THE EURASIA PROCEEDINGS OF HEALTH, ENVIRONMENT AND LIFE SCIENCES

ISRES Publishing

VOLUME 8 ICVALS CONFERENCE
ISSN: 2791-8033
ISBN: 978-625-6959-02-6
ICVALS 2022: 5th International Conference on Veterinary, Agriculture and Life Sciences (ICVALS)
November 17 - 20, 2022
Antalya, Turkey
Edited by: Mehmet Ozaslan (Chair), Gaziantep University, Turkey

ICVALS 2022 NOVEMBER

Volume 8, Pages 1-35 (November 2022)

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)

e-ISSN: 2791-8033

Address: Istanbul C. Cengaver S. No 2 Karatay/Konya/TURKEY

Website: www.isres.org

Contact: isrespublishing@gmail.com

Conference: ICVALS 2022: 5th International Conference on Veterinary, Agriculture and Life Sciences (ICVALS)
Conference website: https://www.2022.icvals.net

Dates: November 17 – 20, 2022
Location: Antalya, Turkey
Edited by: Mehmet Ozaslan

About Editor
Prof Dr. Mehmet Ozaslan
Department of Biology, Gaziantep University, Turkey
Website: mehmetozaslan.com
Email: ozaslanmd@gantep.edu.tr

Language Editor
Assoc. Prof. Dr. Kagan Buyukkarci
Department of English Language Education, Suleyman Demirel University, Turkey
Email: kaganbuyukkarci@sdu.edu.tr

ORGANIZING COMMITTEE

Editorial Policies

ISRES Publishing follows the steps below in the procedings book publishing process.
In the first stage, the papers sent to the conferences organized by ISRES are subject to editorial oversight. In the second stage, the papers that pass the first step are reviewed by at least two international field experts in the conference committee in terms of suitability for the content and subject area. In the third stage, it is reviewed by at least one member of the organizing committee for the suitability of references. In the fourth step, the language editor reviews the language for clarity.

Review Process

Abstracts and full-text reports uploaded to the conference system undergo a review procedure. Authors will be notified of the application results in three weeks. Submitted abstracts will be evaluated on the basis of abstracts/proposals. The conference system allows you to submit the full text if your abstract is accepted. Please upload the abstract of your article to the conference system and wait for the results of the evaluation. If your abstract is accepted, you can upload your full text. Your full text will then be sent to at least two reviewers for review.

The conference has a double-blind peer-review process.

Any paper submitted for the conference is reviewed by at least two international reviewers with expertise in the relevant subject area. Based on the reviewers' comments, papers are accepted, rejected or accepted with revision. If the comments are not addressed well in the improved paper, then the paper is sent back to the authors to make further revisions. The accepted papers are formatted by the conference for publication in the proceedings.

Aims & Scope

Plant and animal health is closely related to human health. In this century, where the human population is rapidly increasing and technology is developing rapidly, the problem of food supply to the increasing population brings plant and animal health to the fore. Nowadays, when concepts such as artificial meat and capsule feeding are discussed, the process of growing plants and animals has begun to be discussed. For this reason, this conference focused on the changes and innovations in the field of Veterinary, Agriculture and Life Sciences.

The aim of the conference is to bring together researchers and administrators from different countries, and to discuss theoretical and practical issues of Veterinary, Agriculture and Life Sciences. At the same time, it is aimed to enable the conference participants to share the changes and developments in the field of Veterinary, Agriculture and Life Sciences with their colleagues.

Articles: 1-4

CONTENTS

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)
ISSN: 2791-8033

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS), 2022

Volume 8, Pages 1-6

ICVALS 2022: International Conference on Veterinary, Agriculture and Life Sciences

Study of the Relationship between Biomass and Fractional Green Canopy Cover of Two Forage Crops Using Canopeo®

Tarek BOUREGA
Ferhat Abbas University

Hakima BOULAARAS
Ferhat Abbas University

Nacer Eddine LOUAHDI
Technical Institue for Field Crops (ITGC)

Haroun BELGUET
Technical Institue for Field Crops (ITGC)

Abstract: Rapid and accurate estimation of biomass is crucial for the assessment of the crop nutrition status and the improvement of crop management strategies. The study was conducted in Setif province (Northeast of Algeria). Canopy cover was calculated using Canopeo® application, at stem elongation, booting, and heading stages for triticale, and V5, R1, and R3 for lentil. The results indicate a high correlation between canopy cover and triticale biomass, at the stem elongation stage with R^2 = 0.98 and 0.89 under no till and conventional till, respectively. The RMSE was 0.1 and 0.06t/ha, under the two modes. The lowest correlation is seen at heading stage with a value of 0.21 and RMSE of 0.65t/ha, under no till mode. For lentil, the high correlations between are observed at R3 (early pod), and R1 (early bloom) stages, with a coefficients of 0.85 and 0.98, under zero till and conventional respectively. The RMSE was 0.17t/ha. This smartphone application is suitable for assessing triticale and lentil biomass; it is rapid, easy, and could replace the destructive sampling method.

Keywords: Correlation, RMSE, Biomass, FGCC, Canopeo®.

Introduction

Rapid and Accurate estimation of biomass is crucial for the assessment of crop nutrition status and the improvement of crop management strategies (Lu et al., 2019). The measurements of crop biomass and height are usually done by direct sampling or using devices such as the rising plate meter, capacitance meter and meter stick (Viljanen et al., 2018). However, this method is not practical for repeated large scale measurements. It consumes considerable time, destructive, labour and resources. Precision farming and high-throughput phenotyping measurements and digital imaging has the potential to provide more information to make more informed management decisions on a canopy scale in real time (Kipp et al., 2014; Barmeier & Schmidhalter, 2017; Elsayed et al., 2018).

In recent years, indirect and non-destructive optical methods based on canopy cover have been used, especially in field environments (Bendig et al., 2014; Chen et al., 2018; Hufkens et al., 2019). It can be measured by processing digital images of the canopy taken directly above the crop. It is generally defined as the proportion of the ground area covered by the vertical projection of the plant canopy (Büchi et al., 2018).

Researchers used this function to measure perspectives on high throughput phenotyping in developing countries and proved that this method could be applicable in specific situations where research budget is tight. It has been widely tested and proven as an accurate, easy and fast method to estimate canopy cover of durum wheat (Casadesús et al., 2007), barley (Neumann et al., 2015), sorghum (Chung et al., 2017), canola (Pandey et al., 2016), onion (Córcoles et al., 2013), and turf grass (Richardson et al., 2001).

There is recent progress in developing application software that permits rapid, user-friendly measurements of canopy characteristics for pasture management and research, like ImageJ, Assess and Canopeo softwares (Büchi et al., 2018; Schindelin et al., 2015; Xiong et al., 2019). According to Patrignani and Ochsner (2015) fractional green canopy cover can be calculated using the mobile device application, Canopeo® (Oklahoma State University, Stillwater, OK), which automatically classifies pixels as green or not green. Canopeo correctly classified 100% and 90% of pixels in images of winter wheat produced under conventional tillage and no-tillage, respectively.

The main objective of this study is to determine if the fractional green canopy cover analysis using Canopeo® is suitable to evaluate the biomass of triticale and lentil. If it is so, this method would replace the hand-collected data to achieve same goal.

Methodology

Site Description

The study was conducted in Saleh bey region (35°87′24″ N, 5°30′83″ E). It is located in the South-West of Sétif province (Northeast of Algeria) with an area of 27400 ha. The climate of the region is arid; it receives an amount of precipitation less than 300 mm per year. Average temperatures are very high in summer (34°C) and low in winter (5°C), but minimum temperatures remain very low until april with a significant risk of late spring frost. It is characterized by limestone soils and a silty-clayey texture, with pH around 8.5, and organic matter around 2.5%.

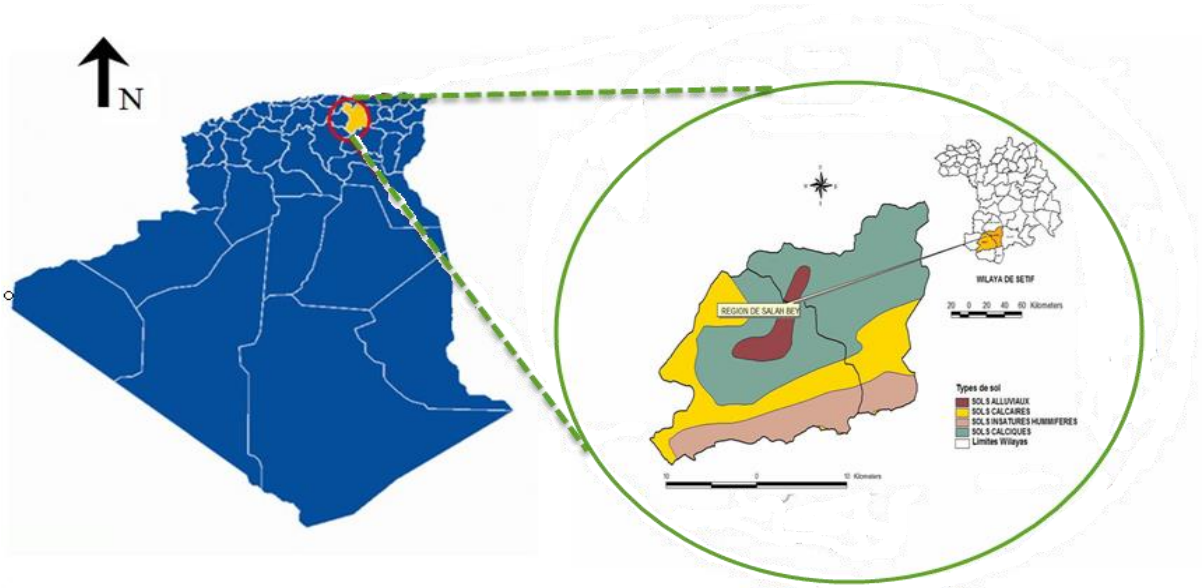

Figure 1. Geographical situation of the study area.

Experimental Design

The experiment was a complete randomized block with one factor (tillage mode), and three repetitions. Destructive above-ground biomass sampling of 0.5 m^2 was carried out within the sampling areas with three repetition of each forage crop (triticale, lentil). Fractional green canopy cover was calculated using the mobile device application, Canopeo®. It is an authomatic color threshold image analysis tool developed in the Matlab programming language, using color values in the red-green-blue (RGB) system. The analysis is based on the selection of pixels according to the ratios of R/G, B/G and the excess green index. The result of the analysis is a

binary image where white pixels correspond to the pixels that satisfied the selection criteria (green canopy) and black pixels correspond to the pixels that did not meet the selection criteria (Patrignani & Ochsner, 2015).

In order to capture three rows of crops, the camera height and length of row was varied based on canopy height (Figure 2). Crops canopy cover was measured, at stem elongation, booting and heading stages for triticale, and V5, R1, R3 stages for lentil. To analyze the relationship between fractional green canopy cover (FGCC), and biomass (BIO), simple linear regressions were calculated using Microsoft Excel (2013) and the Agrimetsoft (2022) on line calculator.

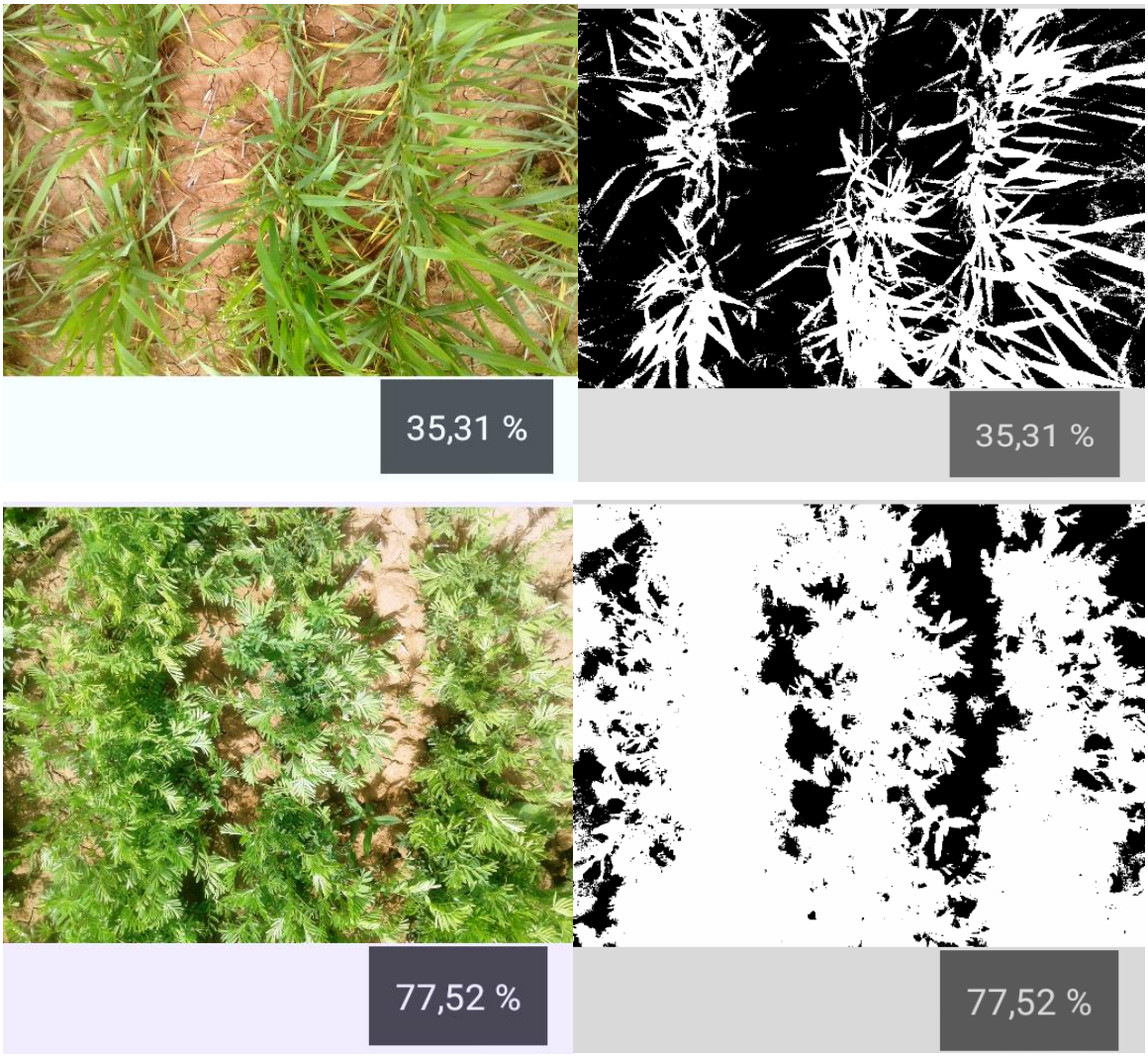

Figure 2. Fractional green canopy cover of triticale (top) and lentil (bottom): original image (left) and Canopeo® processed image (right).

Results and Discussion

The correlation and the root mean square error (RMSE) between fractional green canopy cover, and biomass measurements at three growth stages of forage crops is shown in Table 1. For triticale, the strong correlations between FGCC and BIO are observed at stem elongation stage, with coefficients of 0.99 and 0.89 under no till and conventional till, respectively. The RMSE was 0.1 and 0.06t/ha, under the two modes. The lowest correlation is seen at heading stage with a value of 0.21 and RMSE of 0.65t/ha, under no till mode. This low correlation could be caused by color changes from green to yellowish due to canopy senescence (Chung et al., 2017). For lentil, the high correlations between are observed at R3 (early pod), and R1 (early bloom) stages, with a coefficients of 0.85 and 0.98, under zero till and conventional, respectively. The RMSE was 0.17t/ha. However, a low correlations are seen at the V5 (the first multifoliate leaf has unfolded at the fifth node) stage, with values of 0.31 and 0.37 under no till and conventional till, respectively. This low weak correlation can be

attributed to the confusion of the application between weeds and crops canopy, especially under conventional till, when no weeds control had been made under this till mode.

Table 1. Pearson correlation coefficients and root mean square error (RMSE) between fractional green canopy cover (FGCC) and biomass (BIO), at each measurement stage

		No till		Conventional till	
Crop	Stage	R	RMSE (t/ha)	R	RMSE (t/ha)
Triticale	elongation	0.99	0.1	0.89	0.06
	booting	0.91	0.18	0.71	0.54
	heading	0.21	0.65	0.80	0.4
Lentil	V5	0.31	0.04	0.37	0.07
	R1	0.09	0.08	0.98	0.17
	R3	0.85	0.17	0.42	0.16

V5: the first multifoliate leaf has unfolded at the fifth node; R1: early bloom, one open flower at any node; R3: early pod, pod on nodes 10-13 of the basal primary branch visible.

These results are in accordance with other previous studies. Goodwin et al. (2018) found a good correlation between FGCC and wheat grain yield, with R^2 of 0.45. They mentioned that FGCC measurement is time sensitive and should be conducted at early stages of plant development. Jáuregui et al. (2018) found a positive linear relationship between FGCC and biomass of lucerne, with goodness of fit 0.77 and 0.86 for spring-summer and autumn-winter biomass, respectively. Shepherd et al. (2018) found a linear relationship between canopy cover measured with Canopeo and light interception taken by the line quantum sensor (R^2 = 0.94). (Jia et al., 2014), indicated that canopy cover and aboveground biomass of cotton were closely related (R^2 = 0.74-0.94). (Tomasel et al., 2001), found a high correlation between pixel count and green biomass (r = 0.95). Paruelo et al. (2000) reported that the correlation between FGCC and green grass biomass was 0.87. Louhaichi et al. (2010) and Pask et al. (2012) found a linear relation between digital ground cover and wheat biomass (r^2 = 0.63). The study of Lee & Lee (2013) showed a high correlation between canopy cover and dry weight of wheat (r=0.81). Bumgarner et al. (2012) reported that the correlation coefficients between direct measures of lettuce biomass and WinCAM estimates of canopy cover were 0.71 to 0.95. According to (Büchi et al., 2018), a high correlation was found between visual assessment of canopy cover and Canopeo® image analysis of lentil (0.90), field pea (0.73), and faba bean (0.81). Firatligil-Durmuş et al. (2008), reported that image processing provide a rapid a non-invasive methodology to estimate lentil geometric features and engineering parameters. However, Prabhakara et al. (2015) reported that index saturation, chlorosis, and frost damage may lead to inaccurate estimates of aboveground biomass when using vegetation indices to measure greenness of crops. Another limitation of Canopeo® that can't distinguish between crops and weeds canopy (Jáuregui et al., 2018).

Conclusion

Based on our results, it can be concluded that digital image analysis using Canopeo® is suitable to assess triticale and lentil biomass, it is rapid, easy, and could replace the destructive biomass sampling. However, the measurements are time sensitive. Images should be collected at early stages for triticale, to be more precise and that under the two till modes. However for lentil, the photos should be taken at late stage under no till mode, and at early stages under conventional till mode. The main limitation of Canopeo® application that can't differentiate between crops and weeds canopy.

Scientific Ethics Declaration

The author declares that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the author.

Acknowledgements or Notes

* This article was presented as an oral presentation at International Conference on Veterinary, Agriculture and Life Sciences (www.icvals.net) held in Antalya/Turkey on November 17-20, 2022.

References

Agrimetsoft. (2022). *On line free calculators*. Seoul National University, Korea. Retrieved from https://agrimetsoft.com/calculators/.

Barmeier, G., & Schmidhalter, U. (2017). High-throughput field phenotyping of leaves, leaf sheaths, culms and ears of spring barley cultivars at anthesis and dough ripeness. *Frontiers in Plant Science*, *8*.

Bendig, J., Bolten, A., Bennertz, S., Broscheit, J., Eichfuss, S., & Bareth, G. (2014). Estimating biomass of barley using crop surface models (CSMs) derived from uav-based rgb imaging. *Remote Sensing 6*(11), 10395–10412

Büchi, L., Wendling, M., Mouly, P., & Charles, R. (2018). Comparison of visual assessment and digital image analysis for canopy cover estimation. *Agronomy Journal*, *110*(4), 1289–1295.

Bumgarner, N. R., Miller, W. S., & Kleinhenz, M. D. (2012). Digital image analysis to supplement direct measures of lettuce biomass. *HortTechnology*, *22*(4), 547–555.

Casadesús, J., Kaya, Y., Bort, J., Nachit, M. M., Araus, J. L., Amor, S., Ferrazzano, G., Maalouf, F., Maccaferri, M., Martos, V., Ouabbou, H., & Villegas, D. (2007). Using vegetation indices derived from conventional digital cameras as selection criteria for wheat breeding in water-limited environments. *Annals of Applied Biology*, *150*(2), 227–236.

Chen, D., Shi, R., Pape, J. M., Neumann, K., Arend, D., Graner, A., Chen, M., & Klukas, C. (2018). Predicting plant biomass accumulation from image-derived parameters. *GigaScience*, *7*(2), 1–13.

Chung, Y. S., Choi, S. C., Silva, R. R., Kang, J. W., Eom, J. H., & Kim, C. (2017). Case study: Estimation of sorghum biomass using digital image analysis with canopeo. *Biomass and Bioenergy*, *105*, 207–210.

Córcoles, J. I., Ortega, J. F., Hernández, D., & Moreno, M. A. (2013). Estimation of leaf area index in onion (Allium cepa L.) using an unmanned aerial vehicle. *Biosystems Engineering*, *115*(1), 31–42.

Elsayed, S., Barmeier, G., & Schmidhalter, U. (2018). Passive reflectance sensing and digital image analysis allows for assessing the biomass and nitrogen status of wheat in early and late tillering stages . *Frontiers in Plant Science*, *9*.

Firatligil Durmuş, E., Šárka, E., & Bubník, Z. (2008). Image vision technology for the characterisation of shape and geometrical properties of two varieties of lentil grown in Turkey. *Czech Journal of Food Sciences*, *26*(2), 109–116.

Goodwin, A. W., Lindsey, L. E., Harrison, S. K., & Paul, P. A. (2018). Estimating wheat yield with normalized difference vegetation index and fractional green canopy cover. *Crop, Forage & Turfgrass Management*, *4*(1), 1–6.

Hufkens, K., Melaas, E. K., Mann, M. L., Foster, T., Ceballos, F., Robles, M., & Kramer, B. (2019). Monitoring crop phenology using a smartphone based near-surface remote sensing approach. *Agricultural and Forest Meteorology*, *265*(2018), 327–337.

Jáuregui, J., Delbino, F., Brance Bonvini, M. I., Ballanti, S., Bosio, M., Iglesias, B., Landucci, T., Merki, R., & Berhongaray, G. (2018). Use of Canopeo to determine light interception and yield of alfalfa (Medicago sativa L.) crops. *II World Alfalfa Congress*, *October*.

Jia, B., He, H., Ma, F., Diao, M., Jiang, G., Zheng, Z., Cui, J., & Fan, H. (2014). Use of a digital camera to monitor the growth and nitrogen status of cotton. *The Scientific World Journal*, *2014*, 19–22.

Lee, K.-J., & Lee, B.-W. (2013). Estimation of rice growth and nitrogen nutrition status using color digital camera image analysis. *European Journal of Agronomy*, *48*, 57–65.

Louhaichi, M., Johnson, M. D., Woerz, A. L., Jasra, A. W., & Johnson, D. E. (2010). Digital charting technique for monitoring rangeland vegetation cover at local scale. *International Journal of Agriculture and Biology*, *12*(3), 406–410.

Lu, N., Zhou, J., Han, Z., Li, D., Cao, Q., Yao, X., Tian, Y., Zhu, Y., Cao, W., & Cheng, T. (2019). Improved estimation of aboveground biomass in wheat from RGB imagery and point cloud data acquired with a low-cost unmanned aerial vehicle system. *Plant Methods*, *15*(1), 1–16.

Neumann, K., Klukas, C., Friedel, S., Rischbeck, P., Chen, D., Entzian, A., Stein, N., Graner, A., & Kilian, B. (2015). Dissecting spatiotemporal biomass accumulation in barley under different water regimes using high-throughput image analysis. *Plant Cell and Environment*, *38*(10), 1980–1996.

Pandey, B. R., Burton, W. A., Salisbury, P. A., & Nicolas, M. E. (2016). Non-destructive measurement of canopy cover is an alternative to biomass sampling at anthesis to predict yield of canola-quality Brassica juncea. *Australian Journal of Crop Science*, *10*(4), 482–489.

Paruelo, J. M., Lauenroth, W. K., & Roset, P. A. (2000). Technical note: Estimating aboveground plant biomass using a photographic technique. *Journal of Range Management*, *53*(2),190–193.

Patrignani, A., & Ochsner, T. E. (2015). Canopeo: A powerful new tool for measuring Fractional Green Canopy Cover. *Agronomy Journal*, *107*(6), 2312–2320.

Prabhakara, K., Hively, W. D., & McCarty, G. W. (2015). Evaluating the relationship between biomass, percent groundcover and remote sensing indices across six winter cover crop fields in Maryland, United States.

International Journal of Applied Earth Observation and Geoinformation, *39*, 88–102.

Richardson, M. D., Karcher, D. E., & Purcell, L. C. (2001). Quantifying turfgrass cover using digital image analysis. *Crop Science*, *41*(6), 1884–1888.

Schindelin, J., Rueden, C. T., Hiner, M. C., & Eliceiri, K. W. (2015). The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular Reproduction and Development*, *82*(7–8), 518–529.

Shepherd, M. J., Lindsey, L. E., & Lindsey, A. J. (2018). Soybean canopy cover measured with canopeo compared with light interception. *Agricultural & Environmental Letters*, *3*(1), 180031.

Tomasel, F. G., Paruelo, J. M., Abras, G., Ballarín, V., & Moler, E. (2001). A chromaticity-based technique for estimation of above-ground plant biomass. *Applied Vegetation Science*, *4*(2), 207–212.

Viljanen, N., Honkavaara, E., Näsi, R., Hakala, T., Niemeläinen, O., & Kaivosoja, J. (2018). A novel machine learning method for estimating biomass of grass swards using a photogrammetric canopy height model, images and vegetation indices captured by a drone. *8*(5), 70.

Xiong, Y., West, C. P., Brown, C. P., & Green, P. E. (2019). Digital image analysis of old world bluestem cover to estimate canopy development. *Agronomy Journal*, *111*(3), 1247-1253.

Author Information

Tarek Bouregaa
Ferhat Abbas University
Sétif, Algeria
Contact e-mail: *ibnziad_1983@hotmail.com*

Hakima Boulaaras
Ferhat Abbas University
Sétif, Algeria

Nacer Eddine Louahdi
Technical Institue for Field Crops (ITGC)
El Harrach,Algeria

Haroun Belguet
Technical Institue for Field Crops (ITGC)
El Harrach,Algeria

To cite this article:

Bouregaa, T., Boulaaras, H., Louahdi, N.E., & Belguet, H. (2022). Study of the relationship between biomass and fractional green canopy cover of two forage crops using Canopeo® . *The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)*, *8*, 1-6.

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)
ISSN: 2791-8033

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS), 2022

Volume 8, Pages 7-13

ICVALS 2022: International Conference on Veterinary, Agriculture and Life Sciences

The Effect of PMSG on Follicular Development and Ovulation Time of $PGF_{2\alpha}$ Treated Awassi Ewes

Mustafa Q. HUSSEIN
Jordan University of Science and Technology

Ghaid J. AL-RABADI
Mutah University

Hassan GHOZLAN
Jordan University of Science and Technology

Abstract: The objective was to examine the effects of PMSG administration before or at the time of $PGF_{2\alpha}$ injection on reproductive responses of ewes out-of-season. In late June and early July, 57 anestrus ewes were induced to estrus using CIDR-G devices for 12 days. Six days following device removal ewes were randomly allocated into three treatment groups and each received an i.m injection of 20mg $PGF_{2\alpha}$ (day 0, 0 h). PMSG (500 IU) was administered either 24 h before (group A, n=20) or at (group B, n=19) the time of $PGF_{2\alpha}$ injection. Group C ewes (n=18) served as control. Half of the ewes in each group were exposed to three intact rams at 0 h to be naturally mated and the other half were exposed to three aproned rams and inseminated 50-56 h following 0 h. Ewes were checked for breeding marks at 6-h intervals for 4 days. Progesterone levels from day –1 until day 20 were monitored. Occurrence of estrus was similar among ewes of the three treatment groups and averaged 79%. PMSG-treated ewes had shorter ($P<0.01$) intervals to estrus and ovulation, and a higher ovulation rate than non-PMSG treated ewes. Pregnancy and lambing rates were similar among PMSG- and non-PMSG-treated ewes. Although reproductive responses were similar among artificially-inseminated and naturally-mated ewes, the latter had a higher ($P<0.05$) lambing rate (14.3% vs 41.4%, respectively). Ewes inseminated close to the time of ovulation (7.7 h earlier) produced higher ($P<0.05$) pregnancy rate than those inseminated at a wider interval from ovulation (16.7 h earlier). In conclusion, the $PGF_{2\alpha}$ treatment given during luteal phase was effective in resetting subsequent cyclic activity of ewes. Although it did not increase the number of ewes detected in estrus, PMSG shortened intervals to estrus and ovulation, increased ovulation and induced–estrus pregnancy rates.

Keywords: Awassi, PMSG, $PGF_{2\alpha}$, Follicle, Ovulation, ewes

Introduction

Induced-estrus pregnancy rates of < 40% have been reported out-of season for Awassi ewes using different estrus synchronization protocols (Husein & Kridli, 2002a). Several studies have been conducted to determine reasons responsible for low induced-estrus pregnancy rates (Husein & Kridli, 2002b; Abdullah et al., 2002). These authors reported some contributing factors, which include drought, environmental stress and ram inexperience. Other factors may have been season, temperature, disease, nutrition, management, semen quality, artificial insemination method, insemination time, synchronization protocols and the reproductive condition of the ewe (Husein et al., 1996; Husein

et al., 1998). Although the use of various methods of estrus synchronization utilizing progesterone and PMSG produced a little improvement, the overall fertility rate was still reduced (Husein & Kridli, 2002a).

In sheep, administration of the exogenous $PGF_{2\alpha}$ in cyclic ewes between days 4 and 14 of the estrous cycle causes corpus luteum regression and allows a new follicular phase to start and subsequent return to estrus within 2-3 days (Chamley et al., 1972). The use of $PGF_{2\alpha}$ is limited in anestrous ewes because they do not possess active corpora lutea. The intention of this study, therefore, was to use animals of similar stage of the estrous cycle for an attempt to minimize variation in reproductive responses among ewes. Variable fertility has been reported among ewes induced to estrus using $PGF_{2\alpha}$ alone or any of its analogues (Gordon, 1997). However, when PMSG was incorporated into a $PGF_{2\alpha}$ protocol, estrus exhibition was improved and intervals to onset of estrus were shortened (Trounson et al., 1976) and pregnancy rate and incidence of multiple births were increased (Madani et al., 1984). The objective was to examine the effects of PMSG administration before or at the time of $PGF_{2\alpha}$ injection on reproductive responses of ewes out-of-season.

Materials and Methods

Animals

In late June and Early July, 57 anestrus Awassi ewes weighing 49.0 ± 1.0 kg were used in an experiment conducted at the Agricultural Center for Research and Production at Jordan University of Science and Technology (32°33'N, 35°51'E). All ewes had previously lambed at least once and their last lambing dates ranged from November 6 to January 23. Ewes were offered a diet of 1.2 kg wheat straw and 0.5 kg concentrate mixture (soybean, corn, bran and barley) per ewe per day. Mineral blocks and water were available on an ad libtium basis.

Experimental Design

Ewes were induced to estrus using a 12-day CIDR-G protocol and randomly allocated into three treatment groups in a completely randomized design 6 days after CIDR-G removal. Each was given a 20 mg i.m injection of $PGF_{2\alpha}$ (lutalyse, Pharmacia and Upjohn n.v./s.a. Puurs, Belgium) on July 2 (day 0, 0 h). Ewes in groups A (n=20) and B (n=19) received a single i.m injection of 500 IU PMSG (Sanofi Animal Health, Libourne Cedex, France) 24 h before and at the time of $PGF_{2\alpha}$ injection, respectively. Ewes in group C (n=18) did not receive PMSG and served as control. Ewes in each group were divided into two subgroups and were either naturally-mated (n=29) or artificially-inseminated (n=28). The naturally-mated or artificially-inseminated ewes were isolated into two separate adjacent pens. Immediately after $PGF_{2\alpha}$ injection, three intact and three aproned Awassi rams were turned-in with naturally-mated and artificially-inseminated (AI) ewes, respectively. Ewes were checked for breeding marks at 6-h intervals for 4 days (Figure 1). The AI group ewes were inseminated 50-56 h following 0 h using the Guelph (T-AI) equipment. Average number of spermatozoa per straw was approximately 500 x 10^6.

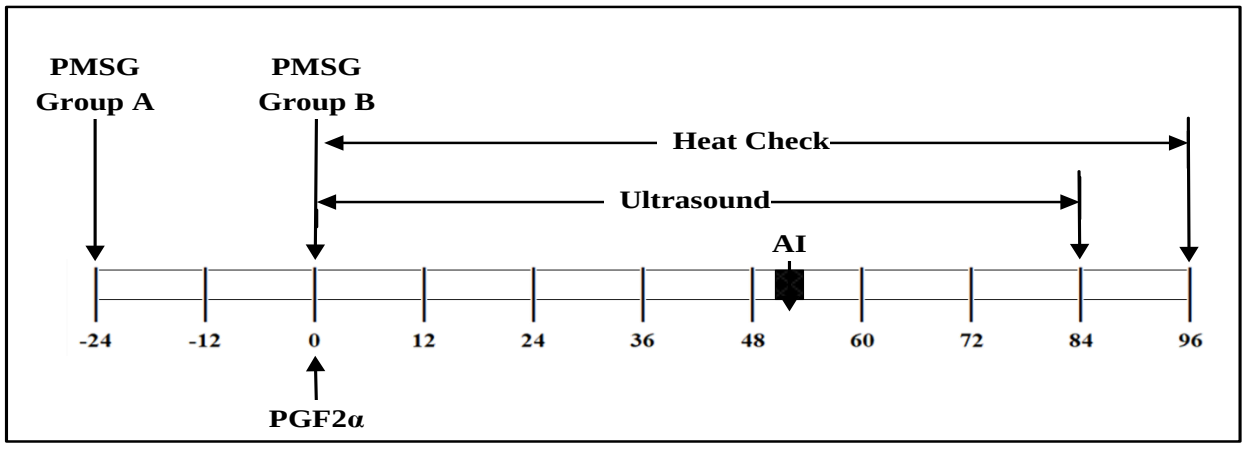

Figure 1. Time line for the treatment groups

Blood Sampling and Progesterone Assay

Blood samples were collected via jugular vein puncture once daily from day −1 until day 6 and on alternate days thereafter until day 20 to verify comparison in progesterone concentrations among treatments and for pregnancy diagnosis. Blood samples (5 ml each) were collected in heparinized tube (5 IU/ml blood) and centrifuged soon after at 3000 rpm for 15 min. Plasma was pipetted and stored at −20 °C until assayed by RIA using Coat-A-Count kit (Diagnostic Products Corporation, DPC, Los Angeles, CA). Sensitivity was 0.1 ng/ml and intraassay CV was 3.4%.

Statistical Analysis

Data were analyzed using SAS/STAT ANOVA procedure (SAS, 2006). Means ± SE are presented in text and tables unless otherwise noted. Effect of PMSG of treatments on incidence of estrus, ovulation, pregnancy, and lambing were analyzed using "Chi square" test. Onset of estrus was considered to have occurred 3 h before observation of the breeding mark. Time to ovulation was considered to have occurred 6 h before disappearance of the large follicles. Effect of PMSG treatments on various intervals were analyzed using least square means (LSM) procedure of the general linear model (GLM). Progesterone levels were analyzed for the effect of treatment and time using the repeated measure procedure of the GLM.

Results

Estrus occurrence did not differ ($P > 0.1$) among groups and was detected in 16/20 (80%), 16/19 (84.2%) and 13/18 (72.2%) ewes in groups A, B and C, respectively. Intervals from $PGF_{2\alpha}$ injection to onset of estrus were shorter ($P < 0.01$) in ewes of groups A (32.4±1.3 h), and B (32.8±1.3 h) than ewes in group C (47.1±1.5 h) (Table 1). Ovulation occurred earlier ($P < 0.01$) in groups A (58.8±2.9 h) and B (61.2±2.9 h) than that (75.0±2.3 h) of group C ewes, with no difference among ewes of groups A and B. Intervals from onset of estrus to ovulation were similar ($P > 0.1$) and averaged 27.9±1.4 h among ewes of the three treatment groups (Table 2).

Table 1. Reproductive responses following $PGF_{2\alpha}$ injection in Awassi ewes treated with PMSG 24 h before (group A), at the time of $PGF_{2\alpha}$ injection (group B) and control (group C)

Parameter	Treatment		
	Group A	Group B	Group C
Number of ewes	20	19	18
Ewes detected in estrus	16 (80%)	16 (84.2%)	13 (72%)
Interval to onset of estrus (h)	32.4 ± 1.3^{a}	32.8 ± 1.3^{a}	47.1 ± 1.5^{b}
Ewes pregnant	11 (55%)	10 (52.6%)	6 (33.3%)
Ewes lambed	4 (20%)	9 (47.4)%	3 (16.7%)

[a, b] Means within row with different superscripts differ ($P < 0.01$)

Table 2. Ovulatory responses following $PGF_{2\alpha}$ injection in naturally-mated Awassi ewes treated with PMSG 24 h before (group A), at the time of $PGF_{2\alpha}$ injection (group B) and control (group C)

	Treatment		
Parameter	Group A	Group B	Group C
Number of ewes	5	5	4
Ewes ovulated	5/5	5/5	4/4
Interval to onset of estrus (h)	31.8 ± 2.4^{a}	32.4 ± 2.4^{a}	47.2 ± 2.7^{b}
Ovulation (h)	58.8 ± 2.9^{a}	61.2 ± 2.9^{a}	75.0 ± 3.2^{b}
Interval from onset of estrus to ovulation (h)	27.0 ± 2.7	28.8 ± 2.7	27.8 ± 3.0
Ovulation rate	1.6 ± 0.2	1.6 ± 0.2	1.0 ± 0.2

[a, b] Means within row with different superscripts differ ($P < 0.01$)
[c, d] Means within row with different superscripts differ ($P < 0.08$)

Number of follicles greater than 5 mm in diameter at 0, 12, and 24 h was similar ($P > 0.1$) among ewes of the three treatment groups. However, number of large follicles at 36 and 48 h was greater ($P < 0.01$) in groups A and B than

ewes in group C. Fewer large follicles were observed at 60 h in groups A and B and no follicles were seen by 72 h. In group C, the number of large follicles dropped after 84 h. Based upon ultrasonic examination, ovulation rate tended (P = 0.08) to be lower in ewes of group C (1.0 ± 0.2) than those of groups A and B (1.6 ± 0.2 and 1.6 ± 0.2, respectively) (Figure 2).

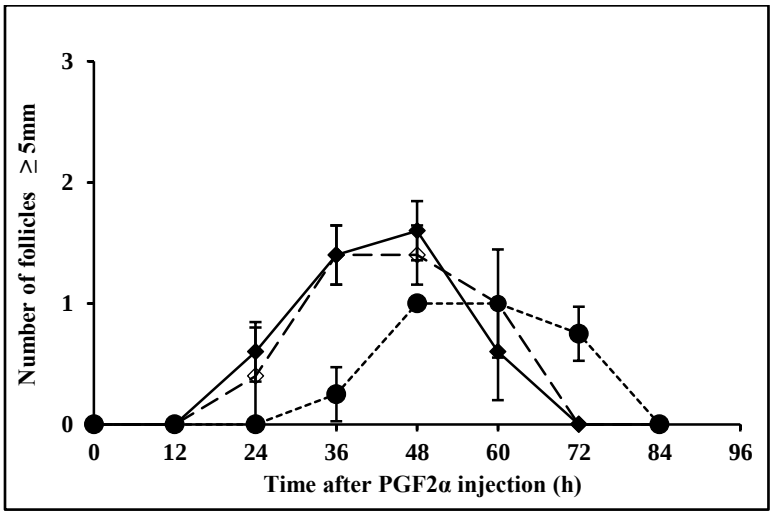

Figure 2. Number of large follicles in groups A (PMSG 24 h before) (♦), B (PMSG at the time) (◊), and C (Control) (●) after PGF2α injection.

Mean plasma progesterone concentrations at the time of $PGF_{2\alpha}$ injection (0 h) were similar (P > 0.5) and averaged 4.2 ± 2.0 ng/ml among ewes of the three treatment groups. Following 0 h, progesterone concentrations rapidly fell in all ewes to ≤ 0.5 ng/ml within 24 h and remained low until day 4. Progesterone concentrations increased gradually after day 4 in all ewes. Apparently, ovulation occurred in all ewes during this period based upon the subsequent rise in progesterone concentrations.

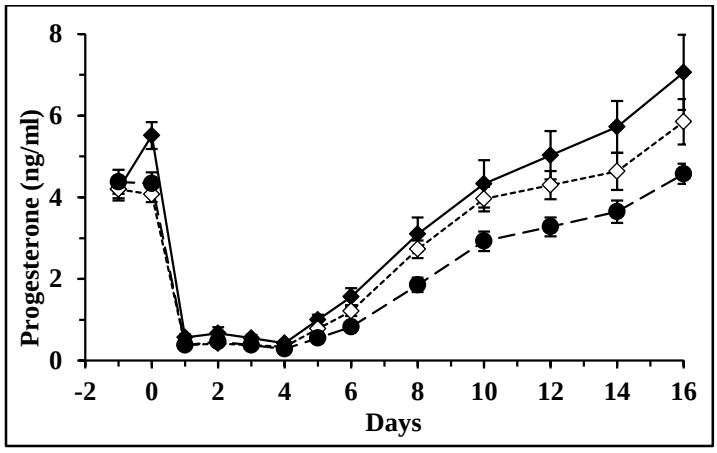

Figure 3. Plasma progesterone concentrations in groups A (PMSG 24 h before, n=20) (♦), B (PMSG at the time, n=19) (◊), and C (Control, n=18) (●) following PGF2α injection until day 16.

Maximum progesterone concentrations were reached on day 16 and averaged 7.1 ± 0.9, 6.0 ± 0.5, and 4.2 ± 0.2 ng/ml for ewes of the three treatment groups (A, B and C, respectively). The PMSG-treated (groups A and B) ewes had higher (P < 0.05) progesterone concentrations between days 4 and 16. Progesterone concentrations remained elevated through day 20 in 11/20 (55%), 10/19 (53 %) and 6/18 (33%) ewes of groups A, B and C, respectively (Table 1). Progesterone concentrations after day 16 dropped spontaneously in 9/20 (45%), 9/19 (47%) and 12/18 (67%) ewes of groups A, B and C, respectively, (Figure 4). After 151 days had elapsed, 4/20 (20%), 9/19 (47%) and 3/18 (17%) of ewes had lambed in groups A, B and C, respectively (Table 1).

Discussion

In the present study, both PMSG treatment 24 h before or at the time of $PGF_{2\alpha}$ injection produced similar estrus responses. Shorter intervals to onset of estrus in these ewes are attributed to faster endocrine responses following PMSG treatment. Earlier endocrine responses in PMSG-treated ewes are the outcomes of earlier estrogen production from the growing follicles (McNatty et al., 1982; Baby et al., 2011; Bartlewski et al., 2011). It is believed that PMSG can markedly increase aromatase activity (Brandt et al., 1988). Similarly, treatment with PMSG was effective in shortening the interval from $PGF_{2\alpha}$ injection to ovulation by about 15 h. These results resembled those reported by Trounson et al. (1976). Quinlivan. (1980) reported that PMSG treatment at the time of progesterone withdrawal resulted in shorter interval to ovulation. Reduced intervals to ovulation have been attributed to the rapid maturation of follicles induced by PMSG prior to the regression of the corpus luteum (Trounson et al., 1976).

Examination of data (Table 2) showed that ovulation occurred in a constant interval from the onset of estrus. Consistency in this interval is due to the fact that LH surge occurs at the onset of estrus (Husein et al., 1997; Shackell et al., 1991; Quirke et al., 1981) as a result of estradiol positive feedback (Khalid et al., 1991) and to the constant interval (21-26) between the preovulatory LH surge and ovulation (Cumming et al., 1973; Baby et al., 2011; Bartlewski et al., 2011).

Treatment with PMSG resulted in earlier appearance of large size (≥5mm) follicles in PMSG- than in non-PMSG-treated ewes. Similar advancement in the time of emergence of large follicles in response to PMSG treatment has been reported (McNatty et al., 1982; Husein et al., 1998a). Advancement in the time of appearance of large follicles has been attributed to the antiatretic effect of PMSG, which results in recruitment of more than one follicle and development of many preovulatory estrogenic follicles from the extent of large follicles pool (McNatty et al., 1982; Junqueira et al., 2019).

Treatment with PMSG at or 24 h before the time of $PGF_{2\alpha}$ injection tended to increase ovulation rates compared to treatment with $PGF_{2\alpha}$ alone. Higher ovulation rates with PMSG treatment have been reported (Henricks and Hill, 1978; Noël et al., 1994; Husein et al., 1998a). This has been attributed to the fact that PMSG has an FSH like activity and results in more follicles to grow and develop (Gordon, 1997a; Baby et al., 2011; Bartlewski et al., 2011).

Mean plasma progesterone concentrations prior to $PGF_{2\alpha}$ injection were similar among ewes of the three treatment groups. Elevated concentrations of progesterone in ewes are a reflection of their cyclicity. After $PGF_{2\alpha}$ injection, concentrations of progesterone fell to ≤ 0.5 ng/ml within 24 h. Thereafter, progesterone concentrations started to increase gradually after day 4. The increase in plasma progesterone concentrations at that time indicates that all ewes had ovulated irrespective to whether or not they exhibited estrus.

Progesterone concentrations in PMSG-treated ewes (Group A and B) were similar during the entire period of the estrous cycle. Progesterone concentrations declined to ≤ 0.5 ng/ml and remained low until day 4. This indicates that $PGF_{2\alpha}$ was effective in inducing luteolysis of corpora lutea, which allowed all ewes to ovulate. After day 4, progesterone concentration started to increase in both groups A and B indicating that new corpora lutea formed and started to secrete progesterone. Progesterone concentrations among ewes of the three treatment groups continued to rise until those reached maximum concentrations on day 16. Higher progesterone concentrations in PMSG-treated ewes (Groups A and B) during the luteal phase has been related to the action of PMSG in advancing the time of ovulation (Jabbour & Evans, 1991) and in producing multiple corpora lutea through increasing ovulation rate (Henricks and Hill, 1978). The overall pregnancy rate was 47.4% for ewes of the three treatment groups and was lower than that reported by Trounson et al. (1976).

Pregnancy rates in this study were higher than those reported earlier in the JUST sheep flock (Husein & Kridli, 2002a, 2002b). Haynes and Haresign (1987) reported that running rams with ewes prior to ram introduction in a breeding program increased the fertility of the ewes. Breeding ewes at the second cycle improved pregnancy rates since the reproductive condition of the ewe at that time may be set better. In addition, ewes treated were of similar stage of the estrous cycle which allows greater proportion of ewes to be in approximately similar or uniform stages of follicular development (Beck et al., 1996).

Uniformity in follicular growth and development enhances pregnancy rate. Proportions of ewes lambed were 20%, 47%, and 17% in groups A, B, and C, respectively. Reasons for overall low lambing rate are due to the fact that half of the ewes were artificially inseminated. Other contributing factors will be discussed in the following part of discussion (subheading 5.5.). Lambing rate in artificially-inseminated ewes was 14.3% compared to 41.4% for the naturally-mated ewes. This could be attributed to the fact that some ewes may have been traumatized due to the T-AI technique.

Conclusion

Results indicate that $PGF_{2\alpha}$ injection during luteal phase (2 to 5 day old corpus luteum) can be effectively used to induce estrus irrespective to whether or not eCG treatment is given. However, eCG administration 24 h before or at the time of $PGF_{2\alpha}$ injection shortened the intervals to onset of estrus and ovulation. eCG treatment resulted in higher progesterone concentrations during luteal phase. Although pregnancy and lambing rate were not influenced by eCG treatment, ewes inseminated near to the time of ovulation had higher pregnancy and lambing rates.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

* This article was presented as an oral presentation at International Conference on Veterinary, Agriculture and Life Sciences (www.icvals.net) held in Antalya/Turkey on November 17-20, 2022.

References

Abdullah, A.Y., Husein, M.Q., & Kridli, R.T. (2002). Protocols for estrus synchronization in Awassi ewes under arid environmental conditions. *Asian-Australasian Journal of Animal Sciences*, *15*(7), 957-962.

Baby, T.E., & Bartlewski, P.M. (2011). Progesterone as the driving regulatory force behind serum FSH concentrations and antral follicular development in cycling ewes. *Reproduction Fertility and Development, 23*(2), 303-310.

Bartlewski, P.M., Baby, T. E., & Giffin, J. L. (2011). Reproductive cycles in sheep. *Animal Reproduction Science,124*(3-4), 259-268.

Chamley, W.A., Buckmaster J.M., Cain M.D., Cerini J., Cerini, M.E., & Goding J.R. (1972). The effect of $PGF_{2\alpha}$ on progesterone, estrogen and LH secretion in seep ovarian transplant. *Journal of Endocrinology, 75*, 253-263.

Gordon, I. (1997). *Controlled reproduction in sheep and goats* (pp. 86-115) (1 st ed.). New York, NY: CAB International.

Husein, M. Q., & Kridli, R. T. (2002a). Reproductive responses of Awassi ewes treated with either naturally occurring progesterone or synthetic progestogen. *Asian-Australasian Journal of Animal Sciences, 15*(9), 1257-1262.

Husein, M. Q., & Kridli, R. T. (2002b). Reproductive responses following royal jelly treatment administered orally or intramuscularly into progesterone-treated Awassi ewes. *Animal Reproduction Science*, *74*(1-2), 45-53.

Husein, M. Q., Ababneh, M. M., Crabo, B. G., &Wheaton, J. E. (1996). Out-of-season breeding of ewes using transcervical artificial insemination. *Sheep & Goat Research Journal, 12*(1), 39-45.

Husein, M. Q., Romano, J. E., Bailey, M. T., Ababneh M. M., Crabo B. G., Godfrey R. W., Head, W. A., &Wheaton, J. E. (1998). Estrus synchronization and pregnancy rate of transcervically inseminated ewes during the breeding season. *Sheep & Goat Research Journal,14*(2), 148-152.

Junqueira, F.B., Souza, J.C., Campos, J.P.A., Faria, L.R., Silva, D.R.D., Ascari, I.J., Lima, R.R., Furusho Garcia, I.F., Nogueira, G.P., & Alves, N.G. (2019). Ovarian follicular development, hormonal and metabolic

profile in prepubertal ewe lambs with moderate dietary restriction and lipid supplementation. *Animal Reproduction Science, 204*,152-164.

Madani, M. O. K., Ali, T. M., Yagoub B. A., & Zawia, M. T. (1984). The influence of $PGF_{2\alpha}$ analogue and PMSG treatment on the lambing performance of Labian fat-tailed ewes. *British Veterinary Journal, 140,* 27-33.

Trounson A. O. Willadsen S. M., & Moor R. M.(1976). Effect of prostaglandin analogue Cloprostenol on oestrus, ovulation and embryonic viability in sheep. *Journal of Agricultural Science (Cambridge) 86*,609-611.

Author Information

Mustafa Hussein
Jordan University of Science and Technology
Irbid, Jordan
Contact e-mail: *huseinmq@just.edu.jo*

Ghaid Al-Rabadi
Mutah University
Al - Karak, Jordan.

Hassan Ghozlan
Jordan University of Science and Technology
Irbid, Jordan

To cite this article:

Hussein, M. Q., Gozlan, H., & Al- Rabadi, G. (2022). The effect of PMSG on follicular development and ovulation time PGF_{2a} treated awassi ewes *The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS), 8,* 7-13.

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)
ISSN: 2791-8033

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS), 2022

Volume 8, Pages 14-27

ICVALS 2022: International Conference on Veterinary, Agriculture and Life Sciences

Response of Morphogenesis and Cell Proliferation to Allelopatic Compounds on Rice Germplasm

Moh Rosyadi ADNAN
State Polytechnic of Jember

Dwi Mai A.I. BUQORI
University of Jember, Jember

Kyung Min KIM
Kyungpook National University

Muhammad Noval KHULUQ
University of Jember, Jember

Muhammad UBAIDILLAH
University of Jember, Jember

Abstract: Allelopathic compounds are chemical substances produced by weeds, including Imperata cylindrica, that can inhibit the absorption of nutrients and cell growth of neighboring plants. Tissue culture technique can be used to determine the effect of media composition on morphogenesis and cell proliferation, as indicated by related genes. Through the process of somatic embryogenesis, cells regenerate into whole plants via several phases initiated by gene expression. Genes are the signal that connects environmental cues and plant cells. The objective of this study was to determine the effect of allelopathic compounds from Imperata cylindrica root extract on the morphogenesis and cell proliferation of indigenous Indonesian rice plant. The used callus was derived from the seeds of Bondoyudo, Caok, Ciliwung and Situbagendit rice varieties. The results of callus induction were selected and transferred to regeneration media that had been treated with I. cylindrica roots extract of 2.5 g/L and 5 g/L. The development of callus was monitored weekly for six weeks. As for the molecular analysis, four-week-old callus was utilized. The results demonstrated that the root extract of Imperata cylindrica had a specific effect on the morphogenesis and proliferation of rice cells depending on concentration and target plant. At a molecular level, the expression of the OsBBM, OsLEA, OsLEC1, OsSERK, and OsWOX4 genes was affected differently by the administration of allelopathic compounds at a concentration of 5 g/L on a molecular level.

Keywords: Allelopathic compounds, Gene expression, Indonesia local rice, Tissue culture, Morphogenesis

Introduction

Tissue culture is the *in-vitro* or aseptic propagation of cells, tissues, or plant organs on artificial media, resulting in the regeneration of entire organs. Initially, tissue culture techniques were used to obtain large quantities of seeds in a short period of time. However, the development of tissue culture techniques has been applied to numerous purposes, including the assembly of high-yielding varieties. According to (Wahyurini 2009), the role of tissue culture techniques is extremely advantageous because it enables the rapid and large-scale production of plants with improved properties that are unaffected by environmental factors. Several factors, including sterile conditions, the composition of the medium, and the use of plant growth regulator (PGR) and suitable explants, have contributed to the success of tissue culture. The optimal combination of basic media and PGR will stimulate cell division during the morphogenesis process. Consequently, tissue culture can be used to determine the interaction between the

- Selection and peer-review under responsibility of the Organizing Committee of the Conference

chemical composition of the media and plant cells. According to (Suparyono & Setyono 1997), tissue culture is an effective and efficient method for determining the response of cells to medium content, primarily because environmental conditions can be controlled in an *in vitro* culture setting. In tissue culture, the composition of the medium influences morphogenesis and proliferation in plants.

Rice (*Oryza sativa* L.) is an important and primary staple crop for a number of nations, including Indonesia. Weeds are a typical problem in rice cultivation (Zarwazi et al., 2016). The presence of weeds on the field will interfere with the growth and development of the rice plants due to competition. Physically, weeds compete with rice plants for nutrients, air, and light as well as growing space, because weeds are capable of growing faster than rice plants. Chemically, the exudate released by weeds inhibits nutrient absorption and cell division. The exudate released by weeds is an allelopathic secondary metabolite. Based on previous research, allelopathic compounds have a profound effect on their targets (Narwal & Sampietro 2009). The presence of allelopathic compounds inhibits the growth and development of plants. One (Menambah pustaka) of the plants that contain allelopathic compounds is reeds, due to the phenolic substances in the plant. Some of the potent allelopathic chemicals were abscisic acid and methyl caffeate which were extracted from cogon grass rhizomes (Suzuki et al., 2015). Another potential allelopathic substance produced by cogon were phenolic and aromatic acid (Hagan et. al., 2013). By preventing nutrient absorption and inhibiting plant cell growth, these substances could stunt the development of other plants (Kurniati et al., 2018). Karmegam et al. (2014) demonstrated that allelopathy could inhibit germination, germination length, total weight, and chlorophyll content in three rice cultivars.

During the phases of morphogenesis and proliferation, the application of allelopathic compounds induces the expression of a number of genes in rice plants. Genes, such as *OsLEA*, *OsLEC1*, *OsSERK*, *OsBBM*, and *OsWOX4* are involved. These genes have an impact on the morphogenesis of rice plant cells. Rice plants that are resistant to allelopathic compounds have a chemical defense mechanism, whereas those that are sensitive to allelopathic compounds lack a chemical defense mechanism, resulting in retardation of growth and development in response to the allelopathic compound (Usman et al., 2016).

Several studies have reported the effect of allelopathic substances on rice plants, but the effect on morphogenesis and cell proliferation levels has not been comprehensively studied. Since the differentiation of explants into plantlets could be precisely followed and analyzed, this phenomenon could be studied using the tissue culture technique of rice callus induction. Therefore, it is possible to use tissue culture method to determine morphogenesis and cell proliferation in response to an allelopathic compound. The objective of this study was to determine the effect of allelopathic substances in the morphogenesis and cell proliferation responses of indigenous rice varieties in Indonesia. This research will provide the foundational knowledge necessary to comprehend the effect of allelopathic substance affects the growth of indigenous rice varieties in Indonesia.

Materials and Methods

Plant Material and Explant Planting

Rice (*Oryza sativa* L). seed varieties of *Bondoyudo*, *Caok*, *Ciliwung*, and *Situbagendit* were used as material in this research. The above-mentioned seeds were surface sterilized using 70% ethanol prior to planting. Our study used 15 rice seeds as explants in each petri dish with two replications. The sterilized rice seeds were then planted on a standard Murashige and Skoog (MS) medium (Macronutrients: NH_4NO_3, KNO_3, $CaCl_2 \cdot H_2O$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4; Iron: Na_2-EDTA, $FeSO_4 \cdot 7H_2O$. Micronutrients: $MnSO_4 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, H_3BO_3, KI, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$) containing 30 g L^{-1} sucrose , 3 g L^{-1} GELRITE agar supplemented with 2 mg/l (2 ppm) 2,4-D (2,4 Doichlorophenoxyacetic acid) (for the callus induction according to Upadhyaya et al. (2015). The medium pH was adjusted to 5.8 prior to autoclaving at 120 ^{O}C and 1.5 atm for 15 minutes. The medium containing explant were maintained at growth chamber at 26 ^{O}C and 20% relative humidity in the dark condition. The seed explant was kept in for two to three weeks to observe callus formation. The weekly examination assessed the percentage of callus formation (%) and morphology of the callus. Visual observations were made using stereomicroscope and the calculations were performed as follows:

$$\text{Callus Induction Percentage} = \frac{\text{Total Number of Callus}}{\text{Number of Explant}} \times 100\%$$

$$\text{Callus size} = \frac{(\text{width}+\text{length})}{2}$$

Callus Regeneration

After two to three weeks of incubation, calli from individual varieties with the best growth performance in the induction media were then transferred into regeneration media to obtain healthy plantlets and intact plant parts consisting of shoots, roots, stems, and leaves that were unharmed. The regeneration medium used MS media with the addition of 2 mg L^{-1} Kinetin and 1 mg L^{-1} NAA. Subsequently, sterilized reed extract was then added to the MS media according to the treatment. The treatments including control, 2.5 g/l, and 5 g/l of reed extract with three replications.

Afterward, we observe the morphological changes of the callus to plantlets. At 2 weeks and 4 weeks of age, the following parameters were observed: callus diameter, percentage of green spot, embryogenic callus morphology (percentage of callus in globular, scutellar, and coleoptile phases), percentage and number of plantlets formed, and plantlet morphology (shoot length, root length, and the number of leaves).

RNA Extraction and Gene Expression Analysis

Callus 4 days after planting (DAP) on regeneration media were used for RNA extraction. Total RNA was extracted from the callus using the *Ribospin™ Plant* kit (GeneAll, Korea). The extracted RNA was used as template to synthesize cDNA using the *ReverTra Ace® qPCR RT Master Mix* kit (Toyobo, Japan). To measure the expression level of target genes (*OsBBM*, *OsLEA*, *OsLEC1*, *OsSERK*, and *OsWOX4*), semi quantitative polymerase chain reaction (PCR) was performed using *GoTaq® Green Master Mix* (Promega, USA*)*. List of primer used in this study was listed in Table 1. The PCR products were then separated in a 2% agarose gel stained by EtBr and visualized with a UV transilluminator (Bio-Rad, Germany). The gel image of DNA bands were then captured and classified according to their thickness.

Table 1. A list of primers used in semi quantitative RT-PCR

Gene	Primer	NCBI reference sequence
OsSERK	Forward: 5’ TGC ATT GCA TAG CTT GAG GA 3’ Reverse: 5’ GCA GCA TTC CCA AGA TCA AC 3’	XM_015794373.2
OsWOX4	Forward: 5’ CGC TAA CGA AAC CAA AGA GG 3’ Reverse: 5’ GGA AGA GCT CCA GGG TCA CT 3’	XM_015779881.2
OsLEC1	Forward: 5’ CGT CGG TGG GAT GCT CAA GTC 3’ Reverse: 5’ GGT GCT CGA AGT TGA CGG TCT 3’	XM_015769434.2
OsBBM	Forward: 5’ CGA TTT ACC GTG GCG TGA CA 3’ Reverse: 5’ CGT GAA GAG CAT CCT GGA CA 3’	XM_026019980.1
OsActin	Forward: 5’ TCC ATC TTG GCA TCT CTC AG 3’ Reverse: 5’ GTA CCC GCA TCA GGC ATC TG 3’	XM_015774830.2

Experimental Design and Statistical Analysis

This research employed a factorial Completely Randomized Design (CRD) method with two treatment factors and two replications. The first factor was rice varieties (*Bondoyudo*, *Caok*, *Ciliwung*, *Situbagendit*). The second factor was allelopathic treatment (control, 2.5 g/l, and 5 g/l) with three replications. The data obtained from the observations included both qualitative and quantitative data. Qualitative data were analyzed descriptively, whereas quantitative data were analyzed using ANOVA; if the results obtained were significantly different, further analysis was conducted using Duncan's Multiple Range Test (DMRT) with a confidence level of 95%.

Results and Discussions

Callus Induction

Induction of callus plays a crucial step in rice plant propagation. To regenerate into plantlets, callus must be of high quality. A high-quality callus that has a greater potential to regenerate into plantlets callus called embryonic callus. Before being transferred to regeneration media, the callus obtained from induction media must undergo a selection phase. Induction media (MS medium containing 2,4-D 2 mg/L) containing the explants were kept in a growth chamber at 27°C in the dark condition. Then, on day seven, all explants were injured and began to expand within the embryo. 14 days after planting (DAP), induction of callus was observed.

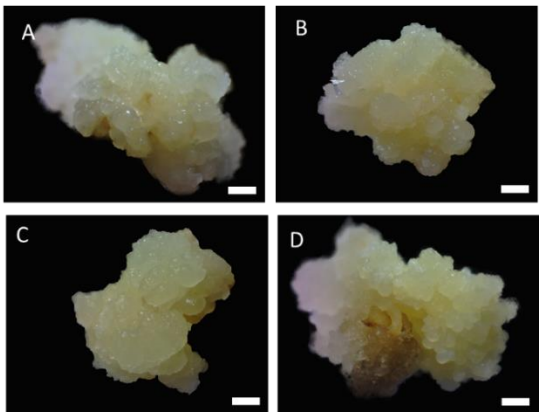

Figure 1**.** Callus morphology of Indonesian local rice on MS medium containing 2,4-D 2 mg/L at 14 days after planting (DAP). (Bar= 1 mm). A: *Bondoyudo*, B: *Caok*, C: *Ciliwung*, D: *Situbagendit*. (Bar scale = 1mm).

Figure 1 depicts the calli morphology at 14 days after planting the seed from *Bondoyudo*, *Caok*, *Ciliwung*, and *Situbagendit* rice as explants. The produced callus appear yellowish-white color at 14 DAP. Callus has a crumbly appearance due to its brittle nature and nodule-covered surface. This observation follows Minarsih et al. (2016), who state that an embryogenic callus has crumbly, nodular, and yellowish-white visual characteristics. Compared to a non-embryogenic callus, an embryogenic callus has a greater ability to regenerate into plantlets, making it an ideal explant for regeneration. In this study, we discovered that the addition of 2,4-D hormone to Murashige and Skoog (MS) medium at a concentration of 2 mg/L is an effective method for inducing embryogenic callus formation in *Bondoyudo*, *Caok*, *Ciliwung*, and *Situbagendit* Rice.

The percentage of explants that were successfully induced to form callus was determined for 14-day-old calluses in the induction medium (Table 2). The percentage was calculated by dividing the number of produced calli by the number of explants planted and multiplying the result by 100%. The callus diameter was then measured using the *Imager Raster* and *Optilab* to determine its size.

Table 2. Percentage and diameter of callus formation on MS media containing 2,4-D 2 mg/L at 14 days after planting

Varieties	Callus induction percentage (%)	Diameter callus (mm)
Bondoyudo	73.5 ± 4.32^{a}	6.61 ± 0.15^{b}
Caok	58.50 ± 0.97^{b}	8.2 ± 0.04^{a}
Ciliwung	$59.89 \pm 2{,}76^{b}$	6.45 ± 0.6^{b}
Situbagendit	51.20 ± 4.16^{b}	8.22 ± 0.13^{a}

Note: Numbers followed by the same letter showed an insignificant difference on 5% DMRT test

The statistical analysis of the percentage of callus induction variables yielded significantly different results, as shown in Table 2. *Bondoyudo* Rice exhibited the highest percentage of callus formation (73.5%), whereas *Situbagendit* Rice exhibited the lowest percentage (51.2 percent). Similarly, the results for the callus diameter variable varied significantly. *Situbagendit* rice produced the largest callus diameter (8.22 mm), followed by *Caok* Rice (8.2 mm), *Bondoyudo* Rice (6 mm), and *Ciliwung* Rice (6 mm) (6.45 mm). The differences in callus formation response showed by rice varieties might reflect several factors that influence the callus induction including the genotype and composition of the media used (Michel et al., 2008).

Bondoyudo rice responded better in callus formation than the other three rice varieties, although the resulting callus diameter was smaller in comparison to Caok and Situbagendit. Caok, Ciliwung, and Situbagendit shared a similar

lower percentage of callus formation in comparison to Bondoyudo rice. The callus diameter also varies among the rice varieties. The callus diameter of Bondoyudo and Ciliwung shared an almost similar size of ± 6 mm, whereas Caok and Situbagendit shared a larger diameter of ± 8.2 mm. Among the 4 rice varieties, Ciliwung rice has a relatively lower percentage of callus formation and callus diameter. This phenomenon is presumably linked to the genotype of each rice variety. Furthermore, each rice seed used as an explant was already accompanied by endogenous growth regulators. The application of the exogenous growth regulator may interact with the plants' endogenous growth regulator. This collaboration of exogenous and endogenous growth regulators result in the formation of specific organs or tissues when exogenous growth regulators are introduced to shape the direction of culture development. Sari et al. (2014) report that the administration of the 2,4-D hormone promoted callus morphogenesis.

Callus Regeneration

The calli obtained at the induction stage were transferred to regeneration media supplemented with the hormones NAA 1 mg/L and Kinetin 2 mg/L, as well as alang-alang root extract as a treatment. There were three types of treatment: P0 as control or without the addition of cogon grass root extract, P1 with the addition of cogon grass root extract 2.5 g/L, and P2 with the addition of alang-alang root extract 5 g/L. To replenish the nutrients in the media, media replacement occurs every two weeks. The transferred calli were incubated for 16 hours of light at 27 ^{o}C (room temperature). During a six-week period, the morphology of a callus was monitored weekly. In the first two weeks, the induced calli were transferred to regeneration media using a Petri dish and at the following week, they were transferred to regeneration media using a tube to facilitate observation of the newly formed plantlets.

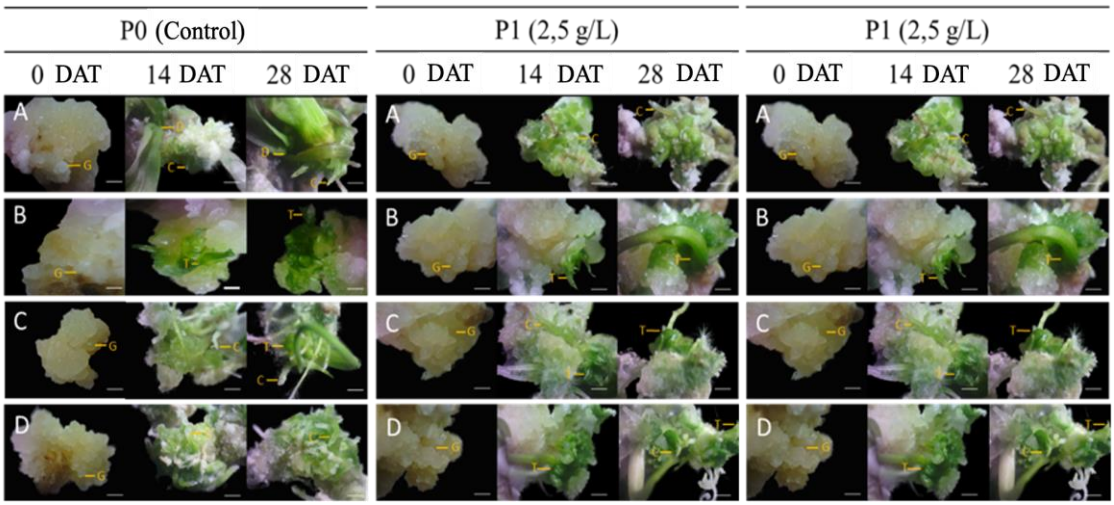

Figure 2. Morphological changes of rice calli after 14 and 28 days grown on the MS medium containing NAA 1 mg/L and Kinetin 2 mg/L. The addition of alang 3a'ytrsIUYTREW-alang extract of P0 = 0 g/L, P1= 2.5 g/L, and P2= 5 g/L. A:*Bondoyudo*, B:*Caok*, C:*Ciliwung*, D: *Situbagendit.*

Plant regeneration can be obtained by somatic embryogenesis, a formation of embryonic cells from somatic cells. Somatic embryogenesis consists of two phases; the induction phase, during which differentiated somatic cells acquire embryogenic competence and proliferate as embryogenic cells; and the expression, during which the embryogenic cells express their embryogenic competence and differentiate into somatic embryos. After the embryogenic induction is completed, the next stages are the globular, scutellar, and coleoptile stage for monocot plants (Mastuti, 2017).

There are several stages of embryogenesis in monocotyledonous plants such as rice, including: pro-embryo, globular, scutellar, and coleoptile. Figure 2 showed the morphological changes 0, 14, and 28 DAP of callus grown on P0, P1, and P2. When the callus was transferred from the induction medium, the surface of each explant was a shiny and dry nodule. This indicated that the callus had reached the pro-embryo mass (PEM) stage. After 4 days of incubation in regeneration media, all local Indonesian rice callus developed green spots, with the exception of *Situbagendit* rice on media treated with 2,5 g/L (P2). Greenspot signified an essential stage in tissue culture because it is an early indicator of callus regeneration (Artadana et al., 2017).

The progression of green spot formation depends on the viability and regeneration of the calli used. At the time of induction, the callus used was two weeks old and was not subcultured. According to Minarsih et al (2016), the long-

term use of 2,4-D hormone causes genetic variations and inhibits regenerative capacity of plants. In general, young explants from meristematic tissue that have undergone dedifferentiation are easier to regenerate into plantlets than older explants (Morrish et al., 1987).

On 14 DAP, callus planted on control media (P0) of all rice emerged green and had entered the coleoptilar phase, characterized by the elongation of the callus surface nodules. *Bondoyudo* rice even demonstrated a marginally superior performance, as it has been successful in producing plantlets with the appearance of leaves. All rice varieties produced shoots when treated with alang-alang root extract at 2.5 g/L (P1) concentration. *Bondoyudo* rice has entered the coleoptilar phase in a solution containing 5 g/L cogon grass root extract (P2). 14 days after planting, *Caok* rice grew more slowly and remained in the scutellar stage. This stage is characterized by the formation of a liver-like cell mass in which the cell's center is lower and the right side left protrudes due to faster cell division (Yadav et al., 2020). *Ciliwung* rice has successfully produced shoots at 14 DAP. In the meantime, the *Situbagendit* rice callus had entered the coleoptilar phase despite the absence of green spots and the occurrence of the albino phenomenon.

Without alang-alang root extract (P0), *Bondoyudo* and *Ciliwung* rice produced shoots after 28 DAP. However, neither *Caok* nor *Situbagendit* rice had yet sprouted. Nonetheless, the growth progression of *Caok* and *Situbagendit* rice improved over the previous fourteen days. In contrast, all explants that were exposed to 2.5 g/L of cogon grass root extract (P1) developed shoots. Under the treatment with 5g/L cogon grass root extract, *Bondoyudo*, *Caok*, and *Ciliwung* were successful in producing shoots, whereas *Situbagendit* failed to produce shoots. The calli of *Situbagendit* rice appeared albino and underwent browning in certain areas.

The response of callus from different rice varieties to treatment with cogon grass root extract during plantlet formation was variable (Figure 2). The genetic factor of the rice calli and the concentration of the cogon grass root extract may affect this result. Compared to the control (P0), the administration of 2.5 g/L (P1) of cogon grass root extract showed no significant difference. However, callus treated with 5 g/L of cogon grass root extract demonstrated a slower regeneration process. According to Yulifrianti et al. (2015), allelopathic compounds inhibit the absorption of nutrients in the medium and the division of plant cells. In addition to its inhibitory effect, a concentration of 5 g/L of cogon grass root extract caused the callus of *Situbagendit* rice to become albino. Albinism is caused by DNA damage in the plastid or nucleus, or by the addition of chemical compounds to the media (Sun et al., 1979).

Table 3. Percentage of callus greenspots during the second and fourth weeks of treatment

Varieties	Treatment	Green Spot Percentage(%)	
		2nd week	4th week
Bondoyudo	P0	91.67 ± 8.33^{a}	100 ± 0
	P1	91.67 ± 8.33^{a}	100 ± 0
	P2	83.33 ± 8.33^{a}	100 ± 0
Caok	P0	91.67 ± 8.33^{a}	100 ± 0^{a}
	P1	91.67 ± 8.33^{a}	100 ± 0^{a}
	P2	83.33 ± 16.67^{a}	91.67 ± 8.33^{a}
Ciliwung	P0	91.67 ± 8.33^{a}	100 ± 0^{a}
	P1	91.67 ± 8.33^{a}	91.67 ± 8.33^{a}
	P2	91.67 ± 8.33^{a}	91.67 ± 8.33^{a}
Situbagendit	P0	91.67 ± 8.33^{a}	100 ± 0^{a}
	P1	91.67 ± 8.33^{a}	100 ± 0^{a}
	P2	0^{b}	8.33 ± 8.33^{b}

Note: Numbers followed by the same letter showed an insignificant difference at 5% DMRT test

The two-week-old calli were transferred to the regeneration media containing various concentrations of allelopathic compounds and incubated for six weeks at 16/8 hours of irradiation (light/dark). During the second and fourth week, observations were performed regarding green-spotted callus. The percentage of green spots in the second week is displayed in Table 3. In the second week, all rice in the P0 (control) and P1 (2.5 g/L) treatments produced 91.67 percent green spots. However, the P2 (5 g/L) treatment reduced green spot appearance on *Bondoyudo* and *Caok* rice to 83.33 percent, whereas *Situbagendit* rice had not yet entered the green spot phase. In contrast, the administration of allelopathic compounds of cogon grass root extract on P1 (2.5 g/L) and P2 (5 g/L) in *Ciliwung* rice had no effect on the formation of green spots at 2 weeks of age. In the fourth week, the treatment of *Bondoyudo* rice with allelopathic compounds of cogon grass root extract at concentrations of 2.5 g/L (P1) and 5 g/L (P2) did not differ from the control (P0). They have all successfully progressed to the green spot phase. This result suggests that the allelopathic compounds affect the formation of green spot differently among the rice varieties. This effect of cogon

grass root extract was likely to be slightly concentration-dependent as seen in *Bondoyudo*, *Caok*, and *Situbagendit*, especially during 2 weeks of culture. *Ciliwung*, on other hand displayed no apparent effect during 2 weeks of culture due to the given treatments. In addition, the effect of P1 and P2 in *Bondoyudo* also appears to be diminished on the 4 weeks of culture. This finding highlights the response and sensitivity or tolerance differences shown among 4 rice varieties.

Treatment of *Caok* Rice with 2.5 g/L cogon grass root extract (P1) had no effect on the formation of green spots; however, treatment with 5 g/L cogon grass root extract (P2) reduced the green spot phase by in to 91.6%. The allelopathic compounds applied at P1 (2.5 g/L) and P2 (5 g/L) in *Ciliwung* Rice could reduce the green spot phase by 91.67 percent. The P1 treatment had no effect on *Situbagendit* Rice, but the P2 treatment drastically reduced the green spot phase by up to 8.33%. The addition of 5 g/L allelopathic compounds to *Situbagendit* callus caused the callus to become albino. The presence of chemical compounds in the medium causes damage to the nucleus or plastids in cells, preventing the formation of green spots in an albino callus (Sun et al., 1979).

Due to photosynthesis induction in the regenerated callus by light exposure, the regenerated calli can produce green spots. It is important to observe the formation of green spots because they are frequently used as an indicator of plant regeneration. The purpose of the plant regeneration parameter is to determine the number of plantlets that will develop from the regenerated callus (Nabors et al., 1982).

Table 4. Percentage of calli that entered globular, scutellar, and coleoptilar phases (%)

Varieties	Treatment	2 weeks			4 weeks		
		Globular	Scutellar	Coleoptilar	Globular	Scutellar	Coleoptilar
Bondo-yudo	P0	0	6.67 ± 6.67	93.33 ± 6.67	0	0	100 ± 0
	P1	0	13.33 ± 6.67	86.67 ± 6.67	0	6.67 ± 6.67	93.33 ± 6.67
	P2	0	20 ± 11.55	80 ± 11.55	0	13.33 ± 6.67	86.67 ± 6.67
Caok	P0	0	6.67 ± 6.67	93.33 ± 6.67	0	0	100 ± 0
	P1	0	6.67 ± 6.67	93.33 ± 6.67	0	0	100 ± 0
	P2	0	26.67 ± 17.64	73.33 ± 17.64	0	20 ± 11.55	80 ± 11.55
Ciliwung	P0	0	13.33 ± 6.67	86.67 ± 6.67	0	6.67 ± 6.67	93.33 ± 6.67
	P1	0	13.33 ± 6.67	86.67 ± 6.67	0	6.67 ± 6.67	93.33 ± 6.67
	P2	0	20 ± 0	80 ± 0	0	13.33 ± 6.67	86.67 ± 6.67
Situ-bagendit	P0	0	33.33 ± 33.33	93.33 ± 6.67	0	0	100 ± 0
	P1	0	13.33 ± 13.33	86.67 ± 13.33	0	6.67 ± 6.67	93.33 ± 6.67
	P2	0	20 ± 11.55	73.33 ± 6.67	0	13.33 ± 13.33	80 ± 11.55

Note: Numbers followed by the same letter showed an insignificant difference at 5% DMRT test

In Table 4, the percentage of callus that entered the globular, scutellar, and coleoptile phases within two and four weeks of subcultivation on regeneration media is displayed. The globular phase is characterized by a spherical shape, which is followed by the formation of a scutellar phase embryo, which is the transitional phase to coleoptile, or the first young shoots to emerge (Zhao et al., 2017).

The induced callus is embryogenic and has reached the pro-embryonic mass phase. One day after being transferred to callus regeneration media, the callus developed and entered the globular phase. All calluses at 2 weeks of age had entered the scutellar and coleoptile phases. Even the percentage of callus that had entered the coleoptile phase was greater than the percentage of callus that had entered the scutellar phase. At four weeks of age, the callus has developed and a greater proportion of callus has entered the coleoptilar phase. According to Table 3, *Bondoyudo* and *Situbagendit* treated with allelopathic compounds of cogon grass root extract grew more slowly. The phase of callus development appeared to be more impeded as the concentration of allelopathic compounds increased. In contrast, the growth of *Ciliwung* and *Caok* rice treated with 2.5 mg/L cogon grass root extract (P1) did not differ from that of rice untreated with allelopathic compounds (P0). However, a concentration of 5 g/L cogon grass root extract (P2) prevented callus formation.

The application of allelopathic compounds had an effect on the explants proportional to the concentration level and the target plant. Table 4 reveals that 2.5 g/L of cogon grass root extract (P1) exhibited allelopathic properties in *Bondoyudo* and *Situbagendit* rice, but showing no effect on *Caok* and *Ciliwung* rice compared to the control (P0) treatment. At a concentration of 5 g/L, allelopathic compounds inhibited the callus development phase in *Bondoyudo*, *Caok*, *Ciliwung*, and *Situbagendit* rice. The allelopathic compounds found in plant cells can inhibit cell division and differentiation, thereby impeding the regeneration process (Cheng & Cheng 2015).

Table 5. Rice callus proliferation at 2 and 4 weeks

Varieties	Treatment	Callus Diameter (mm) 2-week	4-week
Bondoyudo	P0	7.10 ± 0.05[b]	8.14 ± 0.06
	P1	6.66 ± 0.19[a]	8.01 ± 0.10
	P2	6.43 ± 0.25[ab]	7.77 ± 0.11
Caok	P0	7.52 ± 0.06	8.60 ± 0.05
	P1	7.89 ± 0.02	8.65 ± 0.12
	P2	7.19 ± 0.06	7.67 ± 0.08
Ciliwung	P0	7.13 ± 0.09	8.28 ± 0.08
	P1	6.82 ± 0.28	7.83 ± 0.05
	P2	6.42 ± 0.05	6.78 ± 0.04
Situbagendit	P0	7.11± 0.05	8.03 ± 0.17
	P1	7.27± 0.07	8.19 ± 0.01
	P2	6.58 ± 0.14	7.71 ± 0.08

Note: Numbers followed by the same letter showed an insignificant difference at 5% DMRT test

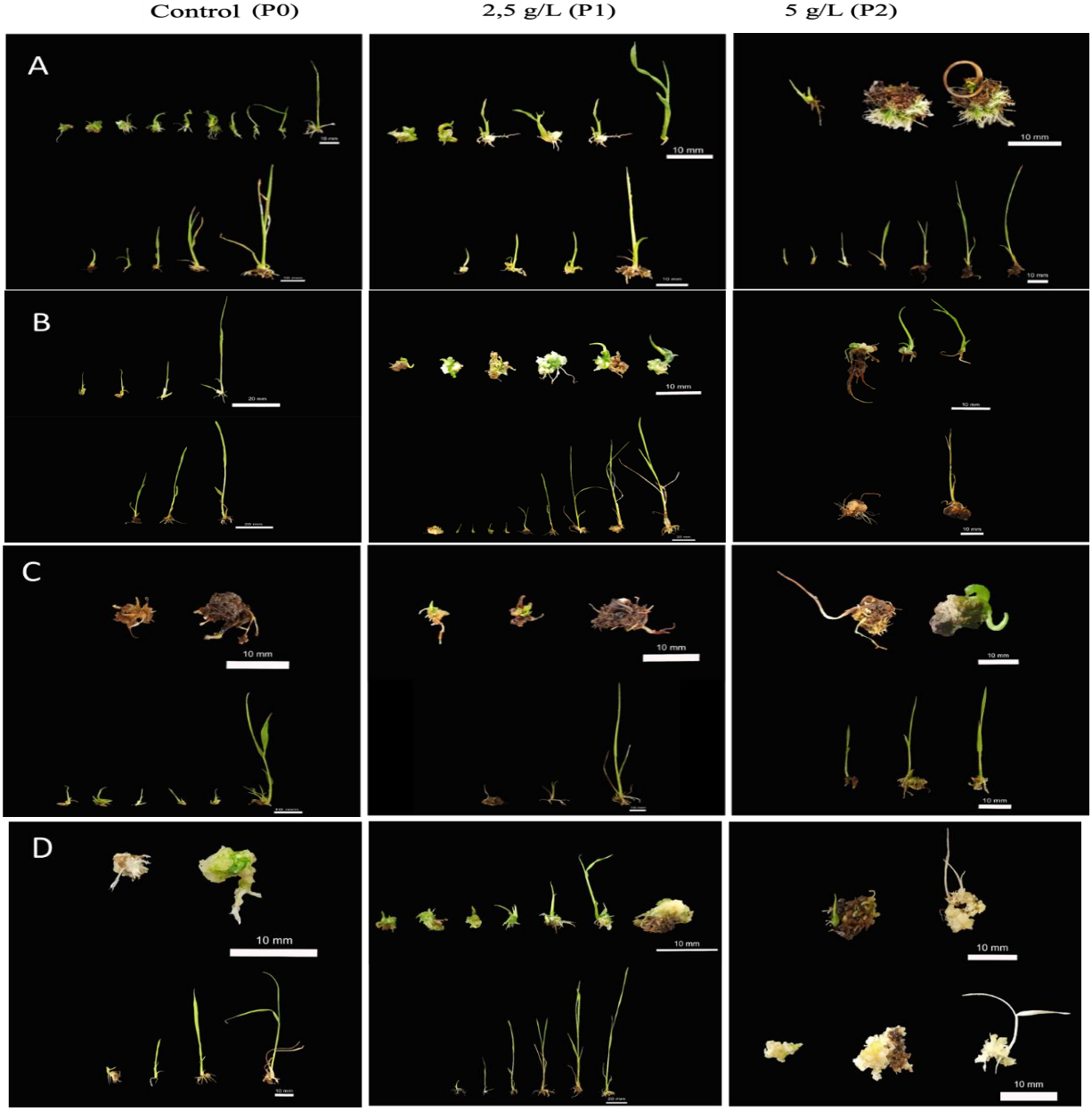

Figure 3. Morphological changes of plantlet emerged from calli at individual explant 42 days of age grown on the MS medium containing NAA 1 mg/L and Kinetin 2 mg/L. The addition of cogon grass root extract into the media were applied as P0 = 0 g/L, P1= 2.5 g/L, and P2= 5 g/L. A: *Bondoyudo*, B: *Caok*, C: *Ciliwung*, D: *Situbagendit*.

Proliferation is the cumulative result of an individual's cell cycle. Proliferation is the capacity of cells to divide, resulting in an increase in cell number. Typically, cells proliferate to increase their number or to replace dead cells. In addition to cell differentiation and death, cell proliferation is a growth and development determinant in plants (Novikova et al., 2013). In the *In vitro* culturing condition, proliferation is tightly regulated by the composition of the culture medium. There are chemical compounds in the medium that can either inhibit or stimulate plant cell proliferation.

It is demonstrated in Table 5, the administration of allelopathic compounds in *Bondoyudo* rice produced a highly significant effect in the second week, with the concentration of allelopathic compounds decreasing callus proliferation. The administration of allelopathic compounds to *Caok*, *Ciliwung*, and *Situbagendit* rice was not statistically significant, but it was observed that the higher the concentration of allelopathy compounds, the more they inhibited the process of cell proliferation. In the fourth week, the progression of callus development in terms of cell proliferation is indicated by an increase in callus diameter over the previous two weeks. Similar to the second week, the proliferation decreased in the fourth week due to the presence of allelopathic compounds.

Table 6. Percentage of plantlet formation during 42 days of age grown on regeneration media with the addition of cogon grass root extract

Varieties	Treatment	Percentage of Plantlet Formation (%) *	Total Plantlet **
Bondoyudo	P0	100 ± 0^{a}	15
	P1	100 ± 0^{a}	9
	P2	75 ± 11.18^{b}	8
Caok	P0	100 ± 0^{a}	6
	P1	100 ± 0^{a}	12
	P2	75 ± 7.91^{b}	3
Ciliwung	P0	50 ± 7.91^{a}	6
	P1	50 ± 13.69^{a}	4
	P2	50 ± 13.69^{a}	3
Situbagendit	P0	100 ± 0^{a}	5
	P1	100 ± 0^{a}	12
	P2	70 ± 9.35^{b}	2

*Average percentage of callus that produced plantlet. Number followed by the same letter showed an insignificant difference at DMRT test 5%. ** Number of plantlets produced from callus

According to Table 6, all explants from the varieties utilized in this study are capable of regenerating into plantlets regardless of treatment. This indicates that the selected explants can be cultured because they have a high capacity for regeneration and plantlet formation. However, when treated with cogon grass root extract at a concentration of 5 g/L (P2), plantlet formation appeared to decrease.

A 100 percent of the callus of *Bondoyudo* rice in the control treatment (P0) produced 15 plantlets, indicating the formation of plantlets. In the presence of 2.5 g/L of cogon grass root extract (P1), all calli were able to produce healthy plantlets. In the subsequent treatment with 5 g/L (P2), 75% of callus has been successfully generated the *Caok* rice in the control treatment (P0) produced six plantlets. In the 2.5 g/L (P1) treatment, all calli generated 12 plantlets. In contrast, the 5 g/L (P2) treatment successfully induced plantlet development in 75% of the planted callus In each treatment, 50% of *Ciliwung* Rice explants produced plantlets. The control treatment produced six plantlets, P1 produced four plantlets, and P2 produced three plantlets. On the control (P0) media, *Situbagendit* rice explants have a 100 percent callus formation rate 75 percent fewer plantlets were produced in the 5 g/L (P2) treatment, namely two plantlets.

The effect of cogon grass root extract concentration on callus affects the proportion of plantlet formation. The higher the concentration, the lower the percentage of plantlet formation, although no effect was found on the formation of plantlets in *Ciliwung* rice. Rice explants also reacted differently to cogon grass root extract. *Bondoyudo* and *Ciliwung* rice responded negatively to an increased plantlet. The greater the concentration, the fewer plantlets are produced. In *Caok* and *Situbagendit* rice, the 2.5 g/L of cogon grass root extract produced a greater total number of plantlets than the control treatment. At 5 g/L of cogon grass root extract, however, the total number of plantlets drastically reduced. The differential effect of allelopathic compounds on plantlet formation was influenced by the target plant's concentration and sensitivity (Gniazdowska et al., 2015). According to Rice (2012), allelochemicals can either inhibit or promote the plant growth, depending on certain concentrations. This was supported by a study by Yar et al. (2020) that low concentrations of allelochemicals can stimulate germination and plant growth.

Table 7. Length of shoot, length of root, and number of leaves of plantlets from each variety from different treatments

Varieties	Treatments	Length of shoot (mm)*	Length of root (mm)*	Number of leaves**
	P0	75.99 ± 10.65a	10.67 ± 0.78a	27
Bondoyudo	P1	43.92 ± 4.55b	8.71 ± 0.66ab	15
	P2	38.8 ± 10.03b	7.57 ± 1.24b	10
	P0	81.69 ± 10.87ab	10.77 ± 1.48a	19
Caok	P1	89.77 ± 26.31a	15.25 ± 2.23a	26
	P2	32.39 ± 5.27b	9.49 ± 2.13a	9
	P0	118.34 ± 3.46a	10.74 ± 1.14a	14
Ciliwung	P1	70.58 ± 22.29b	10.37 ± 1.82a	7
	P2	29.20 ± 5,61c	8.36 ± 1.4a	6
	P0	38.96 ± 11.67b	7.60 ± 0.38ab	15
Situbagendit	P1	106 ± 33.06a	12.78 ± 3.20a	28
	P2	18.34 ± 1.36b	4.24 ± 0.35b	2

*Average percentage of callus that produced plantlet. Numbers followed by the same letter showed an insignificant difference at 5% DMRT test.**Number of leaves that produced in plantlet

Table 7 demonstrates that each type of rice grown on regeneration media responded differently to the treatments. *Bondoyudo* rice responded negatively to shoot length, root length, and leaf number. The shoot length, root length, and number of leaves produced decrease as the concentration of treatments increases (P1 and P2). The length of the shoot was 75.99 mm at the control medium (P0), 43.92 mm at the P1 medium, and 38.8 mm at the P2 medium. P0 root length was 10.67 mm, P1 root length was 8.71 mm, and P2 root length was 7.57 mm. Following a similar trend, the leaves of *Bondoyudo* rice grown in the control (P0), P1, and P2 medium produced 27, 15, and 10 leaves, respectively. Figure 3 demonstrates that the plantlets formed on P2 media emerged from a browning callus, resulting in inferior growth of shoots and number of leaves compared to P0 and P1 media. Nevertheless, *Bondoyudo* callus grown on P2 showed a dense root growth. *Bondoyudo* variety appears to be sensitive to the treatment of cogon root extract in a concentration-dependent manner.

Caok rice grown in P1 media shown a positive effect on shoot length (89.77 mm), root length (15.25 mm), and leaf number (26), compared to control media (81.69 mm, 10.77, and 19 leaves). Meanwhile, the effect of P2 in the callus growth seems to be showing an inhibitory effect. The shoot length, root length, and number of leaves were decreased in comparison to P0 and P1. Moreover, observation of *Caok* callus grown for 42 days revealed that on P2 media, callus exhibited browning and denser root growth than on control (P0) and P1 media (Figure 3). *Caok* variety shown a stimulatory response upon the P1 treatment and inhibitory response upon the P2 treatment.

Ciliwung rice grown in the control medium had the highest values for shoot length (118.34 mm), root length (10.74 mm), and number of leaves (14 strands). Although P1 showed a decrease in comparison to P0, P2 media showed the lowest values for shoot length (29.2 mm), root length (8.36 mm), and number of leaves (6). This indicates the sensitivity shown by *Ciliwung* variety upon the treatment of cogon root extract in a concentration-dependent fashion. Based on plantlet morphology, there were several browning calli in each treatment medium after 42 days. This result may be associated by the endogenous factors working within the rice callus

In comparison to control media, *Situbagendit* rice grown in P1 medium produced the longer shoots (106 mm), roots (12.76 mm), and most leaves (28 strands). The P2 medium yielded the shortest shoot length of 18.34 mm, the shortest root length of 4.24 mm, and few of leaves. Based on the morphology of the callus, *Situbagendit* grown in P2 medium produced white (albino) callus and some browning callus. Albino callus resulted in the development of albino plantlets. Albino is an abnormal condition in which plant cells cannot produce chlorophyll pigment and disrupt the chloroplast membrane's differentiation process. Therefore, albinism causes insufficient photosynthesis and accelerates plant mortality (Kumari et al., 2009).

Gene Expression

In this study, the regulatory genes, such as *OsBBM*, *OsLEA*, *OsLEC1*, *OsSERK*, and *OsWOX4* were analyzed during the development of somatic embryogenesis using the thickness of the band obtained from electrophoresis and PCR results, visualized with a UV-transilluminator. The band was the results of 28-day-old callus RNA from *Bondoyudo*, *Caok*, and *Ciliwung* rice on the regeneration media. Because the callus had visually or morphologically matured after 28 days, it was easier to compare gene expression and morphology.

During the development of somatic embryogenesis, several regulatory genes, including *OsBBM*, *OsLEA*, *OsLEC1*, *OsSERK*, and *OsWOX4*, were analyzed in this study using the band thickness obtained from electrophoresis and PCR results visualized using a UV-transilluminator. The bands were obtained from is the identified RNA of *Bondoyudo*, *Caok*, and *Ciliwung* rice callus grown on the regeneration media for 28 days. The 28-day-old callus was utilized as it was easier to compare the gene expression with callus morphology.

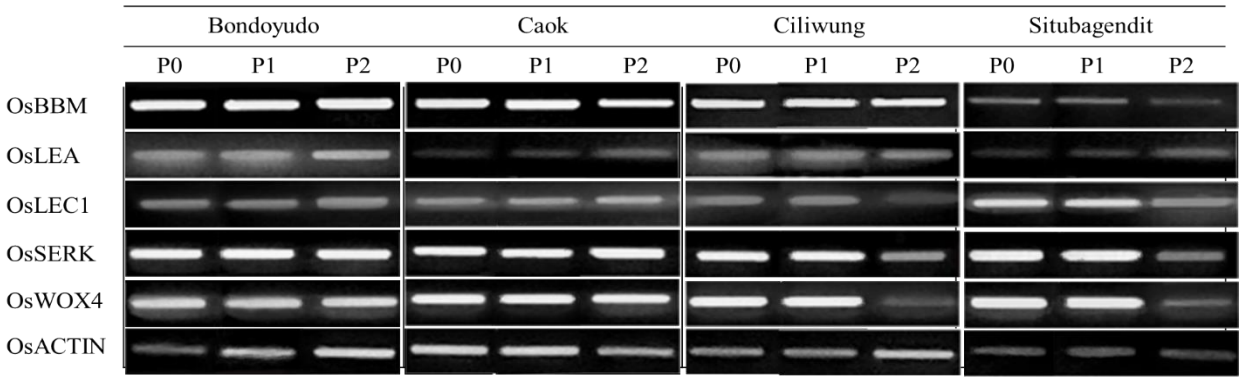

Figure 4. The gene expression of *OsBBM*, *OsLEA*, *OsLEC1*, *OsSERK*, *OsWOX4*. The expression of housekeeping genes, i.e. *OsACTIN*, was used as a reference. RNA used for gene expression analysis were harvested from callus grown for 28 days on the regeneration media.

Our study reveals that the treatment of 2.5 g/L (P1) and 5 g/L (P2) of cogon grass root extract had no effect on the expression level of *OsBBM* gene in *Bondoyudo*, *Caok*, and *Ciliwung* rice. In contrast, the expression of *OsBBM* gene in the *Situbagendit* rice was significantly lower in both control (P0) and treatment (P1 & P2) than in the other three rice varieties. The effect of cogon grass root extract on the expression of *OsBBM* was observed at a concentration of 5 g/L (P2). The band was significantly less intense than P0 and P1. Rice *BABYBOOM* (Os*BBM*) gene regulates somatic embryo induction, cell differentiation, and plant development (Jha & Kumar 2018). *OsBBM* stimulates the expression of Auxin biosynthesis genes which is an essential process in somatic embryogenesis, especially in callus induction (Khanday et al., 2020). In our study, *Situbagendit* showed slower progression of shoot development at 28 DAP in comparison to the other three rice varieties. Given that *OsBBM* also induce the somatic competence (Khanday et al., 2020), the lower expression of *OsBBM* might partially explain the slower progress of shoot development at 28 DAP. The success of callus regeneration to plantlets is essential, since it demonstrates the success of somatic embryogenesis as a cumulative result of the simultaneous collaboration of essential genes in plant development (Figure 2 and 3).

The *OsLEA* gene expression was detected in *Bondoyudo*, *Caok*, *Ciliwung*, and *Situbagendit*, although the intensity of the band differs between rice varieties. The *Bondoyudo*, *Caok*, and *Situbagendit* rice exhibited a slight increase in band intensity between P1 and P2 relative to P0. A slight clear increase of band intensity was observed upon P2 treatment in *Bondoyudo*, *Caok*, and *Situbagendit*. In contrast, the expression of *OsLEA* in *Ciliwung* was comparable between P0, P1, and P3. According to Hong-Bo et al. (2005), the expression of the Late Embryogenesis Abundant (*LEA*) gene indicates the increased allelopathic content of plant media, associated with the regulation of abiotic stresses. According to Cheema et al. (2013), the administration of allelopathic compounds to the growing medium can induce abiotic stress because allelopathic compounds themselves can inhibit plant absorption of nutrients. According to Hong-Bo et al. (2005), *LEA* is a protein that protects the cytoplasm from drought conditions. Based on our results (Figure 4), we hypothesize that cogon grass root extract can increase the *LEA* gene expression, when administered. Therefore, the addition of allelopathic compounds can inhibit the regeneration of plantlets compared to the absence of allelopathic treatment.

Figure 4 demonstrates that the *OsLEC1* gene was expressed differently in each treatment and variety. The *OsLEC1* gene controls the somatic embryo development and embryo maturation (Kumar et al., 2020). The *OsLEC1* gene expression in *Bondoyudo* and *Caok* experience an inconsiderable effect among the control and treatments. Conversely, the *OsLEC1* gene expression was observed at a higher level in P0 and P1 than in P2 in *Ciliwung* and *Situbagendit*. This indicate there is a slight suppression effect of the treatment of cogon grass root extract to the expression of *OsLEC1*, especially at the concentration 5 g/L (P2). According to our findings, the administration of cogon grass root extract has various effects on the expression of *OsLEC1* gene in different rice varieties. Our study pronounces the application of cogon grass root extract has minimum effect on the *OsLEC1* gene expression in *Bondoyudo* and *Caok* rice, but a suppression was found in *Ciliwung* and *Situbagendit* rice under the treatment of 5 g/L (P2). This finding is in the accordance with Gniazdowska et al. (2015), who stated that the effect of allelochemicals is dependent on the concentration and sensitivity of the target plant. The expression of *OsSERK*

gene in *Bondoyudo* and *Caok* rice remained constant in control and under the treatment. However, in *Ciliwung* and *Situbagendit*, the *OsSERK* gene expression was relatively high in P0 and P1, and lower in P2. These results indicate that cogon grass root extract administered at up to 5 g/L concentrations had no effect on the expression of *OsSERK* gene in *Bondoyudo* and *Caok* rice. However, in *Ciliwung* and *Situbagendit*, the expression of *OsSERK* gene under the treatment of P2 was slightly downregulated. Although the *OsSERK* gene mainly expressed in callus during morphogenesis (Hu et al., 2005), it also regulates root development, immune responses, and cell death (Kumar & Van Staden 2019).

The expression of *OsWOX4* gene in four rice varieties was depicted in Figure 4. The expression of *OsWOX4* in *Bondoyudo* and *Caok* were distinct in both control (P0) and treatments (P1 & P2). The treatments of 2,5 and 5 g/L of cogon grass root extract apparently did not alter the level of *OxWOX4* expression in both varieties. Meanwhile, although under P1 treatment *Ciliwung* and *Situbagendit* showed no difference in expression level with respect to the control, the expression of *OsWOX4* was significantly reduced under the treatment of 5 g/L of cogon grass root extract. This study showed that the treatment of cogon root extract affects expression of *OsWOX* differently depends on the variety used. The *OsWOX4* gene has multiple functions, including embryogenesis, root elongation, meristem cell maintenance, and early leaf development (Yasui et al., 2018). In addition, the *OsWOX4* gene influences cell proliferation and root tip elongation, which contribute to the formation of primary root length (Chen et al., 2020).

The gene expression analysis using RT-PCR, visualized in a UV-transilluminator revealed that rice varieties grown *in vitro* and treated with cogon grass root extract express genes at different levels. The expression level of *OsBBM* gene in *Situbagendit* rice was affected by the administration of allelopathic compounds at a concentration of 5 g/L (P2) while the P0 and P1 treatment show less differences. Subsequently, the allelopathic compounds can slightly induce *OsLEA* expression in all rice varieties, with *Ciliwung* as an exception which has the similar level of expression in P1 and P2 treatments compared to the control (P0). Rice varieties differ in their expression of *OsLEC1* gene. *Bondoyudo* and *Caok* rice treated with 2.5 g/L (P1) and 5 g/L (P2) exhibited no effect on the *OsLEC1* gene expression level. In contrast, the expression of *OsLEC1* gene was suppressed in *Ciliwung* and *Situbagendit* under the P2 treatment, whereas under P1 and P0, *OsLEC1* expression was maintained at the same level. The *OsSERK* and *OsWOX4* genes exhibited identical patterns of gene expression. *Bondoyudo* and *Caok* varieties at P0, P1, and P2 expressed both genes at a comparable level. Under the influence of *OsSERK* and *OsWOX4* genes, however, a moderate to obvious reduction in band thickness was observed. This means that the expression of *OsLEC1*, *OsSERK*, and *OsWOX4* genes responds similarly to the treatment of allelopathic compounds of cogon grass root extract, particularly at a concentration of 5 g/L (P2), which caused a significant reduction in expression level among the three genes.

Conclusion

The administration of allelopathic compounds has a specific stimulatory/inhibitory effect depending on the concentration administered and the target plant. In tissue culture, the correct quantity of stimulatory substance can promote the growth and development of cultured plant on the media. The administration of 2.5 g/L allelopathic compounds stimulated the morphogenesis and proliferation of *Caok* and *Situbagendit* rice, except in *Bondoyudo* and *Ciliwung* rice. The administration of 5 g/L allelopathic compounds inhibited morphogenesis and cell proliferation significantly in all four rice varieties. Administration of allelopathic compounds can also have diverse effects on the expression of *OsBBM*, *OsLEA*, *OsLEC1*, *OsSERK*, and *OsWOX4* genes at the molecular level. The expression of essential genes in morphogenesis and cell proliferation may influence the growth and development of plants in their respective media. This research investigates the effect of allelopathic compounds in stimulating or inhibiting the growth and development of plant tissue in culture media. Future research should be carried out to dissect the effect of allelopathic compounds on other rice varieties as well as numerous essential genes.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

* This article was presented as an oral presentation at International Conference on Veterinary, Agriculture and Life Sciences (www.icvals.net) held in Antalya/Turkey on November 17-20, 2022.

References

Artadana, I.B.M., Suhono, G.B.F., Hardjo, P.H., Purwanto, M.G.M., Wang, Y.B., & Supaibulwatana. K. (2017). Plant regeneration induced from mature Embryo-derived callus of Balinese red rice (Oryza sativa Cv. Barak Cenana). *Bali Med J 3*,12–17. https://doi.org/10.15562/bmj.v6i3.710

Cheema, Z.A., Farooq, M., & Wahid, A. (2013). Allelopathy: Current trends and future applications. *Allelopath Curr Trends Futur App*, (pp.l 1–517). https://doi.org/10.1007/978-3-642-30595-5

Chen, R., Xu, N., Yu, B., Wu, Q., Li, X., Wang, G., & Huang, J. (2020). The wuschel-related homeobox transcription factor oswox4 controls the primary root elongation by activating osaux1 in rice. *Plant Sci*, 298. https://doi.org/10.1016/j.plantsci.2020.110575

Cheng, F., & Cheng, Z. (2015). Research progress on the use of plant allelopathy in agriculture and the physiological and ecological mechanisms of allelopathy. *Front Plant Sci*, *6*,1–16. https://doi.org/10.3389/fpls.2015.01020

Gniazdowska, A., Krasuska, U., Andrzejczak, O., & Soltys-Kalina, D. (2015). Allelopathic compounds as oxidative stress agents: yes or no. *Reactive Oxygen and Nitrogen Species Signaling and Communication in Plants, Signaling and Communication in Plants.* (pp. 155–176). 10.1016/j.cropro.2003.09.004

Hagan, D., Jose, S., & Lin, C.H., (2013). Allelopathic exudates of cogongrass (imperata cylindrica): implications for the performance of native pine savanna plant species in the southeastern us. *Journal of Chemical Ecology 39*(2), 312-322.

Hong-Bo, S., Zong-Suo, L. &, Ming-An, S. (2005). Lea proteins in higher plants: structure, function, gene expression and regulation. *Colloids Surfaces B Biointerfaces*, *45*,131–135 . https://doi.org/10.1016/j.colsurfb.2005.07.017

Jha, P., & Kumar, V. (2018). Baby boom (bbm): a candidate transcription factor gene in plant biotechnology. *Biotechnol Lett* 5. https://doi.org/10.1007/s10529-018-2613-5

Karmegam, N., Kalpana, M., & Prakash, M., (2014). Allelopathic effect of aqueous root bark extract of tamarindus indica L. and rhizosphere soil on germination and seedling growth of oryza sativa L . *Int J Curr Microbiol Appl Sci 3*,505–514

Khanday, I., Medellin, C.S., & Sundaresan, V. (2020). Rice embryogenic trigger baby boom1 promotes somatic embryogenesis by upregulation of auxin biosynthesis genes. https://doi.org/10.1101/2020.08.24.265025

Kumar, V., Jha, P., & Van Staden, J. (2020). Leafy cotyledons (lecs): master regulators in plant embryo development. *Plant Cell Tissue Organ Cult, 140*, 475–487 . https://doi.org/10.1007/s11240-019-01752-x

Kumar, V., & Van Staden, J. (2019). Multi-tasking of SERK-like kinases in plant embryogenesis, growth, and development: current advances and biotechnological applications. *Acta Physiol Plant, 41*,1–16 . https://doi.org/10.1007/s11738-019-2819-8

Kumari, M., Clarke, H.J., Small, I., & Siddique, K.H.M., (2009). Albinism in plants: A major bottleneck in wide hybridization, androgenesis and doubled haploid culture. *CRC Crit Rev Plant Sci*, *28*,393–409 . https://doi.org/10.1080/07352680903133252

Kurniati, T., & Daniel, S. (2018). Uji toksisitas dan sifat alelopati ekstrak alang-alang (Imperata cylindrica) terhadap perkecambahan biji padi (Oryza sativa). *J At 3*,54–6

Mastuti, R. (2017). *Dasar-dasar kultur jaringan tumbuhan*. Universitas Brawijaya Press

Méndez-Hernández, H.A., & Ledezma-Rodríguez, M., Avilez-Montalvo, R.N., Juárez-Gómez, Y.L., Skeete, A., Avilez-Montalvo, J., De-la-Peña, C., & Loyola-Vargas, V.M., (2019). Signaling overview of plant somatic embryogenesis. *Front Plant Sci, 10*. https://doi.org/10.3389/fpls.2019.00077

Michel, Z., Hilaire, K.T., Mongomaké, K., Nguessan, A., & Justin, K.Y. (2008). Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (Gossypium hirsutum L.). *Aust J Crop Sci*, *2*,115–125.

Minarsih, H., Suharyo., Riyadi, I., & Ratnadewi, D. (2016). Pengaruh jumlah subkultur dan media sub-optimal terhadap pertumbuhan dan kemampuan regenerasi kalus tebu (Saccharum officinarum L.) (Effect of repeated subculture and suboptimum media on the growth of sugarcane calli (Saccharum officinarum L.)). *E-Journal Menara Perkeb*, *84*, 28–40 . https://doi.org/10.22302/ppbbi.jur.mp.v84i1.219

Morrish, F., Vasil, V., & Vasil, I.K. (1987). Developmental morphogenesis and genetic manipulation in tissue and cell cultures of the Gramineae. *Adv Genet, 24*, 431–499 . https://doi.org/https://doi.org/10.1016/S0065-2660(08)60014-0

Nabors, M.W., Kroskey, C.S., & McHugh, D.M. (1982). Green spots are predictors of high callus growth rates and shoot formation in normal and in salt stressed tissue cultures of oat (avena sativa l.). *Zeitschrift für Pflanzenphysiologie*, *105*, 341–349 . https://doi.org/10.1016/s0044-328x(82)80030-5

Narwal, S. (2009). Allelopathy and allelochemicals. In: Narwal (ed) *Isolation, identification and characterization of allelochemicals/natural products*. Science Publishers, Playmouth, (pp 3–5).

Novikova, G.V., Nosov, A.V., Stepanchenko, N.S., Fomenkov, A.A., Mamaeva, A.S., & Moshkov, I.E. (2013). Plant cell proliferation and its regulators. *Russ J Plant Physiol*, *60*, 500–506 . https://doi.org/10.1134/S102144371304010

Rice, E.L. (2012). *Allelopathy*. Academic Press.

Safitri, F.A., Ubaidillah, M., & Kim, K.M. (2016). Efficiency of transformation mediated by agrobacterium tumefaciens using vacuum infiltration in rice (Oryza sativa L.). *J Plant Biotechnol*, *43*, 66–75 . https://doi.org/10.5010/JPB.2016.43.1.66

Sari, N., Suwarsi, R. E., & Sumadi (2014). Biosaintifika. *J Biol Biol Educ*, *6*, 51–59 . https://doi.org/10.15294/biosaintifika.v6i1.3785

Sun, C.S., Wu, S.C., Wang, C.C., & Chu, C.C. (1979). The deficiency of soluble proteins and plastid ribosomal RNA in the albino pollen plantlets of rice. *Theor Appl Genet*, *55*, 193–197 . https://doi.org/10.1007/BF0026811

Suparyono, & Setyono, A. (1997). Mengatasi Permasalahan Budidaya Padi. Penebar Swadaya, Jakarta

Suzuki, M., Tominaga, T., & Kato-Noguchi, H. (2015). The potent allelopathic substances of cogongrass rhizome extracts. Allelopathic Exudates of Cogongrass (Imperata cylindrica): Implications for the Performance of Native Pine Savanna Plant Species in the Southeastern US

Upadhyaya, G., Sen, M., & Roy, A. (2015). In vitro callus induction and plant regeneration of rice (Oryza sativa L.) var. 'Sita', 'Rupali' and 'Swarna Masuri.' *Asian J Plant Sci Res*, (ISSN 2249-7412) *5*, 24–27

Usman, Purwoko, B.S., Syukur, M., & Guntoro, D.D. (2016). Toleransi galur harapan padi sawah (Oryza sativa L.) pada persaingan dengan gulma echinochloa crus-galli. *J Agron Indones*, *44*, 111. https://doi.org/10.24831/jai.v44i2.13476

Wahyurini, E. (2009). Pengembangan teknologi berbasis bahan Baku lokal. In: *Peran kultur jaringan tanaman dalam pengembangan pangan lokal*. Lembaga llmu Pengetahuan Indonesia LlPl, Yogyakarta, (pp 675–682).

Yadav, H., Malik, K., Kumar, S., & Jaiwal, P.K. (2020). Comparative regeneration in six bread wheat (Triticum aestivum L.) varieties from immature and mature scutella for developing efficient and genotype-independent protocol prerequisite for genetic improvement of wheat. *Vitr Cell Dev Biol - Plant*, *56*, 610–617 . https://doi.org/10.1007/s11627-020-10070-3

Yar, S., Khan, E.A., Hussain, I., Raza, B., Abbas, M.S., & Munazza, Z. (2020). Allelopathic influence of sorghum aqueous extracts and sorghum powder on germination indices and seedling vigor of hybrid corn and jungle rice. *Planta Daninha*, *38*, 1–10 . https://doi.org/10.1590/s0100-83582020380100005

Yulifrianti, E., Linda, R., & Lovadi, I. (2015). Potensi alelopati ekstrak serasah daun mangga (mangifera indica (l .)) terhadap pertumbuhan gulma rumput grinting (cynodon dactylon (l .)) press. *J Protobiont*, *4*, 46–51 . https://doi.org/http://dx.doi.org/10.26418/protobiont.v4i1.8719

Zarwazi, L.M., Chozin, M.A., & Guntoro, D.D. (2016). Potensi gangguan gulma pada tiga sistem budidaya padi sawah. *J Agron Indones*, *44*, 147. https://doi.org/10.24831/jai.v44i2.13481

Zhao, P., Begcy, K., Dresselhaus, T., & Sun, M.X. (2017). Does early embryogenesis in eudicots and monocots involve the same mechanism and molecular players? *Plant Physiol*, *173*, 130–142. https://doi.org/10.1104/pp.16.01406

Author Information

Moh Rosyadi Adnan
Agriculture Production Department, State Polytechnic of Jember, East Java 6812, Indonesia

Dwi Mai A.I. Buqori
Study Program of Agronomy, Faculty of Agriculture, University of Jember, Jember, East Java 6812, Indonesia

Kyung Min Kim
Division of Plant Biosciences, School of Applied Bio Sciences, College of Agriculture and Life Science, Kyungpook National University, Daegu, South Korea

Muhammad Noval Khuluq
Study Program of Agrotechnology, Faculty of Agriculture, University of Jember, Jember, East Java 6812, Indonesia

Muhammad Ubaidillah*
Study Program of Agrotechnology, Faculty of Agriculture, University of Jember, Jember, East Java 6812, Indonesia
Contact: moh.ubaidillah.pasca@unej.ac.id

To cite this article:

Adnan, M.R., Buqori, D.M.A.I., Kim. K. M. Khuluq. M. N., & Ubaidillah, M. (2020). Response of morphogenesis and cell proliferation to allelopatic compounds on rice germplasm. *The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)*, *8*, 14-27.

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)
ISSN: 2791-8033

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS), 2022

Volume 8, Pages 28-35

ICVALS 2022: International Conference on Veterinary, Agriculture and Life Sciences

Walnut Paste: A Healthy Alternative for Nutella Consumers

Eugenia COVALIOV
Technical University of Moldova

Rodica SIMINIUC
Technical University of Moldova

Violina POPOVICI
Technical University of Moldova

Abstract: Nutella is the best-selling chocolate and hazelnut spread in the world. It is known that the main ingredients of Nutella are sugar (55%) palm oil (23%), hazelnuts (14%), cocoa solids and skimmed milk (8%). Currently, worldwide, there is a tendency to make healthier food choices, and the sugar and palm oil from Nutella's formulation are ingredients blamed for causing certain health disorders (obesity, cardiovascular diseases and diabetes). In this order, the purpose of the research was to develop the technology for obtaining walnut paste, since walnuts have proven prophylactic properties, with a low sugar content and without palm oil. The quality of the elaborated walnut paste was determined and monitored during the shelf life in terms of oxidative stability (Acidity index (FFA) and Peroxide value (PV)), total phenols content, antioxidant activity and rheology. The obtained results demonstrated that during 4 months of storage the acidity index of the walnut paste did not register significant changes, reaching maximum values of 0.17±0,01 (Oleic g/100 g), while the peroxide values evolved up to 2.22±0,03 meq/kg oil. The slow evolution of the oxidative parameters can be due to the phenolic compounds in walnut paste that recorded a value of 47.2±0,36 mg GAE/g with an antioxidant activity (DPPH free radical scavenging) of 70±1,02 %. Concerning sensory characteristics, the walnut paste samples were positively appreciated in comparison with Nutella and no significant quality difference was observed after storage for 4 months.

Keywords: Walnut paste, Oxidative stability, Phenolic compounds, Antioxidants

Introduction

The walnut (Juglans Regia L.) fruit is considered one of the most consistent foods, and walnut culture is specified as a strategic direction for human nutrition and included by FAO and WHO in the list of priority plants (Gandev, 2007). Because of the high fat content, walnut kernels were not considered a healthy food until recently. This perception has changed a lot lately, because it has been found that they have a healthy polyunsaturated fatty acid profile, are rich in proteins, vitamins and minerals. Many authors report that walnut kernel contains a large amount of lipids (> 50% of the weight), 11% proteins, 5% carbohydrates and is very caloric (approx. 525 kcal/100 g). Walnut lipids have a high content of unsaturated fatty acids (up to 90%), including polyunsaturated fatty acids (PUFA) (up to 78% of the total fatty acid content), which play an essential role for the proper functioning of the human body. They also contain appreciable amounts of dietary fiber, vitamins (E, B3, B5, B6) and mineral elements (K, P, Mg) (Chatrabnous et al., 2018; B. Liu et al., 2020; Martínez et al., 2010).

The importance of walnut culture is determined by its multi-functional utility that includes food, medicine, dyes, adhesives, cosmetics, oils, furniture and sculpture (Guasch-Ferré et al., 2018; L. Liu & Dai, 2021; Rusu et al., 2020). The interest in walnuts is also determined by the nutritional value, which derives from their unique

composition, with certain nutrients and phytochemicals responsible for multiple beneficial effects of consuming walnuts and derived products (Ni et al., 2022; Ros et al., 2018). The objective of combining walnut kernel with other raw materials is to diversify and improve the nutritional and organoleptic qualities of traditional food products, but also to obtain so-called functional foods. Thus, by using certain strategies in the reformulation of food product matrix, food with a specific composition (eg: reduction of animal fat and sodium content, fortification with various bioactive substances, etc.) and acceptable physico-chemical and organoleptic properties is obtained (Otunola & Martirosyan, 2021). Due to the beneficial effects of walnut consumption on human health demonstrated by numerous researches, there has been increased interest in the development of new food products based on walnuts, such as walnut milk, various fillings for pastry products, walnut flour. Some researchers have tried to produce meat products containing walnut kernel (Ayo et al., 2005; Cofrades et al., 2004). Some studies related to the production of drinks and emulsions using walnut kernels have also been carried out (Gharibzahedi et al., 2012; Ouyang et al., 2022).

The walnut sector is a traditional branch for Republic of Moldova, being favored by the moderate climate, the fertile soils, the possibilities of cultivating the most valuable varieties from the world selection (Zimny, 2012). As in other regions of the world, the market leader of the nut spread industry in Republic of Moldova is the well-known *Nutella* spread. Today, Ferrero's product is a global success. There are studies that mention that on one hand a jar of Nutella is sold worldwide every 2.5 seconds and on the other hand there are health and environmental damage concerns due to the use of palm oil in Nutella spread manufacturing (Cova & D'Antone, 2016; Silva, 2016). Taking into account the fact that walnuts are a local product for the Republic of Moldova and that there are no Moldovan products similar to the "Nutella" spread on the market, the prime objective of this research was to develop a walnut paste with cocoa and chocolate addition that is similar to Nutella using locally grown walnuts.

Materials and Methods

Materials

Walnuts from *Pescianski* variety used in the research were purchased from an orchard in the Republic of Moldova. In walnut paste formulations was also used cocoa, dark chocolate (95% cocoa) and powdered sugar that were bought from a local supermarket in Moldova.

Methods

Preparation of Walnut Paste

After walnuts shelling, the kernel was roasted in a convection oven (Rational SCC 61E, Germany) at 170 °C for 10 min. The roasted walnut kernel was then shaken on a sieve and winnowed in order to remove part of the kernel skin, which, on the one hand, is very rich in polyphenols (Jahanban-Esfahlan et al., 2019), and on the other hand gives the final product a bitter taste (M. Liu et al., 2021). The walnut kernel (83.5%), powdered sugar (10.0 %), dark chocolate (5.0 %) and cocoa (1.5 %) was then blended in a blender (KitchenAid 5KSB4026, United Kingdom) at 8000 rpm until a fine homogeneous paste was obtained. The paste was then distributed in jars, covered and kept in a dark place in order to monitor its quality during storage.

Acidity Index (FFA)

In a 250 ml volumetric flask weighed 5 g of sample with the precision of 0.01 g. Then were added 25 ml of hexane and 25 ml of ethyl alcohol. The potentiometric method consists of adding 0.1N NaOH until the pH of the solution reaches the value of 8.1(*AOCS Official Method Cd 3d-63*, 1999).

$$A(\%\ oleic\ acid) = \frac{28{,}2 \cdot N\ (NaOH) \cdot V}{m}$$

where:
V– volume of sodium hydroxide, [ml]
N – concentration of sodium hydroxide, [mol/dm^3]
m – mass of sample, [g].

Peroxide Value (PV)

In a 250 ml volumetric flask were weighed 2 g of the sample, were added 10 cm^3 of hexane, the analyzed sample was quickly dissolved, then added 15 cm^3 of 15% glacial acetic acid and 1 cm^3 of 50% potassium iodide solution, the obtained solution was mixed for 1 minute and placed in a dark place for 15 minutes. Then was added 75 cm^3 of distilled water, mixed and titrated with sodium thiosulfate solution (5,09 mM) until a pale blue colour appeared and was stable for 5 seconds. The titration was performed in the presence of 1% starch indicator solution (*AOCS Official Method*, 2003).

$$\mathrm{PV} = \frac{(\mathrm{V}_s - \mathrm{V}_{ref}) \cdot \mathrm{N} \cdot 1000}{\mathrm{m}}$$

where:
V_{ref} – volume of titrant used for blank titration, [ml]
V_s – volume of titrant used for sample titration, [ml]
N – normality of sodium thiosulfate solution.

Total Phenolic Content (TPC)

Total phenolic content was performed using a Shimadzu 1800 UV/Vis spectrophotometer, at 765 nm wavelength, using a 10 mm quartz cuvette. The results of the total phenolic content, expressed in mg GA/100g of dry weight, were obtained using the GA calibration curve (y = 0.0041x-0.1331, R^2=0.9924) (Šarolić et al., 2014).

DPPH Antioxidant Activity

The antioxidant activity of the samples was performed using a Shimadzu 1800 UV/Vis spectrophotometer and expressed as % inhibition of DPPH using the following equation (Šarolić et al., 2014):

$$AA\% = \frac{A_0 - A_t}{A_0} \cdot 100\%$$

where:
A_0 - the absorbance of the DPPH solution at the initial time of 0 s;
A_t - the absorbance of the DPPH solution after 30 min of incubation.

Rheological Properties

The viscosity of the nut paste was determined using the BROOKFIELD DVIII Ultra rotary viscometer at 25±0.2 °C.

Sensory Analysis

The sensory analysis of the walnut paste was carried out at the Department of Food and Nutrition, Technical University of Moldova. 20 panelists (aged 20 to 60 years old) rated the quality of the nut paste using a 5-point hedonic scale, from 1 - extremely dislike to 5 - extremely like (Covaliov et al., 2022). Sensory parameters such as taste, color, texture, flavor, spreadability and overall acceptability were evaluated in comparison with Nutella spread.

Statistical Analysis

All the determination were performed in triplicate with exception of sensory analysis as mentioned above. The results are presented as mean ± standard deviation (SD). Student's *t*-test was used for comparison between two means. Physicochemical properties and sensory acceptability results were analyzed with ANOVA software (2020 version); Tukey test (0.05 significance level) was applied for the comparison of mean values.

Results and Discussion

Oxidative Stability

The most common cause of the deterioration of fatty raw materials during storage is lipid peroxidation, which largely depends on the accessibility of oxygen and results in toxic oxidation products (Schwember & Bradford, 2010). The accumulation of peroxides causes the reduction of antioxidant enzymes, their antioxidant capacity and the viability of oilseeds (Bailly et al., 2002).The changes of the acidity and peroxide values of the walnut paste were monitored during storage for 4 months (Table 1). The acidity index is an important quality index for food fats and for food products with advanced fat content. According to Ghasemnezhad and Honermeier (2009), the composition and proportions of free fatty acids is one of the factors that determines susceptibility to fat degradation, because unsaturated fatty acids are much more susceptible to oxidation than saturated fatty acids (Ghasemnezhad & Honermeier, 2009).

Table 1. Physicochemical properties of walnut paste during storage

Storage time, months Parameter	0	1	2	2	4
FFA, Oleic acid g/100 g	0.10±0.01	0.10±0.01	0.13±0.01	0.12±0.01	0.17±0.01
PV, meq/kg	1.17±0.09	1.19±0.05	1.15±0.07	1.81±0.05	2.22±0.11

From the data presented for the oxidative stability indices, no legitimacy is observed in the FFA or PV values during the storage of the walnut paste. Thus, during 4 months of storage, the FFA values oscillate in the range of 0.10-0.17 g Oleic acid/100 g, and the PV values within the limits of 1.15 - 2.22 meq/kg. The oscillations recorded by these parameters can be explained by the fact that at certain stages, some compounds are involved in other reactions, passing into other forms: for example, fatty acids undergo a rearrangement, turning into conjugated dienes, which later, under the action of oxygen, turn into peroxides. Peroxides in their turn will undergo changes thus forming compounds such as aldehydes, ketones, etc., the presence of which degrades the quality of the final product (Abeyrathne et al., 2021; Subotin et al., 2021). According to Ampofo and Grilo (2022), the peroxide value of walnut oil reaches 1.80 meq O_2/kg after 4 months of storage, while FFA ranges within 0.02 – 0.03 Oleic acid g/100g (Ampofo et al., 2022). The difference in FFA content of our walnut paste and the values reported in the previously mentioned study can be linked to the low moisture contents of kernels, thus the lipase activity for FFA formation is limited. Our results are in agreement with the data obtained for the walnut paste (with no addition) accelerated storage study reported by Dordoni et al. (2019). On the other hand, Pourfarza et al. (2020) showed a different behavior of lipids in the case of hazelnut butter production, mentioning a simultaneous increasing trend for both FFA and PV during storage (Pourfarzad & Shokouhi Kisomi, 2020).

Total Phenolic Content and Antioxidant Activity

Current trends in the food field place more and more emphasis on the biologically active potential of certain ingredients due to their effect on human health (Nile & Park, 2014). Polyphenols are categorized as biologically active substances that exhibit free radical scavenging capacity. The total phenolic content and the antioxidant activity of formulated walnut paste is shown in Table 2.

Table 2. Physicochemical properties of walnut paste during storage

Storage time, months Parameter	0	1	2	2	4
TPC, mg GAE/g	47.20±1.23	44.73±0.93	41.43±0.56	37.64±0.65	32.56±0.14
DPPH, %	70.00±1.54	67.31±2.01	61.29±1.32	55.11±0.98	46.65±0.86

The data presented indicate a decrease in the total content of phenolic substances by about 31% after 4 months of storage, and the antioxidant activity, which initially reached a value of 70%, recorded a reduction of 33.35%. The tendency of the values of these parameters to decrease can be explained by the property of polyphenols to be oxidized. Bakkalbaşi et al (2012) reported a walnut phenolic content that ranged within 9.31 – 31.80 mg GAE/g (Bakkalbaşı et al., 2012). In our study the total phenolic content of walnut paste was higher, probably due to the intake of phenolic substances from cocoa, which can reach values up to 106.77 mg GAE/g (Borja Fajardo et al., 2022), and chocolate. In the same context, Dordoni et al. (2019) reported a total phenolic content of walnut paste (with no addition) of 406.9 mg GAE/100 g that showed an upward trend to 465.79 mg GAE/100 g in the 9th day of storage at 60 °C when simulating 2 years storage at 20 °C (Dordoni et al., 2019).

However, it is difficult to compare the values of the total phenolic content obtained in different researches because Folin's method requires the use of different extraction methods and solvents. Multiple researches have demonstrated the direct relationship between the total content of phenolic compounds and the antioxidant activity of vegetable raw materials (Bors & Michel, 2002; Bunea et al., 2012; Gramza et al., 2006). In our study the correlation coefficient is *r=0.99*. This is due to the fact that phenolic compounds actually act as antioxidants contributing to free radical scavenging, thus promoting healthy effects on human body (Chiva-Blanch & Visioli, 2012).

Viscosity

Rheology is the science of the flow and deformation of materials under stress and deformation. In the food industry, rheological data are needed for studying ingredient functionality in product development, determining food texture by correlating with sensory data, immediate or final product control, and process engineering calculation for equipment, etc. (Fischer & Windhab, 2011). The results of rheological properties of developed walnut paste, in terms of viscosity are presented in figure 1.

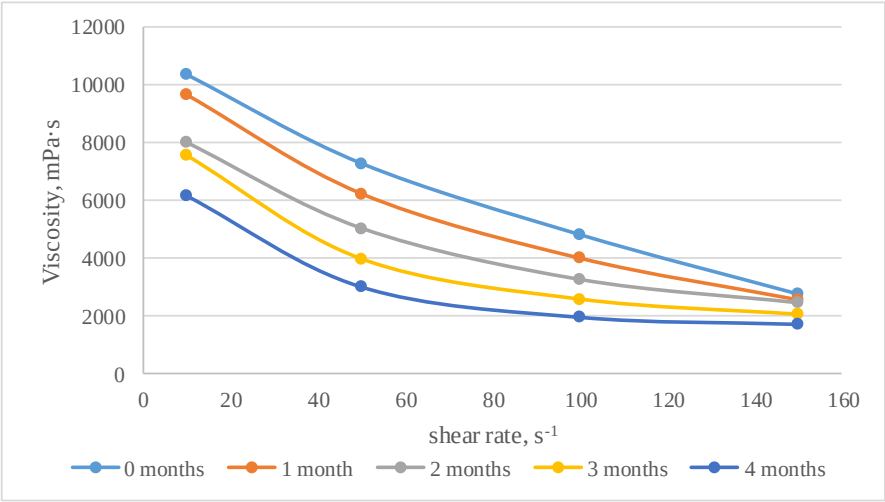

Figure 1. Evolution of apparent viscosity of walnut paste during storage

During the storage period the walnut paste exhibited non Newtonian pseudoplastic behavior, fact confirmed by the values of the apparent viscosities that showed an downward trend when the shear rate increased from 10 to 150 s^{-1} (Rehm et al., 2012). It is also worth mentioning that the reduction of apparent viscosity values during storage is due to the coalescence process of the fat droplets manifested by their fusion and the creation of an oily layer on the surface. Several researches were done in order to avoid the oil separation in nut butters or spreads. In order to avoid the oil separation in penut butter, Gills et al. (2008) tried to use palm oil and hydrogenated vegetable oils. However the results showed that the only factors that affect the quantity of separated oil are temperature and storage time (Gills & Resurreccion, 2000). Ardakani managed to reduse the oil leakage in pistachio butter using two types of emulsifiers: lecithin and mono-di glycerides (Ardakani et al., 2006). Ereifej et al. (2005) states that powdered sugar, pectin and gum arabic manage to decrease oil leakage in halva, however the differences were not significant compared to control sample (Ereifej et al., 2005).

Sensory Analysis

The results of sensory analysis of walnut paste in comparison with *Nutella* spread are summarized in Table 3.

Table 3. Physicochemical properties of walnut paste during storage

Parameter Product	Taste	Flavor	Color	Texture	Spreadability	Overall acceptability
Walnut paste	4.90±0.03	4.90±0.03	5.00±0.06	4.5±0.05	3.7±0.08	4.58±0.05
Nutella	4.50±0.04	4.70±0.05	5.00±0.00	4.8±0.08	5.0±0.00	4.8±0.03

The developed walnut paste had a mean 'overall' acceptability score of 4.58. The panelists deemed the walnut paste between "extremely like" and "neither like nor dislike". This score was lower than the score obtained by Nutella spread (4.8). The acceptability score of walnut paste was in a great measure affected by 'spreadability' compared to Nutella's spreadability. The texture of walnut paste was described as being more fluid than spreadable. The walnut paste had higher scores than the Nutella spread in terms of taste and flavour. Some panelists mentioned that the taste of Nutella is excessively sweet, while the taste of the developed walnut paste was appreciated as moderately sweet with a pleasant flavour of roasted walnuts and an "interesting" aftertaste.

Conclusion

The formulated paste had a high bioactive potential in terms of total phenolic content and antioxidant activity. The investigation of oxidative stability showed that the product is relatively stable during storage, registering slight fluctuations in the value of acidity and peroxide, however, falling within the limits recommended by the normative documents. The sensory analysis showed that the walnut paste was within the acceptance range of the hedonic scale of 4 – 5. Thus, walnuts can be successfully used in the production of a healthy alternative of *Nutella* spread. The developed product would lack an excessive amount of sugar and palm oil, at the same time it would have a good potential on the market of the Republic of Moldova.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements or Notes

* This article was presented as an oral presentation at International Conference on Veterinary, Agriculture and Life Sciences (www.icvals.net) held in Antalya/Turkey on November 17-20, 2022.

*The research was funded by State Project 20.80009.5107.10, nr. PS-62 "Personalized nutrition and intelligent technologies for my well-being", running at Technical University of Moldova

References

Abeyrathne, E. D. N. S., Nam, K., & Ahn, D. U. (2021). Analytical Methods for Lipid Oxidation and Antioxidant Capacity in Food Systems. *Antioxidants*, *10*(10), 1587. https://doi.org/10.3390/antiox10101587

Ampofo, J., Grilo, F. S., Langstaff, S., & Wang, S. C. (2022). Oxidative Stability of Walnut Kernel and Oil: Chemical Compositions and Sensory Aroma Compounds. *Foods*, *11*(19), 3151. https://doi.org/10.3390/foods11193151

AOCS Official Method 8-53. (2003).

AOCS Official Method Cd 3d-63. (1999).

Ardakani, A. Sh., Shahedi, M., & Kabir, Gh. (2006). Optimizing of the process of pistachio butter production. *Acta Horticulturae*, *726*, 565–568. https://doi.org/10.17660/ActaHortic.2006.726.94

Ayo, J., Carballo, J., Solas, M. T., & Jimenez-Colmenero, F. (2005). High pressure processing of meat batters with added walnuts. *International Journal of Food Science and Technology*, *40*(1), 47–54. https://doi.org/10.1111/j.1365-2621.2004.00908.x

Bailly, C., Bogatek-Leszczynska, R., Côme, D., & Corbineau, F. (2002). Changes in activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigour. *Seed Science Research*, *12*(1), 47–55. https://doi.org/10.1079/SSR200197

Bakkalbaşı, E., Yılmaz, Ö. M., Javidipour, I., & Artık, N. (2012). Effects of packaging materials, storage conditions and variety on oxidative stability of shelled walnuts. *LWT - Food Science and Technology*, *46*(1), 203–209. https://doi.org/10.1016/j.lwt.2011.10.006

Borja Fajardo, J. G., Horta Tellez, H. B., Peñaloza Atuesta, G. C., Sandoval Aldana, A. P., & Mendez Arteaga, J. J. (2022). Antioxidant activity, total polyphenol content and methylxantine ratio in four materials of

Theobroma cacao L. from Tolima, Colombia. *Heliyon*, *8*(5), e09402. https://doi.org/10.1016/j.heliyon.2022.e09402

Bors, W., & Michel, C. (2002). Chemistry of the Antioxidant Effect of Polyphenols. *Annals of the New York Academy of Sciences*, *957*(1), 57–69. https://doi.org/10.1111/j.1749-6632.2002.tb02905.x

Bunea, C.-I., Pop, N., Babeş, A. C., Matea, C., Dulf, F. V., & Bunea, A. (2012). Carotenoids, total polyphenols and antioxidant activity of grapes (Vitis vinifera) cultivated in organic and conventional systems. *Chemistry Central Journal*, *6*(1), 66. https://doi.org/10.1186/1752-153X-6-66

Chatrabnous, N., Yazdani, N., & Vahdati, K. (2018). Determination of Nutritional Value and Oxidative Stability of Fresh Walnut. *Journal of Nuts*, *09*(1). https://doi.org/10.22034/jon.2018.540862

Chiva-Blanch, G., & Visioli, F. (2012). Polyphenols and health: Moving beyond antioxidants. *Journal of Berry Research*, *2*(2), 63–71. https://doi.org/10.3233/JBR-2012-028

Cofrades, S., Serrano, A., Ayo, J., Solas, M. T., Carballo, J., & Jimenez Colmenero, F. (2004). Restructured beef with different proportions of walnut as affected by meat particle size. *European Food Research and Technology*, *218*(3), 230–236. https://doi.org/10.1007/s00217-003-0808-y

Cova, B., & D'Antone, S. (2016). Brand Iconicity vs. Anti-Consumption Well-Being Concerns: The Nutella Palm Oil Conflict. *Journal of Consumer Affairs*, *50*(1), 166–192. https://doi.org/10.1111/joca.12082

Covaliov, E., Deseatnicova, O., Resitca, V., Suhodol, N., Carolina, G., & Rodica, S. (2022). Impact of plant additives: Parsley (Petroselinum crispum) leaves and red bell pepper (Capsicum annuum) on the quality of eggless wheat pasta. *Czech Journal of Food Sciences*, *40*(No. 4), 281–289. https://doi.org/10.17221/206/2021-CJFS

Dordoni, R., Cantaboni, S., & Spigno, G. (2019). Walnut paste: Oxidative stability and effect of grape skin extract addition. *Heliyon*, *5*(9), e02506. https://doi.org/10.1016/j.heliyon.2019.e02506

Ereifej, K. I., Rababah, T. M., & Al-Rababah, M. A. (2005). Quality Attributes of Halva by Utilization of Proteins, Non-hydrogenated Palm Oil, Emulsifiers, Gum Arabic, Sucrose, and Calcium Chloride. *International Journal of Food Properties*, *8*(3), 415–422. https://doi.org/10.1080/10942910500267323

Fischer, P., & Windhab, E. J. (2011). Rheology of food materials. *Current Opinion in Colloid & Interface Science*, *16*(1), 36–40. https://doi.org/10.1016/j.cocis.2010.07.003

Gandev, S. (2007). Budding and Grafting of the Walnut (Juglans regia L.) and their Effectiveness in Bulgaria (Review). *Bulgarian Journal of Agricultural Science*, *13*, 683–689.

Gharibzahedi, S. M. T., Mousavi, S. M., Khodaiyan, F., & Hamedi, M. (2012). Optimization and characterization of walnut beverage emulsions in relation to their composition and structure. *International Journal of Biological Macromolecules*, *50*(2), 376–384. https://doi.org/10.1016/j.ijbiomac.2011.12.008

Ghasemnezhad, A., & Honermeier, B. (2009). Influence of storage conditions on quality and viability of high and low oleic sunflower seeds. *International Journal of Plant Production*, *3*(4), 39–48.

Gills, L. A., & Resurreccion, A. V. A. (2000). Sensory and Physical Properties of Peanut Butter Treated with Palm Oil and Hydrogenated Vegetable Oil to Prevent Oil Separation. *Journal of Food Science*, *65*(1), 173–180. https://doi.org/10.1111/j.1365-2621.2000.tb15975.x

Gramza, A., Khokhar, S., Yoko, S., Gliszczynska-Swiglo, A., Hes, M., & Korczak, J. (2006). Antioxidant activity of tea extracts in lipids and correlation with polyphenol content. *European Journal of Lipid Science and Technology*, *108*(4), 351–362. https://doi.org/10.1002/ejlt.200500330

Guasch-Ferré, M., Li, J., Hu, F. B., Salas-Salvadó, J., & Tobias, D. K. (2018). Effects of walnut consumption on blood lipids and other cardiovascular risk factors: An updated meta-analysis and systematic review of controlled trials. *The American Journal of Clinical Nutrition*, *108*(1), 174–187. https://doi.org/10.1093/ajcn/nqy091

Jahanban-Esfahlan, A., Ostadrahimi, A., Tabibiazar, M., & Amarowicz, R. (2019). A Comparative Review on the Extraction, Antioxidant Content and Antioxidant Potential of Different Parts of Walnut (Juglans regia L.) Fruit and Tree. *Molecules*, *24*(11), 2133. https://doi.org/10.3390/molecules24112133

Liu, B., Liang, J., Zhao, D., Wang, K., Jia, M., & Wang, J. (2020). Morphological and Compositional Analysis of Two Walnut (Juglans regia L.) Cultivars Growing in China. *Plant Foods for Human Nutrition*, *75*(1), 116–123. https://doi.org/10.1007/s11130-019-00794-y

Liu, L., & Dai, Y. (2021). Strong adsorption of metolachlor by biochar prepared from walnut shells in water. *Environmental Science and Pollution Research*, *28*(35), 48379–48391. https://doi.org/10.1007/s11356-021-14117-9

Liu, M., Li, C., Cao, C., Wang, L., Li, X., Che, J., Yang, H., Zhang, X., Zhao, H., He, G., & Liu, X. (2021). Walnut Fruit Processing Equipment: Academic Insights and Perspectives. *Food Engineering Reviews*, *13*(4), 822–857. https://doi.org/10.1007/s12393-020-09273-6

Martínez, M. L., Labuckas, D. O., Lamarque, A. L., & Maestri, D. M. (2010). Walnut (Juglans regia L.): Genetic resources, chemistry, by-products. *Journal of the Science of Food and Agriculture*, n/a-n/a. https://doi.org/10.1002/jsfa.4059

Ni, Z.-J., Zhang, Y.-G., Chen, S.-X., Thakur, K., Wang, S., Zhang, J.-G., Shang, Y.-F., & Wei, Z.-J. (2022). Exploration of walnut components and their association with health effects. *Critical Reviews in Food Science and Nutrition*, *62*(19), 5113–5129. https://doi.org/10.1080/10408398.2021.1881439

Nile, S. H., & Park, S. W. (2014). Edible berries: Bioactive components and their effect on human health. *Nutrition*, *30*(2), 134–144. https://doi.org/10.1016/j.nut.2013.04.007

Otunola, G. A., & Martirosyan, D. (2021). Choosing Suitable Food Vehicles for Functional Food Products. *Functional Foods in Health and Disease*, *11*(2), 44. https://doi.org/10.31989/ffhd.v11i2.764

Ouyang, N., Ma, H., Ding, Y., Lu, F., Guo, L., Zhang, X., & Gu, C. (2022). Effect of slit dual-frequency ultrasonic emulsification technology on the stability of walnut emulsions. *Ultrasonics Sonochemistry*, *82*, 105876. https://doi.org/10.1016/j.ultsonch.2021.105876

Pourfarzad, A., & Shokouhi Kisomi, R. (2020). Effect of Lecithin and Mono- and Di-Glyceride on Quality and Shelf Life of Hazelnut Butter: Chemometric Approach. *Polish Journal of Food and Nutrition Sciences*. https://doi.org/10.31883/pjfns/129719

Rehm, B., Haghshenas, A., Paknejad, A. S., Al-Yami, A., & Hughes, J. (2012). Flow Drilling: Underbalance Drilling with Liquid Single-Phase Systems. In *Underbalanced Drilling: Limits and Extremes* (pp. 39–108). Elsevier. https://doi.org/10.1016/B978-1-933762-05-0.50009-7

Ros, E., Izquierdo-Pulido, M., & Sala-Vila, A. (2018). Beneficial effects of walnut consumption on human health: Role of micronutrients. *Current Opinion in Clinical Nutrition & Metabolic Care*, *21*(6), 498–504. https://doi.org/10.1097/MCO.0000000000000508

Rusu, M. E., Georgiu, C., Pop, A., Mocan, A., Kiss, B., Vostinaru, O., Fizesan, I., Stefan, M.-G., Gheldiu, A.-M., Mates, L., Moldovan, R., Muntean, D. M., Loghin, F., Vlase, L., & Popa, D.-S. (2020). Antioxidant Effects of Walnut (Juglans regia L.) Kernel and Walnut Septum Extract in a D-Galactose-Induced Aging Model and in Naturally Aged Rats. *Antioxidants*, *9*(5), 424. https://doi.org/10.3390/antiox9050424

Šarolić, M., Gugić, M., Tuberoso, C. I. G., Jerković, I., Šuste, M., Marijanović, Z., & Kuś, P. M. (2014). Volatile Profile, Phytochemicals and Antioxidant Activity of Virgin Olive Oils from Croatian Autochthonous Varieties Mašnjača and Krvavica in Comparison with Italian Variety Leccino. *Molecules*, *19*(1), 881–895. https://doi.org/10.3390/molecules19010881

Schwember, A. R., & Bradford, K. J. (2010). Quantitative trait loci associated with longevity of lettuce seeds under conventional and controlled deterioration storage conditions. *Journal of Experimental Botany*, *61*(15), 4423–4436. https://doi.org/10.1093/jxb/erq248

Silva, D. F. C. D. (2016). *Innovation in the positioning of Nutella Ferrero's product* [Doctoral dissertation, ISCTE Business School, Instituto Universitario de Lisboa]. https://repositorio.iscte-iul.pt/bitstream/10071/13217/1/Tese%20Daniela%20Cerejo.pdf

Subotin, I., Druta, R., Popovici, V., Covaci, E., & Sturza, R. (2021). Kinetic of Forced Oxidation of Grape Seeds, Walnuts and Corn Germs Oils in the Presence of Antioxidants. *Food and Nutrition Sciences*, *12*(12), 1326–1339. https://doi.org/10.4236/fns.2021.1212097

Zimny, Z. (2012). FDI Potential of the Republic of Moldowa. *SSRN Electronic Journal*. https://doi.org/10.2139/ssrn.2435675

Author Information

Eugenia Covaliov
Technical University of Moldova
168 Stefan ce Mare blvd., Chisinau, Republic of Moldova
Contact e-mail: *eugenia.boaghi@toap.utm.md*

Rodica Siminiuc
Technical University of Moldova
168 Stefan ce Mare blvd., Chisinau, Republic of Moldova

Violina Popovici
Technical University of Moldova
168 Stefan ce Mare blvd., Chisinau, Republic of Moldova

To cite this article:

Covaliov, E., Siminiuc, R., & Popovici, V. (2022). Walnut paste: a healthy alternative for nutella consumers. *The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)*, *7*, 28-35.