Paragraph Font, Font: (Default) Calibri
Formatted: No underline, Font color: Auto
Formatted: Font: Times New Roman, 11 pt, No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: Font: 11 pt
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto

Fig.-1: Heatmap of hierarchical clustering analysis based on non-targeted metabolomic in 5 shallot genotypes of 130 metabolites in non-vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered.

Shallot genotypes in the same group exhibited a similar metabolite profile, and showed a relevant metabolite variability to another group. This was also confirmed by the different color of each heatmap column. The intensity of s~~S~~pecific metabolite ~~compounds~~was ~~were~~ visualized on each genotype column with a bright green color. Hierarchical clustering analysis showed that the genotype had a significant effect on the metabolites composition of the shallot. Each genotype of shallot produces different metabolites that associated to the flowering competency. Hierarchical clustering analysis of shallot metabolites grouped shallot genotypes into three distinct genotypes, in both vernalization treatments (Figure-1 and 2). The result indicated that the presence of specific compounds produced in metabolome processes in the vegetative transition to the reproductive phase in different flowering ability.

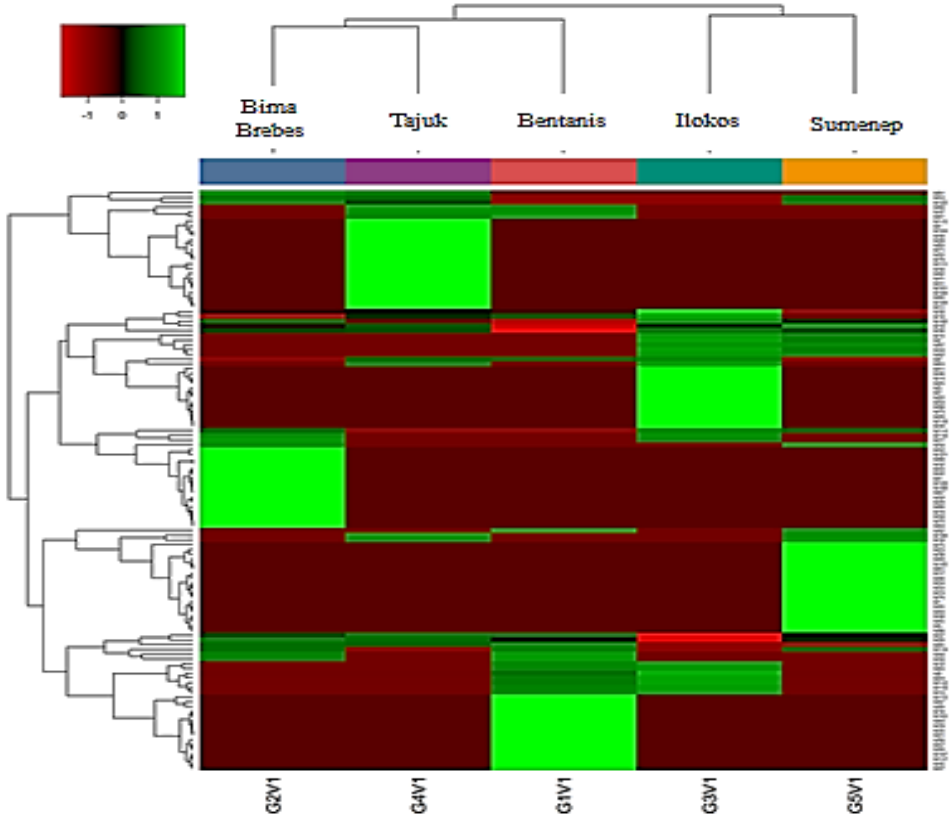


Fig.-2: Heatmap of hierarchical group analysis based on non-targeted metabolomic in 5 shallot local genotypes of 122 metabolites in vernalized bulbs. Color-coded matrix represents the mean values of the metabolite intensity in 5 shallot local genotypes. Metabolite data had been transformed to log2 and mean-centered

Shallot genotypes located in the same group showed that the genotype had the same metabolite profile, and showed significant metabolite diversity with other groups. Visualization with heatmap also confirmed in the presence of different colors from each of the heatmap columns. The genotypes included in the first group were the Bentanis genotype (G1). In the second group, there was the Bima Brebes (G2) and Tajuk genotype (G4), while in the third group there was Ilokos (G3) and Sumenep genotype (G5). The Bentanis

Formatted: No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: No underline, Font color: Auto

genotype separates on its own group, and has close proximity to the Bima Brebes and Tajuk groups. These three genotypes are genotypes that have the ability to generate interest (sensitive to flowering).
Visualization with heatmap is widely used in metabolomic research because it can visualize large amounts of metabolomic data and classify it in sample groups[222120]. ~~S~~intensities of a specific metabolite ~~compounds~~ of each sample of onion are visualized on each genotype column in bright green color.
Mass spectrometry methods can accurately detect compounds including identification of unknown and unexpected compounds[1918]. Identification of the molecular structure is often given without reference compounds, making it difficult to make a real identification[1918]. The genotypes of shallot that have the ability to produce flowers will produce the specific metabolite compounds. Metabolite compounds produced in the transition process of vegetative to generative stage can be an indicator to determine the ability of flowering in shallot. The distribution of metabolomic compounds produced in non-vernalized shallot genotypes is presented in Figure-3.

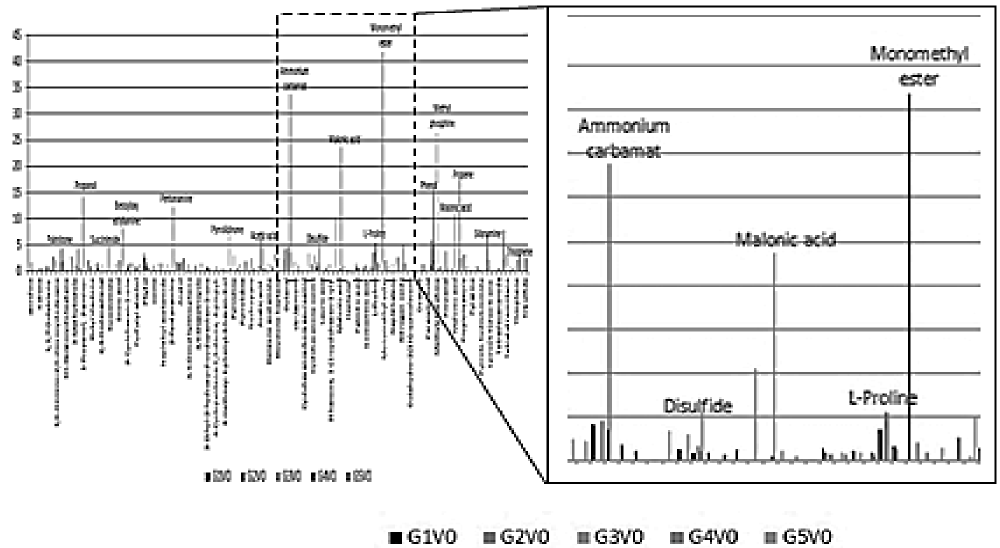


Fig.-3: Metabolite composition in non-vernalizedshallot genotypes. Genotype Bentanis (G1), Bima Brebes (G2), Ilokos (G3), Tajuk (G4) and Sumenep (G5)

The results showed each genotype produced different metabolite compound. In Bentanis genotype showed the presence of monomethyl ester (41.82%) higher than Sumenep genotype (2.12%). The monomethyl ester compound, in the form of Mg-protoporphyrin IX mono-methyl ester cyclase is the catalyst required for the oxidation process of chlorophyll formation in plants[232221]. The Bentanis genotype growsfaster than Sumenep genotype. Bentanis genotype has robust maximum vegetative growth compared to Sumenep genotype at 5 weeks after planting.~~The results in Table 1 show that~~ Morp hological investigation showed that Bentanis genotype ~~h~~was higher ~~plant height~~ (36.96 cm) than of Sumenep genotype (29.82 cm). The presence of monomethyl ester (Mg-protoporphyrin IX mono-methyl ester cyclase) compounds can increase the formation of chlorophyll more optimally, thus increasing the photosynthesis process for the Bentanis genotype growth.
GCMS analysis showed a ~~L~~low concentration of proline (3.55% and 5.56%) was produced in Bentanis and Tajuk genotypes (Figure-3). Proline compounds are generally produced by plants as their responseto stress condition.Bentanis genotype was also produced succinimide compounds at about 4.78%, those can not be found in other genotypes. Succinimide compounds are used as intermediate compounds in the process of forming organic compounds that stimulate plant growth.
The presence of high concentrate of ammonium carbamate compound is found in Bima Brebes genotype. Ammonium carbamate is an important compound in the formation of carbamoyl phosphate and the

Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color:
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color:
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color:
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color:
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color:
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color:
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...

formation of ATP into ADP[24 ~~22~~]. While the presence of disulfide (dipropyl disulfide) compounds was found higher in non-sensitive flowering genotypes, such as Ilokos and Sumenep (1.69% and 5.43%), compared with other genotypes (0.8–0.14%).The content of dipropyl disulfide is an important component of activity as an antimicrobial and antioxidant and gives a distinctive aroma to shallot plant. The sulfur compounds indipropyl disulfide is important the components of these metabolite compounds[25~~24~~~~23~~].The sulfur compounds are known to have important activities as antimicrobials and antioxidants[~~24~~,25,26,27]. Derivative products of allicin compounds such as diallyl disulfide, diallyl trisulfide found in garlic essential oil indicate activity as a good antimicrobial[28 ~~2726~~].

Onion bulbs had the highest concentrations of propanethiol compounds having an aroma 20 times higher than dipropyl disulfide compounds[29 ~~2827~~]. It also found in non-flowering shallot plant, Ilokos and Sumenep genotypes, they produce higher acetic acid compounds (1.04% and 5.98%) compared to the flowering type, the Bentanis genotype (1.02%).

Utilization of sensitive instruments in the metabolomic analysis is an important way to detect the content of plant essential compounds. Plant metabolites consist of highly diverse chemical compounds ranging from inorganic compounds to hydrophilic, volatile alcohol and ketone compounds, amino and non-amino acids, hydrophobic lipids, and complex natural products[19~~18~~].

Utilization of mass spectrometry technology is a great way to solve research and analysis problems in the metabolomic field[19~~18~~]. Metabolomic analysis was succesfully to identify potential compounds extracted directly from shallot genotypes.Th e results showed higher concentrations of acyclic ditherphene alcohol (phytol) compounds in flowering type shallot, Bentanis genotype (8.23%), Bima Brebes (2.2%) and Tajuk (7.52%) than the Ilokos genotype (1.15%) and Sumenep (2.19). The phytol compounds is an indication that vernalization treatment has a positive effect in inducing flowering of shallot genotypes, explaining that these three genotypes are sensitive to vernalization treatment to induce flower formation.Phytol compound is a compound that serves as a precursor of vitamin E and K. The content of these vitamins plays an important role in the plant body as an enzymatic cofactor in the process of metabolism that stimulates rea productive response.The content of vitamin E in the form of tocopherol is an important compound as one of the factors in the context of a cellular antioxidant system[30~~28~~]. The differences of metabolite profile might play a key role in flowering initiation in shallot.

In flowering type of shallot genotypes, Bentanis, Bima Brebes and Tajuk also showed the formation of nitrogen oxide and semicarbazid hydrochlorid which are relatively lower than the non-flowering type, Ilokos and Sumenep. Nitrogen compound is a compound that plays an important role in the vegetative phase of the plant. Once the plant enters the reproductive phase, the nitrogen compounds in the plant body will decrease. In contrast, the Ilokos and Sumenep genotypes show the formation of high nitrogen compounds ie ammonium compounds ammonium carbamat, and nitrogen oxide. The high content of nitrogen compounds in Ilokos and Sumenep genotypes explains that these two genotypes are not sensitive to vernalization treatment, thus not encouraging the flowering formation.

The results also showed that the Sumenep genotype contained high organosulfur compounds compared with other genotypes, namely thiophene (2.31%) and trisulfide (4.25%). The organosulfur is an important compound in giving aroma and spiciness upsurge in shallot, and play an important role as an antioxidant. Essential compounds in *Allium* have strong antioxidant ability. Tests on antimicrobial activity showed that all essential oils on *Allium* were able to inhibit the activity of gram-positive and gram-negative bacteria[9].

Vernalization treatment triggered in the formation of proline compounds in the Sumenep genotype. Vernalization treatment provides a stress condition to the plants that increase the plant mechanism of adaptation by producing proline compounds. Proline accumulation in the plant body is a physiological response to stress, and as a generative tissue development[31 ~~29~~]. Secondary metabolite is the cellular response in plant that receives external pressure.

The metabolite profile of 5 local onion genotypes is also rich in non-volatile phytonutrients, especially the content of phenolic compounds (phenol, 2-propenamide, 3-pyridinamine, N-furfurylidene 2-phenylethyl amine, pyrrolidinedione), amide compounds aziridine-2-carbothioic acid amide) and protein compound (pyroglutamic acid).The essential components, mono and sesquiterpenes, carbohydrates, phenols, alcohols, ether, aldehydes and ketones, are responsible for biological activity and also for their

Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: Font: (Default) Times New Roman, No underline, Font color:
Formatted: Font: (Default) Times New Roman, No underline, Font color: Auto, English (U.K.)
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted ...
Formatted ...
Formatted ...
Formatted ...

fragrance[32~~30~~]. Phenols and polyphenol compoundsplay important roles as antioxidants in plants[33,34,35 ~~31,32,33~~].Research on *Allium rotundum* showed the presence of antioxidant activity of phenol compounds with high concentrations of up to 4%[12]. Onion extract contained the highest of phenol content, at 17.18 mg GAE per gram of fresh weight[36~~34~~], and a phenol content of 114.70 mg GAE per 100 g of different onion samples[37~~35~~].

Metabolite compound is the difference among shallot genotypes. Each plant genotype performs metabolic activities that depend on their genetic conditions and the ability to adapt to its environment. The plant biological activity may imply to the proportion of metabolite containing a plant.

CONCLUSION

A secondary metabolite of 5 genotypes shallot was difference under vernalization treatment. ~~Secondary metabolite is associated to the flowering competency in shallot genotypes.~~Each genotypes produce different specific metabolites which is clustered according to the flowering competency.A high concentration ditherpene alcohol (phytol) found in flowering-sensitive genotypes (genotype of Bentanis, Bima Brebes and Tajuk), with a lower content of proline and nitrogen (ammonium carbamate and nitrogen oxide). While, in flowering-nonsensitive genotypes(genotype Ilokos and Sumenep) were found a higher level of organosulfur compounds (thiophene and trisulfide). Vernalization treatment caused the Sumenep genotype produces the highest proline compound among other genotypes.

ACKNOWLEDGMENT

This research is supported by grants from National Innovation System Research Incentive 2016, Ministry of Research, Technology, and Higher Education, Indonesia.

REFERENCES

1. F.V. Golubev, N.A. Golubkina, and Y.N. Gorbunov. *Appl. Microbiol. Biotechnol*., 39(5): 532–535 (2003). DOI: 10.1023/A:1025465106772.
2. G. Griffiths, L. Trueman, T. Crowther, B. Thomas, and B. Smith. *PTR*. 16(7): 603–615 (2002). DOI: 10.1002/ptr.1222.
3. T. Ritsema , and S. Smeekens. *Curr. Opin. Plant Biol*. 6(3): 223–230 (2003)
4. H. Mohammadi-Motlagh , A. Mostafaie, and K. Mansouri. *Arch. Med. Sci*., 1: 38–44 (2011)
5. Marlin, A. Maharijaya, A. Purwito, and Sobir. *SABRAO J. Breed. Genet*.50 (3) 313-328 (2018). www.sabraojournal.org
6. E.J. Finnegan, D. Bagnall, C. Helliwell, D. Rouse, C. Sheldon, M. Tadege, W.J. Peacock, and E. Dennis. Vernalization. Encyclopedia of Life Sciences Macmillan Publishers Ltd, Nature Publishing Group. (2001).www.els.net.
7. J. Song, A. Angel, M. Howard, and C. Dea. *J. Cell Sci*., 125 (16): 3723–3731 (2012).
8. Y. Iijima. *Metabolite*.4: 699–721 (2014).
9. D. Mnayer, A. Fabiano-Tixier, E. Petitcolas, T. Hamieh, N. Nehme, C. Ferrant, X. Fernandez, and F. Chemat. *Molecules*. 19: 20034–20053 (2014).
10. H. Xiao, and K.L. Parkin. *Phytochem*. 68(7): 1059–1067 (2007).
11. M.A. Vazquez-Prieto, and R.M. Miatello. *Mol. Asp. Med*. 31(6): 540–5 (2010).
12. A.A. Dehpour, M. Yousefian, S.A. Jafary Kelarijani, M. Koshmoo, S. Mirzanegad, V. Mahdavi, S.E. Mousavi, E. Shirzad, M. Afzali, M.J. Javad Bayani, J.E. Olyaei, and M.K. Yahyapor. *Adv. Environ. Biol*., 6(3): 1020–1025 (2012).
13. V. Lanzotti, F. Scala, and G. Bonanomi. *Phytochem Rev*., 13: 769–791 (2014).
14. N. Martins, S. Petropoulos, and I.C.F. Ferreira. *Food Chem*.15(211): 41–50 (2016).
15. R.Y. Galingging, Sobir, S.I. Aisyah, and A. Maharijaya. *Rasayan J. Chem*., 11(2), 575 - 581(2018). DOI: 10.7324/RJC.2018.1123001
~~15.~~16. O. Fiehn. *Plant Mol. Biol*. 48: 155–171 (2002).
~~16.~~17. H. Wu, O.J. Gu, S. Chen, X. Liu, Y. Zhou, X. Zhang, and X. Xu. *J. Pharm. Biomed. Anal*., 72: 267–291 (2013).

Formatted: Font: 11 pt
Formatted ...
Formatted ...
Formatted ...
Formatted: Font: 11 pt
Formatted ...
Formatted ...
Formatted: Font: (Default) Times New Roman, Condensed by 0.15 pt
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted: Font: No underline, Font color: Auto
Formatted ...
Formatted: Font: No underline, Font color: Auto
Formatted: Font: No underline, Font color: Auto
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...

~~17.~~18. T. Soininen. Ph.D. Thesis, University of Eastern Finland. Finland (2014).
~~18.~~19. S.G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, and J. Nielsen, *Mass Spectrom. Rev.*, 24: 613– 646 (2005).
20. P.P. Durgawale, S.A. Hendre, and R.S. Phatak. *Rasayan J. Chem*., 8(3), 271 – 278 (2015).
~~19.~~21. N.C.J.P. Lekshmi, S. Viveka, M.B. Viswanathan, G. Manivannan, and T.M. Shobi. *Nanobio Pharma. Tech.*, (2014). DOI: 10.13140/2.1.3278.7523.
~~20.~~22. S.M. Huang, W. Toh, P.I. Benke, C.S. Tan, and C.N. Ong. *Metabolomics.* 10:1084–1093 (2014).
~~21.~~23. Y.S. Wong, and P.A. Castelfranco, *Plant Physiol.*,79(3): 730–733 (1985).
~~22.~~24. N. Baker, J. Harbinson, and D.M. Kramer. *Plant Cell Environ*,30 1107–1125 (2007)
~~23.~~25. P. Rattanachaikunsopon, and P. Phumkhachorn P, *Afr. J. Microbiol. Res*, 3: 747–750 (2009).
~~24.~~26. A. Chehregani, F. Azimishad, and A.H. Haj. *Int. Agri. Biol.*,9(6): 873–876 (2007)
~~25.~~27. C.L. Ye, D.H. Dai, and W.L. Hu. *Food Control*.30: 48–53 (2013).
~~26.~~28. J.W. Kim, J.E. Huh, S.H. Kyung, and K.H. Kyung. *Food Sci. Biotech*, 13: 235–239 (2004).
~~27.~~29. M.M. Lokke, M. Edelenbos, E. Larsen, and A. Feilberg. *Sensor*s.12: 16060–16076 (2012).
~~28.~~30. *M.G. Traber, J. Atkinson, and Atkinson. Free Radical Biol. Med. 43 (1): 4–15 (2007).*
~~29.~~31. N. Verbruggen, and C. Hermans. *Amino Acids*, 35: 753–759 (2008).
~~30.~~32. E.A. El-Wakil, M.M. El-Sayed, and E.E. Abdel-Lateef. *Int. J. Pharma Sci. Res. IJPSR*, 6 (3) (2015).
~~31.~~33. S.A.B.E. Van Acker, D.J. van den Berg, N.J.L. Tromp, D.H. Griffioen, W.P. van Bennekom, and W.J. van der Vijgh. *Free Radical Biol. Med.*, 20 (3): 331– 342 (1996).
~~32.~~34. M.A. Alvarez, N.B. Debattista, and N.B. Pappano. *Folia Microbiol.*,53(1): 23–8 (2008).
~~33.~~35. A.P. Mukne, V. Viswanathan, and A.G. Phadatare. *Pharmacognosy Rev.*,5(9): 13–18 (2011)
~~34.~~36. X. Lu, J. Wang, H.M. Al-Qadiri, C.F. Ross, J.R. Powers, J. Tang, and B.A. Rasco, *Food Chem*,129: 637–644 (2011).
~~35.~~37. J. Yang, K.J. Meyers, J. van der Heide, and R.H. Liu. *J. Agr. Food Chem.*52: 6787–6793 (2004).

[RJC-4034/2018]

Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted: Font:
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted: Font:
Formatted ...
Formatted: Font:
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...
Formatted ...

Author's Queries (To be sent with Galley Proofs)

1. Biological names must be italic trough out the manuscript(if any).
2. Check the Title, Names of Author(s), their affiliations and E-mail of Corresponding Author once again.
3. Check the complete Manuscript once again for Grammar Check.
4. MOST Important: References must be strict as per the STYLE of the journal (Please refer Guidelines and Sample paper), which may otherwise cause unnecessary delay in publication of your paper. Also,Mention DOI with references, wherever possible.
5. Check the terms highlighted in the Galley Proofs, if it is correct? If not, please correct the same throughout the manuscript in Red/Blue ink, so that it could be visible to us.
6. Check/ correct all Figure and Table numbers and their proper citation/position/alignment in the text.

Marlin UNIB <marlin@unib.ac.id>

Galley Proofs: RJC-5356/2019

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 29 Juli 2019 pukul 12.22
Kepada: Marlin UNIB <marlin@unib.ac.id>

Thank you for your mail.
[Kutipan teks disembunyikan]

Marlin UNIB <marlin@unib.ac.id>

Your paper has been published Online First in RJC, Vol.12, No.3, July- September, 2019

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 3 Agustus 2019 pukul 14.15

Bcc: marlin@unib.ac.id

Dear Contributor,

Congratulations!

Your paper has been published *OnlineFirst* in **RJC, Vol.12, No.3, July - September, 2019**. Please visit www.rasayanjournal.com for downloading your articles directly from the **Current Issue**. The hard copy (If you paid for that) will be reached to you when published.

We are happy to share with you that, **RASĀYAN J. Chem.** (A **Scopus , Elsevier** Indexed Journal) is also included in the list of recommended journals released by **UGC** (the highest recognition body for academic affairs in India), which highlights the worldwide acceptance and recognition of the quality and content of the journal. **You are requested to share this important information with your contacts** and suggest the journal to your colleagues to submit their valuable manuscripts for **RASĀYAN J. Chem.** (A **Scopus , Elsevier** Indexed Journal).

Thanks for your cooperation and support.
Looking forward.

--

Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)

www.rasayanjournal.com | www.rasayanjournal.co.in

Contact: +91 9001699997, +91 9414202678

Marlin UNIB <marlin@unib.ac.id>

Your paper has been published Online First in RJC, Vol.12, No.3, July- September, 2019

marlin <marlin@unib.ac.id> 3 Agustus 2019 pukul 17.40
Kepada: "RASĀYAN J. Chem." <rasayanjournal@gmail.com>

Dear Editors

Thank you very much for publication information. I'm so excited and I will suggest RJC to my colleagues.

Sincerelly
Dr. Marlin

Sent from my Samsung Galaxy smartphone.
[Kutipan teks disembunyikan]

Marlin UNIB <marlin@unib.ac.id>

Book Your Copy: RJC, Vol.12, No.3, July- September, 2019

RASĀYAN J. Chem. <rasayanjournal@gmail.com> 27 September 2019 pukul 14.00

Cc: RASAYAN JOURNAL <rasayanjournal@gmail.com>
Bcc: marlin@unib.ac.id

Dear Contributor,
The Hard Copy of the **RJC, Vol.12, No.3, July- September, 2019** is going to be released soon.
We Congratulate you for your recently published paper in this issue. We are sure that It'll be a valuable research for the readers/ researchers world wide. It would be a great networking tool if you and your friends cite this paper in the future research publications. It'll surely enhance your credibility and readership both worldwide.

If you want to have Hard copy of the Journal, in which your paper published, please book your copy by paying **USD 100** (which includes Registered Air mail Charges), **latest by 11th October 2019**. After that we'll not be able to send the hard copies of the journal issue.

Also you can suggest/advice your friends and Library to purchase a copy of the Published paper.
You are requested to send the Filled attached Payment Cum Address Slip along with the Receipt of the payment.

We hope that you had enjoyed the publication process through out. Please let us know about your valuable feedback also in the provided space in the attachment.
Thanks for your Cooperation and Support.

Prof. Sanjay K. Sharma, FRSC
Editor, Rasayan J., Cherm.

--
Best Regards,

***Editor,* RASĀYAN J. Chem.**

(SCOPUS, Elsevier indexed, Since 2008)
www.rasayanjournal.com | www.rasayanjournal.co.in
Contact: +91 9001699997, +91 9414202678

_Payement Cum Address Slip.docx
130K

Marlin UNIB <marlin@unib.ac.id>

RECEIPT.

RASĀYAN_Accounts <rasayan.finance@gmail.com>
Kepada: marlin@unib.ac.id

26 Oktober 2019 pukul 20.25

Receipt of your Payment

Dear Author,

Greetings from RASĀYAN Journal of Chemistry.

Thanks for contributing your valuable manuscript(s) in RASĀYAN Journal of Chemistry. We value your interest in our publication and hope, you have also enjoyed publishing with us.

Please find herewith attached receipt of your payment made against publication/procession charges for your manuscript(s).

We welcome your more manuscripts for future issues as well.

Thanks again for publishing with us.

With Kindest Regards-

Kunal Sharma

Assistant Executive, Accounts
RASĀYAN Journal of Chemistry.

+91 9001699997

5356.pdf
2290K

M.N ⇒ 5356/2019,

RASĀYAN Journal of Chemistry

(An SCOPUS Indexed International Journal of Chemical Sciences, Since 2008)
ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)
www.rasayanjournal.com | www.rasayanjournal.co.in
E-mail: rasayanjournal@gmail.com

Receipt No.: 711

Date : 30/5/19.

RECEIPT OF PAYMENT

Received with thanks, from Prof./ Dr./ Mr./ Ms. Marlin. the sum of Rs. 27,633. (In words, Rupees twenty seven thousand Six hundred thirty three), being an **ARTICLE PROCESSING CHARGE (APC)** for Manuscript No.: RJC-5356/2019 or **ANNUAL SUBSCRIPTION CHARGE** of **RASĀYAN Journal of Chemistry** for year X through Cheque / Demand Draft No.: SWIFT or NEFT / SWIFT / WUMT/ Cash, dated 28/5/19

Thanks for your Contribution / Subscription.

~~For~~ **RASĀYAN Journal of Chemistry**

23, Anukampa, Janakpuri, Opp. Heerapura Power Station, Ajmer Road, Jaipur-302 024 (India).
t: +91 9001699997, +91 9414202678

M.N ⇒ 5356/2019,

RASĀYAN Journal of Chemistry

(An SCOPUS Indexed International Journal of Chemical Sciences, Since 2008)
ISSN: 0974-1496 (Print) | ISSN: 0976-0083(Online)
www.rasayanjournal.com | www.rasayanjournal.co.in
E-mail: rasayanjournal@gmail.com

Receipt No.: 711

Date : 30/5/19.

RECEIPT OF PAYMENT

Received with thanks, from Prof./ Dr./ Mr./ Ms. Marlin. the sum of Rs. 27,633. (In words, Rupees twenty seven thousand Six hundred thirty three), being an **ARTICLE PROCESSING CHARGE (APC)** for Manuscript No.: RJC-5356/2019 or **ANNUAL SUBSCRIPTION CHARGE** of **RASĀYAN Journal of Chemistry** for year X through Cheque / Demand Draft No.: SWIFT or NEFT / SWIFT / WUMT/ Cash, dated 28/5/19

Thanks for your Contribution / Subscription.

~~For~~ **RASĀYAN Journal of Chemistry**

23, Anukampa, Janakpuri, Opp. Heerapura Power Station, Ajmer Road, Jaipur-302 024 (India).
t: +91 9001699997, +91 9414202678