OPEN ACCESS

African Journal of **Biotechnology**

February 2021
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

About AJB

The African Journal of Biotechnology (AJB) is a peer reviewed journal which commenced publication in 2002. AJB publishes articles from all areas of biotechnology including medical and pharmaceutical biotechnology, molecular diagnostics, applied biochemistry, industrial microbiology, molecular biology, bioinformatics, genomics and proteomics, transcriptomics and genome editing, food and agricultural technologies, and metabolic engineering. Manuscripts on economic and ethical issues relating to biotechnology research are also considered.

Indexing

CAB Abstracts, CABI's Global Health Database, Chemical Abstracts (CAS Source Index) Dimensions Database, Google Scholar, Matrix of Information for The Analysis of Journals (MIAR), Microsoft Academic, Research Gate

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journals of Biotechnology is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

All articles published by African Journal of Biotechnology are licensed under the Creative Commons Attribution 4.0 International License. This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited. Citation should include the article DOI. The article license is displayed on the abstract page the following statement:

This article is published under the terms of the Creative Commons Attribution License 4.0
Please refer to https://creativecommons.org/licenses/by/4.0/legalcode for details
about Creative Commons Attribution License 4.0

Memberships and Standards

Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.

All articles published by Academic Journals are licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited.

Crossref is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

Similarity Check powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

CrossRef Cited-by Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of CrossRef Cited-by.

Academic Journals is a member of the International Digital Publishing Forum (IDPF). The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.org

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editor-in-Chief

Editorial Board Members

Table of Content

Vol. 20(2), pp.43-50, February, 2021
DOI: 10.5897/AJB2020.17281
Article Number: D332F8566038
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

In vitro daylily (*Hemerocallis* species) bract multiple shoot induction

Kanyand Matand[1*], Meordrick Shoemake[2] and Chenxin Li[3]

[1]Center for Biotechnology Research and Education, School of Agriculture and Applied Sciences, Langston University, P. O. Box 1730, Langston, OK 73050, USA.
[2]Department of Agriculture and Natural Resources, School of Agriculture and Applied Sciences, Langston University, P. O. Box 1730, Langston, OK 73050, USA.
[3]Department of Plant Biology, College of Biological Sciences, University of California, Davis, CA 95616, USA.

Received 21 November 2020; Accepted 27 January 2021

A bract is a non-inflorescence structure that exists in many plant species. In daylilies, the bract is biologically functional and is the object of the present tissue culture study. After sterilization, bract explants were treated with 6-benzylaminopurine (BA) and thidiazuron (TDZ) that were used individually or in combination in Murashige and Skoog (MS) nutrients medium, under room environmental conditions, to study its capacity to induce shoots *in vitro*. The results were successful. Both direct and indirect shoot organogenesis were observed. Although variably, all nineteen cultivars that were investigated induced multiple shoots. TDZ was the most potent chemical stimulus for shoot organogenesis. The results also showed no significant correlation between shoot conversion potential and genotype or treatment.

Key words: Daylily, tissue culture, plant organogenesis, bract, *Hemerocallis*, plant regeneration, thidiazuron.

INTRODUCTION

Daylilies are a monocot of great socio-economic and research values that thrive primarily because of its floral beauty and landscaping applications (Hansen, 2007; Rodriguez-Enriquez and Grant-Downton, 2013; Cui et al., 2019; Li et al., 2020). They are also increasingly justified for medicinal usage and pharmaceutical studies (Du et al., 2014; Farcas et al., 2019). Like many other crops of economic value, daylilies are explored for consistent *in vitro* mass multiplication for functional purposes. The application of *in vitro* micropropagation approaches is of a special consideration in daylilies, because the crop's most economically valued aspect (floral beauty) requires faithful reproduction of the parental phenotype that usually guides customers' choice. Accordingly, *in vitro* tissue culture is among the most effective approaches to reliably deliver customers' expectation in a timely manner and in large-scale. Despite some progress (Matand et al., 2020), daylilies remain very challenging for *in vitro* micropropagation, especially, *de novo* plant regeneration. Unfortunately, there is an incorrect perception that *in vitro* plant organogenesis is easily attainable in daylilies. This idea is often promoted by sporadic reports of protocols that are frequently inconsistent or difficult to reproduce. Consequently, it has hindered efforts to improve the crop

*Corresponding author. E-mail: kmatand@langston.edu. Tel: (405) 466-6131.

Figure 1. Inflorescences on the field experiment plants. It also shows a cluster of flower buds with a display of bract samples.*Identifies non-sterile bract units.

utilizing contemporary technologies like genetic transformation and/or CRSPR/Cas9 editing. Therefore, it is important to expand efforts broadening the studies of various tissues or cell types for totipotency until efficient and convenient reproducible protocols are available for broad applications across *Hemerocallis* species.

This study evaluates bract cells' totipotency. The bract is a genetically inherent, non-floral structure that exists in many plant species (Bowman and Jones, 1982; Whipple et al., 2010; Chuck et al., 2010; Chandler, 2014). It performs vital functions ranging from attracting pollinators to protecting inflorescences (Luke, 1968; Rose, 2016). Its prominence in daylilies lends it the suitability of a potentially reliable explant for *in vitro* tissue culture applications. The understanding of its cellular totipotency could also lead to developing an alternative plant regeneration protocol *via* protoplasts, considering that bract tissues are soft and amenable to protoplasts generation which would be compatible, for example, with CRSPR/Case 9 technology.

MATERIALS AND METHODS

Plant genotypes

The cultivars that were studied included 'Atlanta Dove Child', 'Dark Star', 'Water Birds', 'Realms of Glory', 'Look', 'Atlanta Full House', 'Intricate Art', 'Empire State', 'Tail Feather', 'Creepy Crawlers', 'Siloam Virginia Henson', 'Orange Slices', 'Coyote Moon', 'Rococo', 'Grape Velvet', 'Gay Hearted', 'Science Stealer', 'Bright Banner', and 'Alias'.

Explant preparation and cultural conditions

Young inflorescences were freshly collected from 19 cultivars of daylilies in the Langston University (Oklahoma, U.S.A) collection (Figure 1). These were sectioned into subunits and surface-sterilized with 35% sodium hypochlorite solution (commercial Clorox bleach) for 8 min; then, rinsed with sterile distilled water three times. The whole bract was used as explant and cultured individually in test tubes, one unit per test tube containing 5 ml of culture medium. When needed, the tip of the bract tissue was trimmed to ensure that the explant fits properly in the recipient tube. It was previously determined that trimming tips in this manner did not affect the explant response.

Overall, five test tubes with explants (five replicates) were randomly assigned to individual treatments. Each tube was applied as an experimental unit for observations. Three levels of 6-Benzylaminopurine (BA) (0, 1, and 3 mg/l) defined treatment groups, while 1 mg/l thidiazuron (TDZ) and 0 mg/l growth regulator culture media were used as a positive and negative control treatments, respectively. In total, the experiment applied 5 treatments. Treatments were used alone or in combination in the Murashige and Skoog (MS) nutrients medium. The MS medium consisted of MS salts and vitamins (Murashige and Skoog, 1962), 20 g/l sucrose, and 4 g/l phytagel. Cultures were transferred to fresh media monthly. After at least 45 days of culture, responding explants grew bigger and were sub-cultured into Magenta 7 (GA7) containers with corresponding treatments. All containers were wrapped with parafilm and incubated at 8-h photoperiod at room environmental conditions. During the culture, the room temperature varied from 18.3 to 35.5°C, and the humidity from 21 to 55%. The light sources were regular fluorescent tubes (GE 10773, 60 Watt,

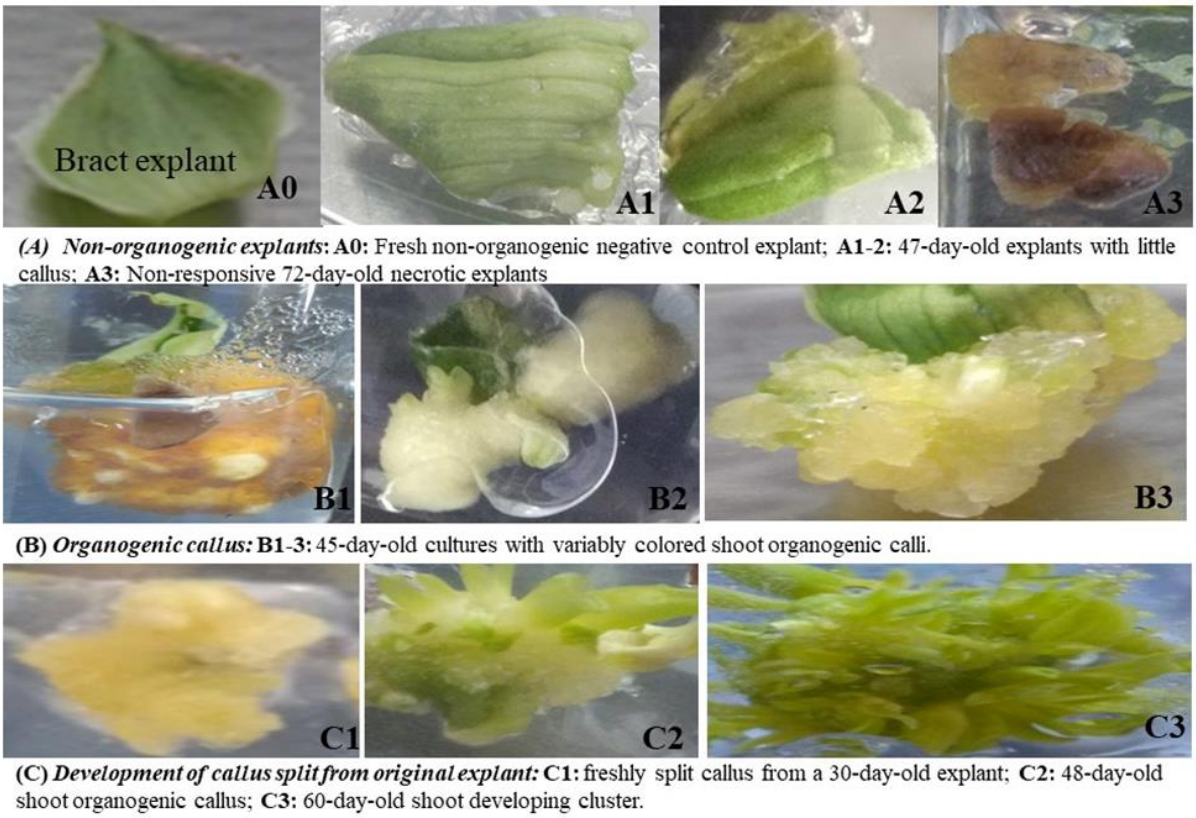

Figure 2. Bract morphogenesis showing variable callus and indirect shoot organogenesis.

48 Inch, T12 Linear Fluorescent, 4100K, 60 CRI) Base, High Output Tube (F48T12/CW/HO/GE)). The final pH of the medium was adjusted to 5.8 - 6.0. The media were autoclaved at 121°C for 20 min. All chemicals were purchased from Sigma Co. (St. Louis, MO, USA).

Data observations and collections

Observations and data collections were conducted daily on individual explants (experimental units) starting from the first week of culturing, for a ninety day-period. Observations included callus, shoot primordium, bud and shoot, and root inductions. Primordia and buds and shoots were counted on individual explants using a binocular light microscope (Stereomaster microscope, Fisher Scientific, U S A). However, global morphogenic responses were pictured using an AT&T GoPhone photo camera. For convenience, only morphogenic responses observed directly from original explants were recorded for statistical analysis. Repetitive organogenic responses following the splitting of responding original explants were not included in the analysis. Shootlets of at least 2.5 cm long were split and transferred onto MS medium without growth regulators for root induction.

Experimental design and statistical analysis

The study used the randomized factorial design. Data were analyzed using linear models with interactions among factors. The analyses were performed with R and R Studio software (version 3.6.0, 2019). The statistical package used for analysis of variance was *emmeans* (Searle et al., 1980) and plotting and graphing were achieved with the software *ggplot2* (Wickham, 2016). The statistical significance of differences among sample means was tested with the Tukey test at 5% level.

RESULTS

The present report pioneers successful shoot organogenesis in daylily bract tissue, demonstrating that it can be reliably applied as a primary explant for *in vitro* propagation. The results of callus, bud and shoot, and root inductions are presented in Figures 1 to 6.

Callus and indirect shoot formation

Within five days of culture, differential cell divisions were observed in cut wound of explants cultured. However, within ten days and thereafter, treated explants developed greater mitotic activities with significant callus formation than negative control explants (Figure 2). Overall, explants cultured on control medium without growth regulators did neither induce organogenesis (shoots or roots) nor callus and subsequently necrotized (Figure 2A). Callus was variably colored, friable, and shoot organogenic (Figure 2B). Indirect shoot

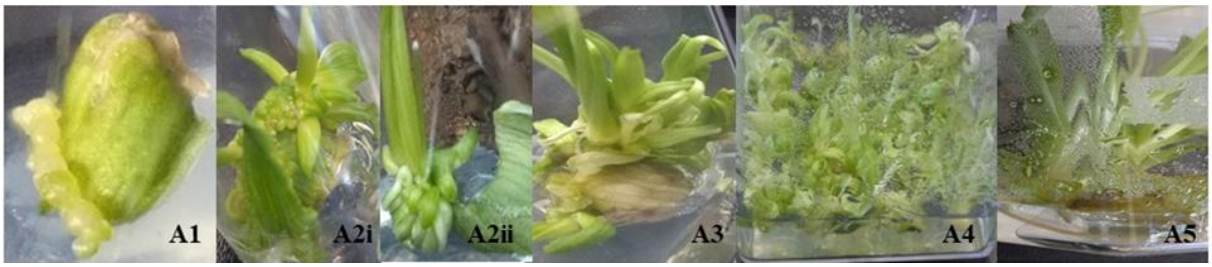

Figure 3. Direct shoot organogenesis and development. A: 21-day-old early multiple shoot bud formation at the bract cut base; A2: 33-day-old mid multiple shoot development, across the wounded area (A2i) or in partial wounded area (A2ii); A3: 45-day-old multiple shoots, ready for splitting to root; A4: 47-day-old freshly split shoots on full-strength basal MS medium for rooting; A5: Rooted shoots ready for greenhouse acclimatization.

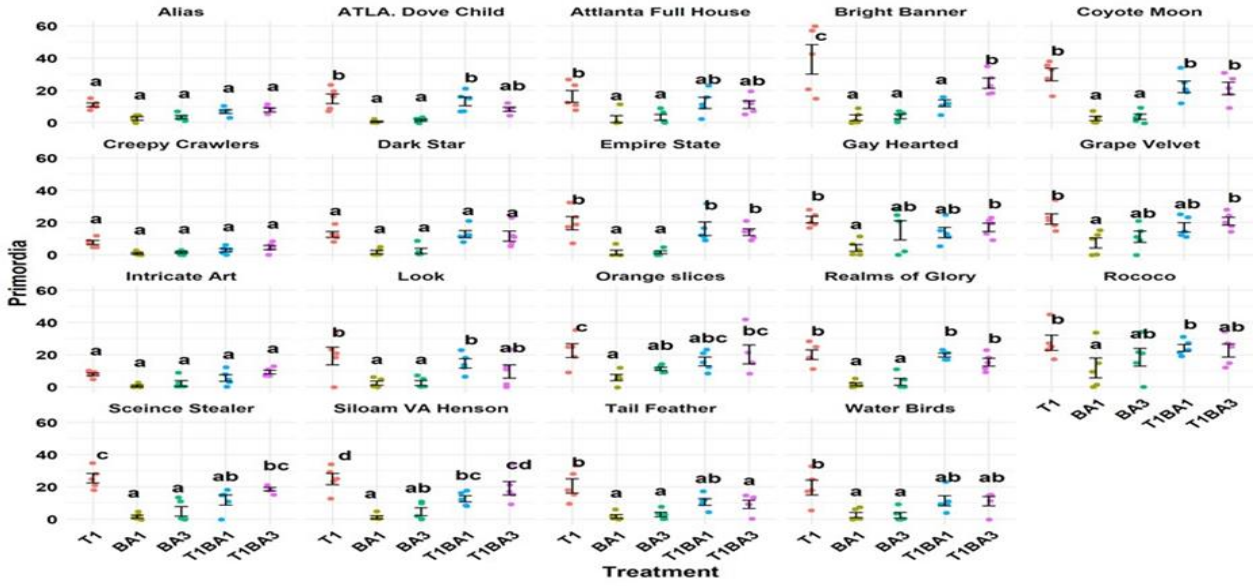

Figure 4. Interactive responses of shoot primordia (Primordia) to growth regulators (T0=0 mg/L TDZ, T1=1mg/L TDZ, BA0=0=mg/L, BAI=1 mg/L BA, and BA3=3 mg/L BA), explant and varieties; five replicates per treatment; bars are mean standard error; and mean differences were tested at 5% level of significance with Tukey test using R and R studio software. Because of the lack of response to T0 and BA0, no related data are presented in Figure 4.

organogenesis occurred within at least 40 days of culture. Callus splitting and sub-culturing speed up multiple shoot formation (Figure 2C) and could potentially extend repetitive multiple shoot induction, indefinitely.

Direct shoot organogenesis

Induction of shoot primordia

Direct shoot organogenesis occurred without callus intervening phase (Figure 3A1) and was more prominent than indirect shoot organogenesis that overall occurred only in less than 5% of explants cultured. Direct shoot formation was typically preceded by the protuberance of a group of cells that bulged in the basal wounded area of the bract explant. Those cellular bulges are termed *shoot primordia* that characteristically developed across (Figure 3 A2i) or partially (Figure 3 A2ii) wound areas where buds and shoots subsequently formed and were an important indicator of the level of shoot organogenesis potential in individual explants. Shoot primordia were observed within two weeks of culture. However, not all primordia developed into buds and shoots, successfully. The amount of induced shoot primordia in individual explants ranged from 0 to 60 and was genotype dependent (Figure 4). The greatest number of shoot primordia (60) was induced in the cultivar ‘Bright Banner’. The average shoot primordia ranged from 0.6 (‘Intricate’) to 39.2 (‘Bright Banner’) per explant. The five top cultivars with

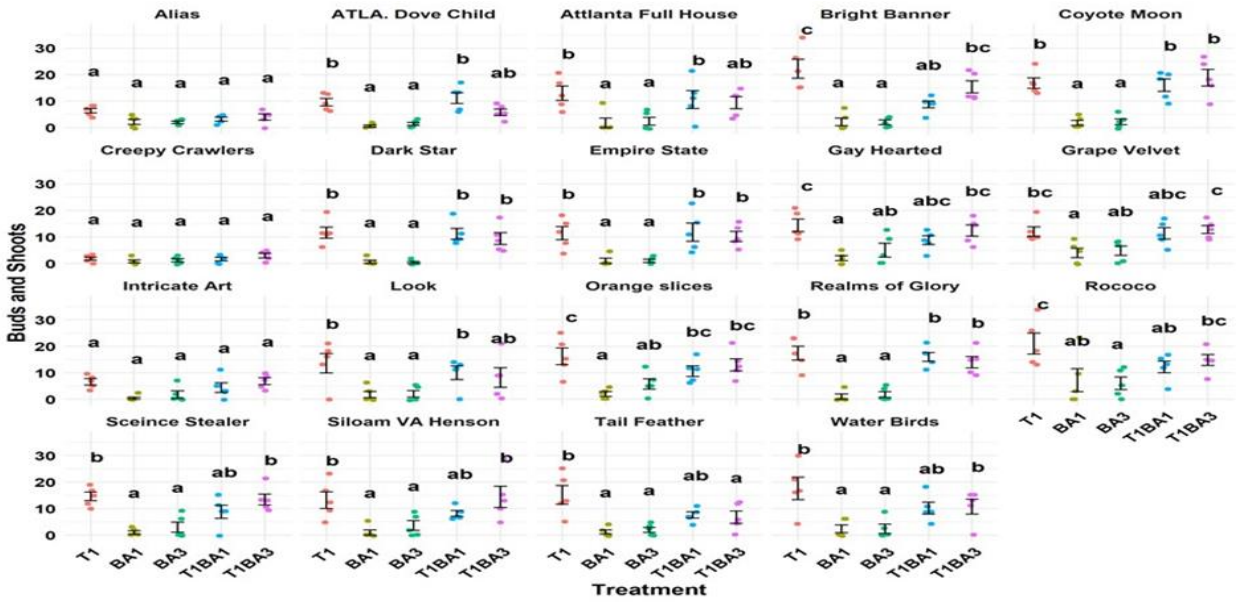

Figure 5. Interactive responses of shoot buds and shoots development to growth regulators (T0=0 mg/L TDZ, T1=1 mg/L TDZ, BA0=0=mg/L, BAI=1 mg/L BA, and BA3=3 mg/L BA), explant and varieties; five replicates per treatment; bars are mean standard error; and mean differences were tested at 5% level of significance with Tukey test using R and R studio software. Because of the lack of response to T0 and BA0, no related data are presented in Figure 5.

greatest average shoot primordia included 'Bright Banner' (39.2), 'Coyote Moon' (29.8), 'Rococo' (27.4), 'Scene Stealer' (25.4), and 'Siloam Virginia Henson' (24.8).

Bud and shoot development

The success of the study was predicated on the potential of the cultivars to induce *in vitro* multiple shoots. The development of buds and shoots from primordia was apparent within three weeks of culture. Accordingly, the proportion of buds and shoots that successfully developed from individual explants is reported in Figure 5. Although statistically variable, the study overall showed that all nineteen cultivars that were studied induced multiple adventitious shoots based on both genotypic and treatment differences. Developmental stages of direct shoot organogenesis are pictured in Figure 3. The number of buds and shoots formed per individual explants ranged from 0 to 34. The greatest amount of buds and shoots was observed in the cultivars 'Bright Banner' and 'Rococo'. The five top performing cultivars in buds and shoots development were 'Bright Banner' and 'Rococo' (34), 'Water Birds' (30), 'Siloam Virginia Henson' (29), and 'Coyote Moon' (27). Ipso facto, the five top cultivars with greatest average buds and shoots per explant were 'Bright Banner' (22.2), 'Coyote Moon' (18.8), 'Water Birds' (17.6), 'Realms of Glory' (17.4), and 'Orange Slices' (16.2). Six cultivars formed at least 20 buds and shoots per explant in at least two treatments, while 17 out of 19 cultivars formed at least 10 buds and shoots per explant in at least two treatments, during the study period.

When considering the influence of growth regulator treatments on shoot organogenesis, TDZ was the most potent chemical stimulus. The greatest buds and shoots numbers per explant of the five top cultivars were influenced by TDZ treatments. Overall, 100% of the cultivars studied responded more effectively to TDZ than BA non-combination treatments. The cultivars 'Alias' and 'Creepy Crawlers' were overall the least respondents across treatments. The ceiling numbers of buds and shoots per explant in these two cultivars were less than ten.

Conversion of buds and shoots from shoot primordia

The proportion of shoot primordia that developed into buds and shoots successfully was estimated using the following formula and data are presented in Figure 6.

Shoot conversion rate (%) = Cultivar no. of buds and shoots/Cultivar no. of shoot primordia × 100

The estimate of successful shoot conversion rate from primordia is consequential in predicting more accurately expected results of current cultivars, when applied under similar cultural conditions. The general conversion rate ranged from 0 to 100%. Accordingly, the five top cultivars with greatest average conversion rates include 'Water Birds' (93.65%), 'Realms of Glory' (91.59%), 'Coyote Moon' (90.12%), 'Dark Star' (90.25%), and 'Atlanta Dove

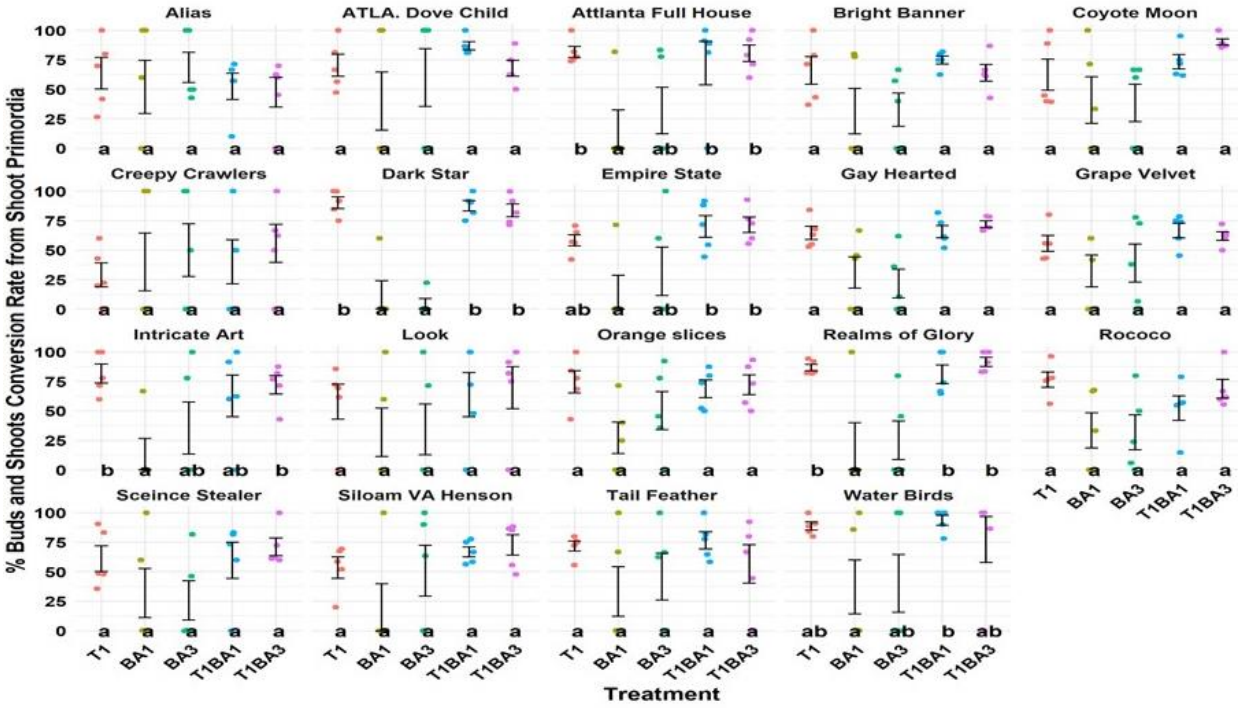

Figure 6. Interactive responses of shoot buds and shoots conversion rates (%) to growth regulators (T0=0 mg/L TDZ, T1=1mg/L TDZ, BA0=0=mg/L, BAI=1 mg/L BA, and BA3=3 mg/L BA), explant and varieties; five replicates per treatment; bars are mean standard error; and mean differences were tested at 5% level of significance with Tukey test using R and R studio software. Because of the lack of response to T0 and BA0, no related data are presented in Figure 6.

Child'. The general response for shoot conversion across cultivars was significantly effective. At least 11 cultivars maxed out the shoot conversion rate in at least three treatments. To highlight the effectiveness of this protocol, each studied cultivar converted buds and shoots form primordia at the minimal rate of 70% in at least three treatments. Based on individual non-combination treatments, the perfect conversion rate was recorded in response to the treatments 1 mg/l TDZ, 1 mg/l BA, and 3 mg/l BA in at least 8, 10, and 9 cultivars, respectively. Similarly, the perfect conversion rate was also observed in 9 and 8 cultivars in response to 1 mg/l TDZ and BA, and 1 mg/l TDZ and 3 mg/l BA combination treatments, respectively.

Root formation and hardening

Separating shootlets that have reached at least 2.5 cm long from the clump (Figures 2C3/3A3 and 3A4) and culturing it on a full-strength MS medium without growth regulators was successful in inducing roots, under the same cultural conditions. More than 95% of shootlets formed roots (Figure 3A5). Lids of the receptacles containing rooted shoots were removed to acclimate the plantlets to the ambient pressure and other environmental conditions, for three days prior to moving it to potted soil, Berger Germinating Mix, BM2 (www.berger.ca), in the greenhouse. All plants grew healthy and normally.

DISCUSSION

This study has successfully pioneered the application of bract as the main explant for micropropagation of daylilies, for all functional purposes. Because callus and shoot formation occurred only at the base cut of the explant, it underpins the concept that stressful wounding releases chemical signals among which are those that induce morphogenesis (Ikeuchi et al., 2013; Chen et al., 2016; Fehér, 2016). Similar observation has previously been reported in globe artichoke bract tissue culture during which shoot organogenesis occurred in the lower region of the bract explants (Ordas et al., 1990). Despite that callus and/or organs may occur naturally in plants or tissue culture without exogenous growth regulators (Chen et al., 2016; Ikeuchi et al., 2019; Zhang et al., 2019), the addition of plant hormones was essential for obtaining positive responses in this study, because the lack of it in the negative control explants' nutrients medium resulted in necrosis without morphogenesis.

It is clearer that either direct or indirect shoot organogenesis can result from applying daylily bract tissue in *in vitro* culture. Remarkably, despite variations

shoot organogenesis occurred across genotypes and was more effective in response to TDZ treatments. This establishes the bract as a reliable explant for daylily *in vitro* propagation. The application of TDZ in previous tissue culture studies has often resulted in very effective responses across plant species and superior performances over different other growth regulators (Huang et al., 2020; Chen et al., 2020). Because of the differential level of difficulty to micro-propagate plant species, there is an exhaustive effort to explore a spectrum of plant tissues, including the bract, for their totipotency potential. Accordingly, the bract tissue has been explored in different plant species for its capacity to induce *in vitro* mass adventitious plants. Although previous results varied, ranging from mere protoplast in banana (Matsumoto et al., 1988) and callus in gladiolus (Bajaj et al., 1983) development to complete organismal development in banana and globe artichoke (Smitha et al., 2017; Comino et al., 2019), the interest in bract tissue culture is growing. The present results are encouraging and show similarities to standard explants that are commonly applied in tissue culture. For example, the use of bract tissue has led to plant development via both direct and indirect shoot organogenesis in different plant species (Ordas et al., 1990; Ninghui et al., 2001; Comino et al., 2019). Other studies have shown that plants can be produced in bract tissue culture via somatic embryogenesis (Divakaran and Nair, 2011; Smitha et al., 2017). Considering that this investigation tested a larger number of genotypes, the results are therefore more extensive. Thus, the contribution of individual explants was better estimated to enable efficient activity planning and predicting of expected results for cost-efficiency, when current genotypes are applied under similar conditions.

Conclusion

The present study has established, for the first time, that daylily bract tissue can be reliably and effectively applied as the principal explant for daylily micropropagation. Considering the extent and diversity of the samples and across the board shoot organogenic success under unrestricted lab environmental conditions, it is anticipated that this protocol will be reproducible more convincingly in broad environmental conditions.

CONFLICT OF INTERESTS

All authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Raysun and the Langston University Department of Agriculture and Natural Resources students Kelviante Murray and Alexia Thurmond, for their assistance in the field and laboratory during the conduct of the studies. This work was supported by the USDA National Institute of Food and Agriculture (NIFA), Evans-Allen project accession number: 1012702.

REFERENCES

Bajaj YPS, Sidhu MMS, Gill APS **(**1983**).** Some factors affecting the *in vitro* propagation of gladiolus**.** Scientia Horticulturae 18**(**3**):**269-275, https://doi.org/10.1016/0304-4238(83)90031-6.

Bowman DT, Jones JE (1982). Inheritance Studies of Bract Size in Cotton. Crop Science 22(5):1041-1045. doi:10.2135/cropsci1982.0011183X002200050035x.

Chandler JW (2014). Patterns and Polarity in Floral Meristem and Floral Organ Initiation. Critical Reviews in Plant Sciences 33 (6):457-469. DOI: 10.1080/07352689.2014.917531.

Chen L, Sun B, Xu L, Liu W (2016). Wound signaling: the missing link in plant regeneration. Plant Signaling and Behavior 11(10):e1238548. doi:10.1080/15592324.2016.1238548

Chen S, Xiong Y, Xincheng Y, Pang J, Zhang T, Wu K, Ren H, Jian S, Teixeira da Silva JA, Xiong Y, Zeng S, Guohua M (2020). Adventitious shoot organogenesis from leaf explants of *Portulaca pilosa* L. Scientific Reports 10(1):3675; DOI: 10.1038/s41598-020-60651-w.

Chuck G, Whipple C, Jackson D, Hake S (2010). The maize SBP-box transcription factor encoded by tasselsheath4 regulates bract development and the establishment of meristem boundaries. Development 137(8):1243-1250 DO 10.1242/dev.048348.

Comino C, Moglia A, Repetto A, Tavazza R (2019). Globe Artichoke Tissue Culture and Its Biotechnological Application. In: Portis E, Acquadro A, Lanteri S (eds) The Globe Artichoke Genome. Compendium of Plant Genomes. Springer, Cham. https://doi.org/10.1007/978-3-030-20012-1_3.

Cui H, Zhang Y, Shi X, Gong F, Xiong X, Kang X, Xing G, Li S (2019). The numerical classification and grading standards of daylily (*Hemerocallis*) flower color. PLOS ONE 14(6):e0216460. https://doi.org/10.1371/journal.pone.0216460.

Divakaran SP, Nair AS (2011). Somatic embryogenesis from bract cultures in diploid *Musa acuminata* cultivars from South India. Scientia Horticulturae 131:99-102.

Du B, Tang X, Liu F, Zhang C, Zhao G, Ren F, Leng X (2014). Antidepressant-like effects of the hydroalcoholic extracts of Hemerocallis citrina and its potential active components. BMC Complementary and Alternative Medicine 14:326. doi: 10.1186/1472-6882-14-326.

Farcas AD, Mot AC, Zagrean-Tuza C, Ticolea M, Sevastre B, Kulak M, Silaghi-Dumitrescu R, Parvu A (2019). Remarkable rutin-rich Hypericum capitatum extract exhibits anti-inflammatory effects on turpentine oil-induced inflammation in rats. BMC Complementary Medicine and Therapies 19(1):289. doi: 10.1186/s12906-019-2680-8.

Fehér A (2016). Somatic embryogenesis — Stress-induced remodeling of plant cell fate. Biochimica et Biophysica Acta., 1849:385-402. doi:10.1016/j.bbagrm.2014.07.005.

Hansen L (2007). Gardening fact sheet: Daylily Basics. Harris County Cooperative Extension, The Texas A&M University System, Houston, Texas. http://harris-tx.tamu.edu/hort.

Huang H, Wei Y, Zhai Y, Ouyang K, Chen X, Bai L (2020). High frequency regeneration of plants via callus-mediated organogenesis from cotyledon and hypocotyl cultures in a multipurpose tropical tree (*Neolamarkia Cadamba)*. Scientific Reports 10:4558. https://doi.org/10.1038/s41598-020-61612-z

Ikeuchi M, Favero DS, Sakamoto Y, Iwase A, Coleman D, Rymen B, Sugimoto K (2019). Molecular Mechanisms of Plant Regeneration. Annual Review of Plant Biology 70(1):377-406.

Ikeuchi M, Sugimoto K, Iwase A (2013). Plant Callus: Mechanisms of Induction and Repression. The Plant Cell 25(9):3159-3173 DOI: 10.1105/tpc.113.116053.

Li S, Ji F, Hou F, Cui H, Shi Q, Xing G, Weng Y, Kang X (2020). Characterization of *Hemerocallis citrina* Transcriptome and Development of EST-SSR Markers for Evaluation of Genetic Diversity and Population Structure of *Hemerocallis* Collection. Frontiers in Plant Science 11:686. https://doi.org/10.3389/fpls.2020.00686.

Luke WJ (1968). Studies on modification of the cotton floral bract as a controlling factor in cotton boll rots. LSU Historical Dissertations and Theses, p. 1450. https://digitalcommons.lsu.edu/gradschool_disstheses/1450.

Matand K, Shoemake M, Li C (2020). High frequency *in vitro* regeneration of adventitious shoots in daylilies (Hemerocallis sp) stem tissue using thidiazuron. BMC Plant Biology 20:31 https://doi.org/10.1186/s12870-020-2243-7.

Matsumoto K, Crepy L, Teixeira JB, Ferreira Fr (1988). Isolation, culture and fusion of banana bract protoplast. In: International Rice Research Institute (IRRI) and Academia Sinica (eds) Genetic manipulation in crops. Cassell Tycooly Philadelphia pp. 414-415.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

Ninghui R, Ronghua W, Xian W, Zhijiang S, Hongwei W (2001). Technical study on tissue culture from bract explant of dwarf Euphorbia pulchrrima. Acta Agriculturae Universitatis Henanensis 35(3):239-240.

Ordas RJ, Tavazza R, Ancora G (1990). *In vitro* morphogenesis in the globe artichoke (*Cynara scolymus* L.), Plant Science 71(2):233-237 https://doi.org/10.1016/0168-9452(90)90013-E.

Rodriguez-Enriquez MJ, Grant-Downton RT (2013). A new day dawning: *Hemerocallis* (daylily) as a future model organism. AoB Plants 5:pls055. https://doi.org/10.1093/aobpla/pls055.

Searle SR, Speed FM, Milliken GA (1980). Population marginal means in the linear model: an alternative to least squares means. The American Statistician 34(4):216-21.

Smitha PD, Binoy KR, Nair AS (2017). Identification of SERK gene from bract derived embryogenic and non-Embryogenic calli of four diploid banana cultivars from South India. Journal of Agricultural Studies 5:161-169.

Sun JF, Huang SQ (2011). White bracts of the dove tree (Davidia involucrata): Umbrella and pollinator lure? Arnoldia 68(3):2-10.

Whipple CJ, Hall DH, DeBlasio S, Taguchi-Shiobara F, Schmidt RJ, Jackson DP (2010). A conserved mechanism of bract suppression in the grass family. Plant Cell 22(3):565-78. doi: 10.1105/tpc.109.073536.

Wickham H (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. ISBN 978-3-319-24277-4; https://ggplot2.tidyverse.org.

Zhang G, Zhao F, Chen L, Pan Y, Sun L, Bao N, Zhang T, Cui C-X, Qiu Z, Zhang Y, Yang L, Xu L (2019). Jasmonate-mediated wound signalling promotes plant regeneration. Nature Plants 5:491–497.

Vol. 20(2), pp.51-65, February, 2021
DOI: 10.5897/AJB2020.17279
Article Number: 0C8756D66058
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Response of French bean genotypes to *Colletotrichum lindemuthianum* and evaluation of their resistance using SCAR markers

Antony Kimani Kamiri[1]*, Edith Esther Arunga[1], Felix Rotich[1] and Reuben Otsyula[2]

[1]Department of Agricultural Resource Management, University of Embu, P.O Box 6-60100, Embu, Kenya.
[2]Non-Ruminant Research Institute, Kenya Agricultural and Livestock Research Organization, Kakamega, P.O. Box 169-50100, Kakamega, Kenya.

Received 10 November, 2020; Accepted 10 December, 2020

Common bean anthracnose caused by *Colletotrichum lindemuthianum* is one of the major biotic constraints to production of French beans (*Phaseolus vulgaris* L.) in Kenya. This study aimed at screening French bean genotypes in relation to their response to common bean anthracnose in order to identify potential sources of germplasm for breeding. The genotypes were tested in three sites (Kakamega, Mwea and Kutus) where results revealed a significant genotype by environment interaction effect ($p \leq 0.05$) which emphasizes the strong influence of the growing conditions on the expression of host resistance. Physiological characterization identified a total of 14 distinct races out of 16 successfully plated isolates, revealing a very high diversity of *C. lindemuthianum* in Kenya. Six races have not been reported in previous studies in the country and are considered as new races, that is, races 84, 141, 246, 515, 576 and 768. Andean race 401 was the most virulent race with a virulence index of 67% among the genotypes. Molecular analysis using six sequence characterized amplified region (SCAR) markers revealed polymorphism among the genotypes. The SCAR markers SBB-14, SH-18 and SAB-03 have shown to be useful for marker assisted selection (MAS) of the target resistance genes. This study has also identified locally improved breeding lines as potential donors for resistance breeding to *C. lindemuthianum* in Kenya.

Key words: Anthracnose, sequence characterized amplified region (SCAR), marker assisted selection (MAS).

INTRODUCTION

French bean (also referred to as the snap or green bean) is valued for its production of tender, thin pods containing small seeds (Singh et al., 2015). It is a type of common bean (*Phaseolus vulgaris* L.), a crop grown widely as a source of protein (Broughton et al., 2003; Blair et al., 2007). The immature legumes have pods that are very tender and thin, with small seeds, features that make them to be considered as vegetables (Allaire and Brady,

2010). French bean is valued for its protein (23%); the pods are also rich in calcium (Ca), phosphorous (P) and iron (Fe). They are packed with dietary fiber which contains high levels of vitamin A, a strong antioxidant that fights against high cholesterol, heart disease and cancer. They also contain vitamin B6 (pyridoxine), thiamin (vitamin B-1), and vitamin C (Anderson et al., 2009). Locally in Kenya the green leaves are also used as vegetables (Mwaninki, 2017).

Anthracnose caused by the Ascomycete *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scrib is among the main diseases of the common bean (*Phaseolus vulgaris* L.) in Kenya (Balardin et al., 1997). The seed-borne pathogen is a major production constraint affecting common beans worldwide, especially in areas that experience high relative humidity and moderate temperatures (Singh and Schwartz, 2010; Conner et al., 2019). Severe infections of anthracnose in common bean can lead to almost complete crop loss because the pathogen produces masses of conidia that are capable of being easily disseminated to healthy tissues (Fernández et al., 2000; Sharma et al., 2008; Padder et al., 2017). Successful dissemination of the spores to the pods produces lesions that reduce the marketable yield of French beans (Singh et al., 2015). From the pods, the inoculum that is deposited on the seed acts as a main source of inoculum in the subsequent crop (Melotto and Kelly, 2000).

Management of the pathogen can be achieved through clean seed programs although this is not a viable option for many farmers in Africa because they lack well organized dry bean seed production systems (Ferreira et al., 2013). The French bean seed systems are well organized resulting in low incidences of anthracnose transmission through seed, although inoculum transmission from neighboring dry bean farms is likely to occur in susceptible cultivars. In this regard, majority of farmers in Kenya mainly rely on fungicides to reduce production and post-harvest losses to this disease (Wahome et al., 2011). The continued use of chemicals leads to emergence of disease resistant pathogen races, increased production costs and negative effect on the environment and human health (Burkett-Cadena et al., 2008). Several other control measures have been applied including cultural methods such as crop rotation, intercropping, elimination of plant debris, adjustment of planting dates, use of compost, and blending heterogeneous cultivars which have shown to reduce disease severity (Deeksha et al., 2009). However, these management practices come with several challenges and therefore, the use of host plant resistance is the most economical and environmentally sustainable method for controlling anthracnose of beans.

The successful development of anthracnose resistant cultivars depends on the understanding of the levels of variability in the pathogen and in the host. Populations of *C. lindemuthianum* comprise a collection of races, as recognized by their interaction with known major genes for host resistance (Batureine, 2009). A set of twelve differential varieties have been established as a means of characterizing the race structure of any given population of *C. lindemuthianum*; some of these races have arisen in the host's Andean gene pool, and others in the Mesoamerican gene pool (Melotto and Kelly, 2000; Gonçalves-Vidigal et al., 2009). Currently, nearly 250 races have been identified, 74 of which have been recorded in Kenya (Mogita et al., 2011a; Musyimi, 2014; De Lima et al., 2017). The dynamic race structure of *C. lindemuthianum* populations has implications for the deployment of resistance genes, particularly in Africa where the majority of farmers are unable to purchase pathogen-free seeds or fungicides (Otsyula et al., 2004; Mogita et al., 2011b).

Anthracnose resistance in common bean is governed by monogenic independent genes that are identified by the *Co* symbol (Kelly and Vallejo, 2004), although quantitative resistance *loci* (QRL) has also been reported (Oblessuc et al., 2014; González et al., 2015). Seventeen numbered genes (*Co-1* to *Co-17*) and other unnumbered genes (*Co-u*, *Co-w*, *Co-x*, *Co-y*, *Co-z*, *CoPv02c*$^{3\text{-}X}$, *CoPv02c*$^{7\text{-}X}$, *CoPv02c*$^{19\text{-}X}$, *CoPv0c*$^{2449\text{-}X}$ and *CoPv09c*$^{453\text{-}C}$) have been mapped in common bean (Geffroy et al., 2008; Lacanallo et al., 2010; Ferreira et al., 2013; Campa et al., 2014; Coimbra-Gonçalves et al., 2016; Zuiderveen et al., 2016). The resistance in all the *loci* is dominant except for the recessive *co-8* gene. Furthermore, co-localization of the anthracnose resistance gene and genes for angular leaf spot and rust resistance have been reported offering additional resistance to other pathogens (Gonçalves-Vidigal et al., 2011; Sousa et al., 2015; Valentini et al., 2017).

The mapping of the *Co* genes has majorly been achieved using molecular markers which offer an opportunity for marker-assisted selection (MAS) to enhance anthracnose resistance in common bean. A number of sequence characterized amplified polymorphism (SCAR) markers, random amplified polymorphic DNA (RAPD) markers and single nucleotide polymorphism (SNP) markers that are tightly linked to anthracnose resistance genes have been reported (Young and Kelly, 1997; Alzate-Marin et al., 2000; Melotto and Kelly, 2000; Garzón et al., 2008; Vallejo and Kelly, 2009). These markers offer new opportunities

*Corresponding author. E-mail: antonykimani22@gmail.com.

for the identification of genes for resistance and have been used successfully in common bean breeding programs (Miklas et al., 2003; Ragagnin et al., 2003; Njuguna, 2014). This study was therefore undertaken to determine the response of Kenyan French bean germplasm to *C. lindemuthianum* under natural environment, to determine their resistance to local races and analyze these entries for their banding patterns to molecular markers that are linked to specific anthracnose resistance genes. This information is important to unlock opportunities for the development of French bean cultivars with complex resistance through MAS.

MATERIALS AND METHODS

Plant material

The germplasm panel was made up of 33 entries comprising 16 commercial French bean genotypes commonly grown in Kenya, 3 local breeding lines, 2 landraces and the 12 standard differential cultivars for *C. lindemuthianum*. The French bean seed was sourced from various seed companies, research organizations and the National Gene bank of Kenya; while the seeds for the differential cultivars were sourced from the International Center for Tropical Agriculture (CIAT-Uganda). The differential cultivars were used as checks in the study because their gene pools and resistance genes have been documented (Kelly and Vallejo, 2004; Ferreira et al., 2013).

Field screening experimental sites

The plant materials were planted at three sites (Mwea, Kutus and Kakamega) in Kenya, chosen to provide a contrasting set of environments. Mwea and Kutus represent French bean growing zones in Kenya, while Kakamega represented a bean disease hot spot site based on previous studies in Kenya (Mogita et al., 2011b; Arunga et al., 2012; Kimno et al., 2016). The three sites experience a bimodal pattern of rainfall: the long rains season from March to June and the short rains season from September to December. The Kakamega site, located at the Non-Ruminant Research Institute (34°46´E, 0°16´N, elevation of 1555 meters above sea level), receives an annual rainfall of 1950 mm per year while the local mean temperature is 21°C. The experiments in Mwea were set up at the Kenya Agricultural and Livestock Research Organization (KALRO) Center in Mwea (37°20´E, 0°37´S; elevation of 1159 meters above sea level), with a total annual mean rainfall of about 850 mm and a mean air temperature of 22°C. The experiments in Mwea and Kakamega were set up during the long and short rain seasons in 2017. During the short season, the disease incidence in Mwea was extremely low and therefore, another site (Kutus), representing another French bean growing zone was included during the long rains of 2018. Kutus and Mwea sites are found in Kirinyaga County, a major French bean production region in Kenya. The Kutus site (35°37'E, 0°01´S; elevation of 1287 meters above sea level) climate is classified as tropical. The average annual temperature is 21°C and receives 1095 mm rainfall annually.

Experimental layout and data analysis

The experiments were set out using a randomized complete block design, with three replicates included in each environment. Each entry was represented by a 4.8 m long single row at a spacing of 45 cm between rows and 15 cm between plants. The dry bean cultivar GLP 585 was used as a guard row. Disease severity was assessed on a whole plot basis, and was quantified using the 1-9 CIAT scale as described by Pastor-Corrales et al*.,* (1998), in which a score of 1-3 is taken to indicate a high level of resistance, 4-6 a moderate level of resistance and 7-9 susceptibility (Table 1). A disease severity index (DSI) was calculated as follows: $(n_1 + 2*n_2 + 3*n_3 + 4*n_4 + 5*n_5 + 6*n_6 + 7*n_7 + 8*n_8 + 9*n_9)/N$, where 1 through 9 represent the disease score of individual plants and n_1 through n_9, the number of plants exhibiting a specific severity score (Ombiri et al., 2002). N represents the total number of plants examined per entry. The disease severity index (DSI) data collected from the field was subjected to analysis of variance (ANOVA) using routines implemented in SAS 9.4 software for Windows (Version 9.4. Cary, NC: *SAS* Institute Inc; 2014), considering four environments (Kakamega season 1, Kakamega season 2, Mwea and Kutus) and 34 entries as fixed factors. Means were compared using the Tukey's honestly significant difference metric at a confidence level of 95%.

Physiological characterization of *C. lindemuthianum*

A total of 16 diseased leaf samples with signs of attack, that is, lower leaf surface with brick red to purplish red discoloration on the leaves and dark brown eyespots on the pods (Figure 1a) were collected from the four contrasting enviroments. Furthermore, purposeful sampling was employed to collect eight more samples from farmers' fields around the study sites making a total collection of 16 samples. These samples were transferred to University of Embu Research Laboratory for isolation as described by Pastor-Corrales et al. (1998).

Isolates of *C. lindemuthianum* collected from the field were cultured on Potato Dextrose Agar (PDA) media (Figure 1b). For single spore isolation, successful colonies were plated on tap water agar (TWA) for 3 days and single hyphae picked under a stereo microscope and cultured on new PDA media. Colonies that failed to sporulate on PDA were cultured on V8 media to enhance sporulation. Incubation was done for 21 days at 22°C in alternating 12 h of light and darkness. Inoculum was prepared from 21 days old monospore cultures by adding 5 ml of distilled water on the surface of the culture and scrapped gently with a brush. Thereafter, the spore suspension was sieved using a cheese cloth and the inoculum was adjusted to a concentration of 1.2×10^6 spores/ml using a hemocytometer (Mahuku et al., 2002).

For race identification, screening of differential cultivars was carried out in a screen house. For each of the isolate, 12 differential cultivars were sown in seedling trays of five seedlings per row in three replicates. The growing media was made up of loam soil, manure and sand in the ratio of 2:1:1, respectively. Fourteen days after sowing, plants were inoculated with monosporic cultures of *C. lindemuthianum*. Using a small sprayer, the spores were sprayed on the stem and leaves of the plants until runoff. After inoculation, the plants were incubated and maintained in a moist chamber (20–22˚C, 95% relative humidity) for three days, and thereafter, maintained at 20-28˚C for 7 days. After this period, the disease symptoms were scored visually for anthracnose severity index on the leaves using a CIAT scale of 1-9 (Pastor-Corrales et al., 1998). Identification of the races was based on the susceptibility of each differential cultivar to the isolates of the pathogen using a binary nomenclature as described by Pastor-Corrales (1991). In summary, each differential cultivar has an assigned number (2^n), where n corresponds to the order number of the cultivar within the 12 standard bean differential series (Table 2). The race designation

Table 1. The CIAT disease evaluation scale (1-9) for assessing severity of *C. lindemuthianum.*

Reaction rating	Category	Description
1	Resistant	No visible symptom
2	Resistant	1% of the leaf veins affected, visible only on the lower leaf surface
3	Resistant	3% of the leaf veins affected, visible only on the lower leaf surface
4	Moderately resistant	1% of the leaf veins affected, visible on both surfaces of the leaves
5	Moderately resistant	3% of the leaf veins affected, visible on both surfaces of the leaves
6	Moderately resistant	Leaf veins affected, visible on both leaf surfaces, and the presence of some lesions on stems, branches, and petioles
7	Susceptible	Necrotic spots on most of the leaf veins and in a large part of the adjacent mesophyll tissue, which ruptures, as well as the presence of abundant lesions on the stem, branches, and petioles
8	Susceptible	Necrotic spots on almost all the leaf veins and very abundant on stems, branches, and petioles, leading to ruptures, leaf shedding, and reduction of plant growth
9	Susceptible	Most of the plant is dead

Figure 1. Field infected pods (A) and Isolates plated on PDA media (B).

was obtained as the sum of all binary numbers of cultivars with susceptible reactions.

Screening French bean accessions for resistance to different races of *C. lindemuthianum*

Identified races in the differential cultivars were re-isolated to obtain monosporic cultures by plating the samples on TWA and single hyphae obtained under microscope. Each single hyphae was plated on PDA and V8 media for sporulation for 21 days at 22°C in alternating 12 h of light and darkness. In order to screen the French bean genotypes, a modified detached leaf technique was used as described by Tu (1986). The detached leaf technique was earlier used to confirm the races of the collected samples and results obtained were similar to the analysis based on whole plants and therefore the method was suitable for screening the French bean accessions. The modified detached leaf technique involved inoculating detached leaves in clean Petri dishes in the laboratory. The procedure started with preparing clean plastic Petri dishes by lining them with a thin layer of cotton that was moistened with sterile water. In the evening hours (cool weather), young trifoliate leaves were carefully excised from three weeks old seedlings by pinching

Table 2. List of twelve anthracnose differential cultivars and their characteristics.

S/N	Differential cultivar	Resistant gene (s)	Binary no.	Gene pool	Growth habit[a]
1	Michelite	*Co-11*	1	Middle American	II
2	MDRK	*Co-1*	2	Andean	I
3	Perry Marrow	*Co-1*^{3}	4	Andean	II
4	Cornell 49-242	*Co-2*	8	Middle American	II
5	Widusa	*Co-1*^{5}, *Co-3*^{3}	16	Andean	I
6	Kaboon	*Co-1*^{2}	32	Andean	II
7	Mexico 222	*Co-3*	64	Middle American	I
8	PI 207262	*Co-4*^{3}, *Co-3*^{3}	128	Middle American	III
9	TO	*Co-4*	256	Middle American	I
10	TU	*Co-5*	512	Middle American	III
11	AB 136	*Co-6, co-8*	1024	Middle American	IV
12	G2333	*Co-3*^{5,} *Co-4*^{2}, *Co-5*^{2}	2048	Middle American	IV

Growth habit[a]: I = Determinate; II = Indeterminate bush; III = Indeterminate bush with weak main stem and prostrate branches; IV = Indeterminate climbing habit.

with a finger at the petiole. They were then placed in triplicates in Petri dishes with the lower sides of the leaves facing up. The monosporic spore suspension was sieved using a cheese cloth, and the inoculum was adjusted to a concentration of 1.2 x 10^6 spores/ml using a hemocytometer for each race. The inoculum was sprayed gently onto the leaves using a hand sprayer. The Petri dishes were then placed on the research laboratory tables (University of Embu) at room temperature (25 ± 2°C) for 10 days. The leaves were moistened daily using sterile water to prevent the leaves from wilting. The leaves were assessed on the 10th day post-inoculation for anthracnose severity by scoring the pathogen reaction from three trifoliate leaves (Figure 2) on a scale of 1-9 (Pastor-Corrales et al*.*, 1998).

Molecular analysis of French bean genotypes using SCAR markers

Seeds of the French bean accessions and the differential cultivars were sown in plastic pots in a greenhouse maintained at 25 ± 5°C at the University of Embu, Kenya. The growth media comprised of loam soil, manure and sand in the ratio of 2:1:1, respectively. Each pot was supplied with Diammonium Phosphate (DAP) fertilizer. The pots were watered daily to field capacity. For DNA extraction, three-week old young leaves for each French bean genotype and differential cultivars were collected. DNA was extracted according to Mahuku and Riascos (2004). The DNA samples were then amplified using six SCAR markers that are linked to anthracnose genes that have some levels of resistance in Kenya (Table 3). A total PCR reaction volume of 10 µl was used composing of 5 ηg/ul DNA, 20uM of each specific reverse and forward primer, 5x Bioline MyTaq Reaction buffer (5 mM dNTPs, 15 mM, $MgCl_2$, stabilizers and enhancers), 1.2 units of *Taq* DNA Polymerase (Bioline) made up to the volume using molecular grade water. PCR reactions were performed using the following regime: an initial hybridization step at 95°C for 1 min; followed by 35 cycles of denaturation step at 95°C for 30 s, annealing step ranging from 60-65°C for 1 min (Table 5), a third extension step at 72°C for 2 min, followed by a final extension at 72°C for 5 min. After amplification, a volume of 2 µl of 6x DNA loading dye (New England Biolabs-NEB) was added to each amplicons and resolved on 1.2% agarose gel containing ethidium bromide, run in 1x Sodium borate buffer at 100 volts for 2 h. The DNA bands were viewed under ultraviolet light and analyzed as binary data by recording 1 and 0 for presence and absence of the marker, respectively.

RESULTS AND DISCUSSION

Reaction of French beans and differential cultivars to anthracnose under field conditions

According to the ANOVA, there were significant differences ($p \leq 0.05$) in the severity of anthracnose infection both among the genotypes and environments. The significant genotype by environment interaction effect emphasizes the strong influence of the growing conditions on the expression of host resistance which is agreement with work conducted by Kiptoo et al. (2020). The highest mean DSIs were experienced at Kakamega (long rains season: 4.0, short rains seasons: 2.64), with a lower disease pressure experienced at Mwea (1.71) and Kutus (1.64). In the Kakamega long rains season experiment, 10 of the 17 French bean genotypes revealed moderate resistance (mean DSI 4-6) while 4 were completely susceptible (7-9). Only 3 genotypes showed resistance (1-3) (Table 4). The entries which showed consistency with respect to resistance were the landrace GBK032921, three breeding lines (T19, MU#2 and MU#13) and two commercial French bean cultivars (Julia and Monel). Among the differential cultivars, Perry Marrow (*Co-1*^{3}), Kaboon (*Co-1*^{2}), Widusa (*Co-1*^{5}, *Co-3*^{3}), TU (*Co-5*), AB136 (*Co-6*, *co-8*) and G2333 (*Co-4*^{2}, *Co-5*^{2}, *Co-3*^{5}) showed the most consistent level of resistance across the four environments. Differentials of the

Figure 2. Detached leaf technique A=infected leaves of a susceptible sample B=un-infected leaf sample from a resistant sample.

Table 3. List of SCAR markers linked to anthracnose resistance genes in common bean that were used in the study.

SCAR marker[a]	*Locus*	Size (bp)	Parental control genotype	Chromosome	Distance (cM)	Tm (°C)[b]	Sequence
SY20	*Co-4*	830	TO	Pv08	0.0	65	F:AGCCGTGGAAGGTTGTCAT R:CCGTGGAAACAACACACAAT
SH-18	*Co-4²*	1150	G2333	Pv08	4.2	65	F:CCAGAAGGAGCTGATAGTACTCCACAAC R:GGTAGGCACACTGATGAATCTCATGTTGGG
SAS-13	*Co-4²*	900	G2333	Pv08	0.4	65	F:CACGCACCGAATAAGCCACCAACA R:CACGGACCGAGGATACAGTGAAAG
SBB-14	*Co-4²*	1050/ 1150	G2333	Pv08	5.9	65	F:GTGGGACCTGTTCAAGAATAA TAC R:TGGCGCACACCATCAAAA AAGGTT
SAB-03	*Co-5*	450	TU	Pv07	5.9	65	F:TGGCGCACACATAAGTTCTCA CGG R:TGGCGCACACCATCAAAA AAGGTT
SZ-20	*Co-6*	845	AB136	Pv08	7.1	60	F:ACCCCTCATGCAGGTTTTTA R:CATAATCCATTCATGCTC ACC

[a]Source: Bean Improvement Cooperative; [b] Annealing temperature based on Laboratory troubleshooting at University of Embu

Mesoamerican gene pool exhibited high anthracnose resistance compared to those of the Andean gene pool. Similar results have been observed for other common bean pathogens in Kenya and East Africa (Kimno et al., 2016; Chilagane, 2017). The resistance exhibited across the different environments by differential cultivars Widusa, AB136 and G2333 is likely to reflect their harbouring of several *Co* genes, since their resistance is effective against multiple races of the pathogen (Alzate-Marin et al., 1997; Gonçalves-Vidigal et al., 1997; Young et al., 1998; Alzate-Marin et al., 2000; Vallejo and Kelly, 2009; De Lima et al., 2017). It is therefore important to consider gene pyramiding in resistance breeding against anthracnose.

The French bean varieties currently grown in Kenya expressed little resistance to anthracnose. Nevertheless,

Table 4. Response of French bean genotypes to *C. lindemuthianum* grown under field conditions in four environments.

S/N	Genotypes	Kakamega	Kakamega	Mwea	Kutus
		Long rains	Short rains	Long rains	Short rains
1	Amy	5.0^{a-c}	2.0^{b-d}	2.0^{a}	2.0^{ab}
2	Belcampo	5.3^{a-c}	2.0^{b-d}	1.0^{a}	2.0^{ab}
3	Blazer	5.7^{a-c}	2.0^{b-d}	1.0^{a}	4.0^{a}
4	Boston	4.3^{a-c}	2.7^{b-d}	3.0^{a}	3.0^{ab}
5	Edge	7.0^{ab}	3.7^{a-d}	1.7^{a}	3.0^{ab}
6	Enclave	7.0^{ab}	3.7^{a-d}	1.7^{a}	3.3^{ab}
7	Fanaka	5.0^{a-c}	2.7^{b-d}	1.0^{a}	2.3^{ab}
8	Goal	3.0^{a-c}	2.3^{b-d}	1.7^{a}	2.3^{ab}
9	Hawaii	4.0^{a-c}	2.0^{b-d}	1.7^{a}	2.0^{ab}
10	Julia	2.7^{a-c}	1.5^{d}	1.0^{a}	2.0^{ab}
11	Konza	4.5^{a-c}	2.0^{b-d}	1.0^{a}	2.0^{ab}
12	Lomami	6.7^{ab}	3.0^{b-d}	1.5^{a}	1.5^{ab}
13	Monel	1.7^{bc}	2.0^{b-d}	1.3^{a}	2.3^{ab}
14	Source	3.7^{a-c}	2.0^{b-d}	1.0^{a}	2.7^{ab}
15	Tahoe	7.0^{a}	2.4^{a}	1.0^{a}	2.3^{ab}
16	Venda	4.3^{a-c}	3.5^{a}	2.0^{a}	-
17	GBK 032921	2.3^{bc}	1.3^{d}	1.0^{a}	2.0^{a}
18	GBK 032952	2.7^{a-c}	5.0^{a-c}	1.5^{a}	2.0^{a}
19	T19	3.3^{a-c}	2.0^{b-d}	1.0^{a}	3.0^{ab}
20	MU#03	2.0^{bc}	3.0^{b-d}	1.5^{a}	2.0^{a}
21	MU#13	2.7^{a-c}	1.7^{cd}	1.0^{a}	1.7^{a}
22	Michelite	2.0^{b-d}	2.0^{a}	1.7^{a}	2.0^{b-d}
23	MDRK	3.3b^{cd}	2.5^{a}	2.0^{a}	3.3b^{cd}
24	Perry Marrow	1.3^{d}	-	1.7^{a}	1.3^{d}
25	Cornell 49-242	7.0^{a}	2.7^{a}	2.3^{a}	7.0^{a}
26	Widusa	1.3^{d}	2.0^{a}	2.0^{a}	1.3^{d}
27	Kaboon	2.0^{b-d}	1.0^{a}	1.7^{a}	2.0^{b-d}
28	Mex 222	3.3^{b-d}	3.5^{a}	3.3^{a}	3.3^{b-d}
29	PI 207262	5.3^{ab}	1.5^{a}	1.7^{a}	5.3^{ab}
30	TO	5.3^{ab}	2.3^{a}	2.0^{a}	5.3^{ab}
31	TU	1.0^{d}	-	2.0^{a}	1.0^{d}
32	AB136	1.3^{d}	1.0^{a}	1.5^{a}	1.3^{d}
33	G2333	1.0^{d}	1.0^{a}	1.0^{a}	1.0^{d}
	Means	4.0^{a}	2.7^{b}	1.8^{c}	1.6^{c}
	P value	*<.0001*	*<.0001*	*0.0425*	*0.7798*

Means sharing the same letter are not different at P ≤ 0.05 according to Tukey's test. S/N 1-16 = commercial French beans, 17-18 = landraces, 19-21 =breeding lines & 22-33 = differential cultivars. (-) = missing data due to confounding effects of other diseases.

the resistance expressed by some commercial cultivars, landraces and local breeding materials could be exploited as a basis for breeding French beans because, in French beans, yield is a complex trait brought about by additional pod quality traits (Singh et al., 2015). Furthermore, the differential cultivar Widusa, which is also a French bean from Europe (Drijfhout and Davis, 1989), can be exploited as a resistant donor in Kenya. Widusa is classified as an Andean differential cultivar but pedigree evidence suggests that it could be of Mesoamerican origin (Gonçalves-Vidigal and Kelly, 2006), supporting the importance of Mesoamerican genes in anthracnose resistance in Kenya. Further, ANOVA depicted significant environment main effect and a significant genotype by

Table 5. Characterization of *C. lindemuthianum* isolates using the differential cultivars used to *identify races.*

Isolate	Differential Cultivars[a]												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Race**
K1[b]	S[c]	R[d]	R	R	R	R	S	R	R	R	R	R	65
K2	R	S	R	R	R	R	R	R	S	S	R	R	768
K3	S	R	S	S	S	R	R	S	S	R	R	R	141
K4	S	R	R	R	R	R	S	R	R	R	R	R	65
K5	R	R	R	R	R	R	R	R	R	R	R	R	0
K7	R	R	R	R	R	R	R	R	S	R	R	R	256
E1	R	R	S	R	S	R	S	R	R	R	R	R	84
E2	R	S	R	R	R	R	R	R	R	R	R	R	2
E3	R	R	R	R	R	R	S	R	R	S	R	R	576
Kt1	S	S	R	R	R	R	R	R	R	S	R	R	515
Kt2	S	R	R	R	S	R	R	S	S	R	R	R	401
Kt3	S	R	R	S	R	R	S	R	R	R	R	R	73
Kt4	R	S	S	R	R	S	R	R	R	R	R	R	38
M2	S	R	R	R	S	R	S	R	R	R	R	R	81
M3	S	R	R	R	R	R	S	R	R	R	R	R	65
M4	S	S	S	R	S	S	R	R	R	R	R	R	55

[a]Differential cultivars: 1-Michelite, 2-MDRK, 3-Perrymarrow, 4-Cornel 49-242, 5-Widusa, 6-Kaboon, 7-Mex 222, 8-PI 207262, 9-TO, 10-TU, 11-AB136, 12-G2333. [b]Collected from: K-Kakamega, E-Embu, Kt-Kutus, M-Mwea. [c]S- Susceptible scale 4-9. [d]R-resistant scale 1-3.

environment interaction effect which emphasizes the strong influence of the growing conditions on the expression of host resistance. This is likely driven by the effect of the environment on the growth and dispersal of the pathogen. The high DSIs observed at the Kakamega site during the long rains season probably reflect the prevalence of warm, humid conditions coupled with high rainfall during the growing season. The ideal conditions for the growth of the *C. lindemuthianum* pathogen are an air temperature range of 13-26°C, the availability of abundant moisture and frequent rainfall which encourages the dispersion of conidia (Schwartz et al*.*, 2005; Sharma et al*.*, 2008). In contrast, Mwea and Kutus sites experience a much lower rainfall and are generally less humid, conditions which are less favourable for the development of anthracnose. These results therefore support the studies by Kimno et al. (2016) who recommended Kakamega as a hotspot for screening common bean diseases.

Physiological characterization of *C. lindemuthianum*

Evaluation using specific races was considered important in this study given the confounding effects brought about by other pathogens as observed in the field. Race characterization will also help one to understand the composition of races in the country to assist in breeding for resistance since anthracnose exhibits a vertical/qualitative form of resistance (Miklas et al*.*, 2006). The current study identified 14 distinct races from 16 successfully plated isolates revealing a high level of diversity of *C. lindemuthianum* out of which 43% were considered new races in Kenya (Table 5). Similar results have been identified in the neighboring country, Tanzania where 96% of the 42 races were considered new obtained from 50 isolates (Mpeguzi et al*.*, 2020). Previous studies conducted in Kenya by Musyimi (2014) identified 12 races most of them being different from the races that were obtained in the other studies too. Race 65 may be considered as widespread as it was identified in Kakamega and Mwea. Races 65 and 73 have also been documented to be prevalent in Brazil (Vieira et al*.*, 2018). Furthermore, race zero (0) was also identified in the present study as it could not infect any of the twelve anthracnose differential cultivars as previously reported in Kenya (Mogita et al*.*, 2011b) and Uganda (Nkalubo, 2006).

There is a need therefore, to review the current set of differential cultivars to include a susceptible cultivar in the differential set that can distinguish this race especially in Eastern Africa which is considered to be a secondary center of diversity for common beans (Allen and Edje, 1990; Wortmann, 1998; Sperling, 2001).

The race composition in the current study was predominantly Andean (70%). This could be probably due

to a large number of French bean genotype used in the study that have been reported to be largely of the Andean origin (Arunga et al*.,* 2015, Arunga and Odikara, 2020). In addition, the common bean composition in the country has been reported to be of Andean origin as well (Asfaw et al*.,* 2009). This supports the work carried out by Baradin and Kelly (1998) where they discovered that races isolated from Andean cultivars have a broader virulence on germplasm of the Andean gene pool and a reduced virulence on Mesoamerican varieties, which suggests a co-evolution between the pathogen and the host. Among the differential cultivars, the most affected cultivar was Michelite having been overcome by seven (7) of the 14 isolates with cultivars AB136 and G2333 being the most resistant as they were not affected by any of the isolates. The susceptibility observed in cultivar Michelite has also been reported to be situated on the lower tire of the differential cultivars as a susceptible check that also succumbed to seven isolates. Similar to field results, high resistance was observed in the Mesoamerican genotypes as compared to the Andean genotypes with the exemption of the cultivar Kaboon *(Co-1).* Race characterization revealed that cultivar MDRK succumbed to five races including race 768, first to be reported in the country. Resistance exhibited by the cultivars AB136 and G2333 is likely to reflect their harbouring of several Co genes.

Virulence of 14 races of *C. lindemuthianum* on French bean genotypes

The evaluated French bean genotypes showed different virulence reactions to the 14 *C. lindemuthianum* races used in this study (Table 6). Races 401 and 84 were the most virulent across all the genotypes while race 768 was the least virulent race. Among the French bean cultivars, Lomami was the most susceptible cultivar as it was infected by 86% of the races; whereas cultivar Boston, breeding lines MU#3, MU#13 and T19 were the most resistant. The results for the breeding lines demonstrated that the greenhouse/laboratory results were not very different from the field experiment. The breeding lines were resistant to most Mesoamerican races except for Andean races 2, 38 and 84. These breeding lines were obtained from one breeding program which utilized resistance genes from both gene pools including dry beans (Arunga et al*.*, 2015). This probably resulted in the introgression of the Mesoamerican genes into the French bean background.

Among the differential cultivars, the high susceptibility observed in cultivar Michelite was as expected given that the cultivar is situated on the lower tire of the differential cultivars as a susceptible check. Similar to field results, race characterization revealed high resistance among the differential cultivars of the Mesoamerican origin compared to those of the Andean origin with the exemption of the cultivar Kaboon. Cultivar Kaboon was only susceptible to two Andean races in the current study, that is, races 33 and 55. Susceptibility to race 55 has been reported in previous studies (Alzate-Marin et al., 1997). According to Melotto and Kelly (2000), the major dominant resistance gene present in the cultivar Kaboon is an allele of the *Co-1* gene, called *Co-1*^{2}. The authors revealed the importance of this gene in common bean breeding programs, as it gives resistance to both Andean and Central American races recently identified in Michigan. Similarly, cultivar Kaboon was resistant under field conditions across the four environments. Given the great composition of the French bean genotypes in Kenya are of the Andean origin, this cultivar can be used for gene introgression to improve resistance to *C. lindemuthianum*. Andean differential genotypes possess locus (*Co-1*), the only most utilized locus in the cultivars of this origin conferring resistance to *C. lindemuthianum* (Melotto and Kelly, 2000; Zuiderveen et al*.*, 2016). This could be the bottleneck facing genotypes within this gene pool. The resistance exhibited by the cultivars AB136 and G2333 is likely to reflect their harbouring of several *Co* genes.

Further, the study employed a better, simple and rapid screening method/technique to ensure efficient selection of resistant materials. Detached leaf technique has been deployed in the past as an alternative to conventional screening techniques in screen houses and has been reported to be successful with similar results (Rezene et al*.*, 2018). This is particularly important where seeds are limited and there is a need to preserve the whole plant for further evaluation. The method can also be carried out in the lab reducing the need for installation of screen houses. In common beans, the method has been used in screening for ALS and anthracnose (Tu, 1986; Rezene et al*.*, 2018). The technique has also been employed in other crops such as maize against southern corn leaf blight (Aregbesola et al*.*, 2020), soybean against white mold (Twizeyimana et al*.*, 2007) and *Fusarium* in wheat (Browne and Cooke, 2004).

Molecular markers analysis

All the six (6) SCAR markers used in this study generated polymorphisms between each parent and the resistant and susceptible genotypes which were specific for the *loci* to which they are linked. However, only SAB-03, SZ-20 and SH-18 markers were allele-specific (Table 7). The marker SY-20$_{830bps}$ a dominant marker reported to be very specific and tightly linked to the *Co-4 locus* at 0.0 cM (Costa et al*.*, 2010), could not discriminate the other alleles at this *locus* among the 12 differential set. This is

Table 6. Response of French bean genotypes, Landraces and Breeding lines to *C. lindemuthianum* races identified in Kenya.

S/N	Genotype	*Colletotrichum lindemuthianum races*														
		0	**2**	**38**	**55**	**65**	**73**	**81**	**84**	**141**	**256**	**401**	**515**	**576**	**768**	**RI%**
1	Amy	S	S	R	R	R	R	S	R	S	R	S	R	R	R	64
2	Belcampo	R	R	S	R	R	S	R	S	R	R	R	R	S	R	71
3	Blazer	R	S	S	R	R	R	R	R	R	S	S	S	R	R	57
4	Boston	R	R	R	S	R	R	R	R	R	R	S	R	R	R	86
5	Edge	R	R	R	S	R	R	R	S	R	R	S	S	R	R	71
6	Enclave	S	R	R	S	R	R	R	R	R	R	S	S	S	R	64
7	Fanaka	R	S	S	R	R	S	R	R	S	R	S	R	R	R	64
8	Goal	R	R	R	R	S	R	R	S	S	S	S	R	R	R	64
9	Hawaii	S	S	S	R	R	S	R	S	S	S	S	R	R	R	43
10	Konza	S	S	S	R	R	R	R	S	R	S	S	R	S	R	50
11	Lomami	S	S	R	S	S	S	S	S	S	S	S	S	R	S	14
12	Monel	S	R	R	S	S	R	R	R	R	R	S	S	R	S	57
13	Source	S	S	R	S	R	R	R	S	R	R	R	R	S	R	64
14	T19	R	R	R	S	S	R	R	R	R	R	R	R	S	R	86
15	MU#3	S	R	R	R	R	S	R	R	R	R	R	R	R	R	79
16	MU#13	R	S	S	R	R	R	R	S	R	R	R	R	R	R	79
17	GBK 032921	S	R	R	R	R	S	R	S	R	S	R	R	R	R	71
18	GBK 032952	S	R	S	R	S	S	R	S	S	R	S	R	R	R	57
Virulence Index (%)		42	42	37	32	21	37	16	53	32	32	63	26	26	11	

[a]Physiological races classified according standard system of classification. [b]RI = Resistance index. [c]Disease reactions: R = Resistant, S = susceptible. [d]VI = Virulence Index.

in agreement with similar work done by Beraldo et al., (2009). Figure 3C shows the clear band of cultivar TO (*Co-4*) (No 9), the tagged *locus* for the marker and the thicker bands of the alternative alleles possessed by cultivars PI 207262 (*Co-4*^{3}) and G2333 (*Co-4*^{2}) No 8 and12 respectively. This is in agreement with Vieira et al. (2018) who did similar work in Brazil. The marker however, did not detect the possible gene for resistance among the French bean genotypes used in this study.

Linkage to the *locus Co-6* in chromosome Pv 07 was evaluated using the marker SZ-20$_{845\ bps}$ linked at a distance of 7.1 cM derived from the differential cultivar AB 136 (Queiroz et al., 2004). The marker was only identified with the differential cultivar AB 136 revealing its specificity (Figure 3D). Cultivars Mitchelite (*Co-11*), MDRK (*Co-1*), Kaboon (*Co-1*^{2}), TO (*Co-4*), TU (*Co-5*) and G2333 (*Co-3*^{5}, *Co-4*^{2}and *Co-5*) amplified an unspecific band close to the *locus*. Among the French bean genotypes, the marker was detected in three cultivars Edge, Blazer and T19 (Tables 6 and 7). The locus *Co-6* has been known to offer broad-based resistance to most of the Andean races in the Latin America (23, 31, 64, 69, 73, 81, 89 and 453) but less effective against races of Mesoamerican origin (Gonçalves-Vidigal et al., 1997;Gonçalves-Vidigal et al., 2001). Thus, the *Co-6* locus can be utilized in breeding programs for French beans in Kenya after successful

Table 7. Evaluation of genotypes using SCAR markers.

S/N	Genotype	SCAR marker					
		SY-20	SZ-20	SAB-03	SBB-14**	SH-18	SAS-13
1	Amy	0	0	0	0	0	1
2	Belcampo	0	0	0	0	0	1
3	Blazer	0	0	1	0	0	0
4	Boston	0	0	1	0	0	1
5	Edge	0	1	0	0	0	1
6	Enclave	0	0	1	0	0	1
7	Fanaka	0	0	1	0	0	0
8	Goal	0	0	0	0	0	0
9	Hawaii	0	0	0	0	0	0
10	Julia	0	0	0	0	0	0
11	Konza	0	0	0	0	0	0
12	Lomami	0	0	0	0	0	0
13	Monel	0	0	0	0	0	0
14	Source	0	0	0	0	0	1
15	Tahoe	0	0	0	0	0	1
16	Venda	0	0	0	0	0	0
17	GBK 032 921	0	0	0	0	0	0
18	GBK 032 952	0	0	1	0	0	0
19	MU#03	0	0	1	0	0	1
20	MU#13	0	0	1	0	0	1
21	T19	0	0	0	0	0	1
22	Michelite	0	0	0	0	0	0
23	MDRK	0	0	0	0	0	0
24	Perry Marrow	0	0	0	0	0	1
25	Cornell 49-242	0	0	0	1	0	1
26	Widusa	0	0	0	0	0	0
27	Kaboon	0	0	1	0	0	0
28	Mex 222	0	0	0	0	0	0
29	PI 207262	1	0	0	0	1	0
30	TO	**1***	0	0	1	0	1
31	TU	0	0	**1***	0	0	1
32	AB136	0	**1***	0	1	0	1
33	G2333	1	0	1	**1***	**1***	**1***

S/N 1-16 = commercial French beans,17-18= landraces, 19-21 = breeding lines and 22-33 = differential cultivars. SBB-14** Co-dominant marker where 1-1150_{bps}, 0-1050_{bps}. 1*parent genotype; 1- presence of gene 0-absence of gene.

marker validation. The SCAR marker SAS-13_{900bps} tightly linked to the *Co-4*^{2} locus at 0.4 cM (Young et al*.,* 1998; Kelly et al*.,* 2003), was detected in two differential cultivars PI 207262 (*Co-4*^{3}) and G2333 (*Co-4*^{2}) specific to the tagged locus and one unspecific amplification in differential cultivar Widusa (*Co-1*^{5}, *Co-3*^{3}) (Figure 3A). Of the nine French bean genotypes under evaluation identified by the *only* local French bean breeding lines were consistent under field evaluation and on the reaction with specific races. Recent studies have shown that SAS-13_{900c} is not specific for *Co-4* allele (Awale and Kelly, 2001; Alzate-Marin et al., 2007) but, has been known to detect *Co-4* locus consistently regardless of the type of allele as was confirmed in this study.

The SCAR marker SBB-$14_{1050/1150bps}$ a codominant marker also linked to the gene *Co-4*^{2} revealed consistency in detecting the *Co-4 locus* among the three differential cultivars PI 207262 (*Co-4*^{3}) to (*Co-4*) and

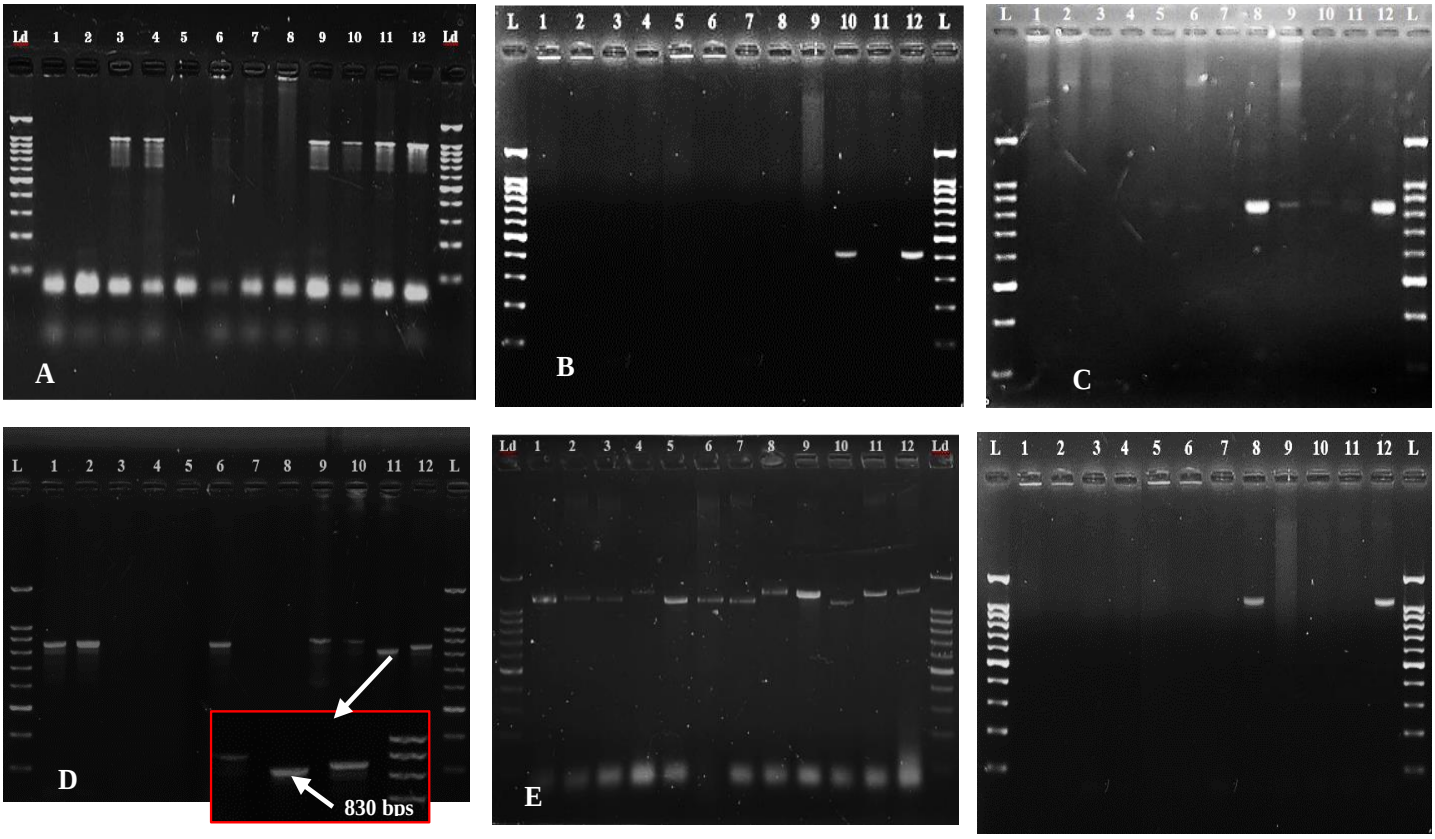

Figure 3. Amplification of molecular markers associated with the anthracnose resistance genes *Co-4*, *Co-5* and *Co-6* in differential cultivars; (A) SCAR marker SAS-13_{900bps} for *Co-4*^{2}, (B) SCAR marker SAB-03_{400bps} for *Co-5*, (C) SCAR marker SY-20_{830bps} for *Co-4*, (D) SCAR marker SZ-20_{850bps} for *Co-6*, (E) SCAR marker SBB-$14_{1050/1150\ bps}$ for *Co-4*^{2} and (F) SCAR marker SH-$18_{1100bps}$ for *Co-4*^{2}. L/Ld- 100bps DNA Ladder. Cultivars (1) Michelite, (2) MDRK, (3) Perry marrow, (4) Cornel 49-242, (5) Widusa, (6) Kaboon, (7) Mex222, (8) PI 207262, (9) TO, (10) TU, (11) AB136, (12) G2333.

G2333 (*Co-4*^{2}) (Figure 3E). However, amplification of cultivars Cornell 49-242 (*Co-2*) and AB136 (*Co-6* and *Co-8*) was unusual and none of the other genotypes were detected. The marker SH-$18_{1100bps}$ a dominant marker revealed consistency in detecting the *Co-4*^{2} *locus* among the differential cultivars PI 207262 (*Co-4*^{3}) and G2333 (*Co-4*^{2}); however, none of the other genotypes was identified with the marker. The *Co-4 locus* is the most utilized *locus* in common bean for anthracnose resistance. Its allele (*Co-4*^{2}) has been shown to provide greater resistance as compared to the original *Co-4* and *Co-4*^{3} allele and is among the broadest-based resistance genes described in common bean (Balardin and Kelly, 1998; Silvério et al., 2002; Vaz Bisneta and Gonçalves-Vidigal, 2020).

SAB-03_{400bps} maker that tags the gene *Co-5* at a distance of 5.9 cM (Vallejo and Kelly, 2001) was detected in the anthracnose differential cultivars TU (*Co-5*) and G2333 (*Co-5*) as expected (Figure 3B). The marker was detected in eight other genotypes, that is, five French beans cultivars, two breeding lines and one local cultivar (Table 7). Only the local breeding lines in the MU series revealed consistency with the marker both in the field and response to specific races.

The *Co-5* gene has not been widely deployed in the past in resistance breeding programs but, has been identified as one of the most effective genes in a survey of races of *C. lindemuthianum* from Central America and Mexico that is predominantly Mesoamerican (Balardin et al., 1997). However, the combination of *Co-5* and *Co-4* has been seen as the most effective in providing broadest resistance to *C. lindemuthianum* races. Thus, the comparative advantage of the limited use of *Co-5* in breeding makes the *locus* even more valuable to breeders for gene pyramiding bean programs.

SCAR markers tightly linked to anthracnose resistance genes offer another means to detect specific genes and for marker assisted selection as has been revealed in this study. Combination of *Co-4*^{2} and *Co-5* has been shown to offer the highest and broad resistance spectrum to

anthracnose as revealed in cultivar G2333 which agrees with the worked done by Sousa et al., (2015). The consistency of the local breeding lines with the markers could be traced to the fact that these breeding lines were obtained from one breeding program which utilized resistance genes from both gene pools including dry beans (Arunga et al., 2015). This may have led to the introgression of the Mesoamerican resistant genes into the French bean background that has been known to offer broader resistance as compared to the Andean genotypes. Four of the six SCAR markers tested in the present work (SH18 and SBB-14 for *Co-4*^{2}, SAB-03 for *Co-5*, and SY-20 for *Co-4*), have shown to be useful for assisted selection of the target resistant genes. They were specific for the *loci* to which they are linked, even though some did not discriminate alleles from the same *locus*, except for SH18, which was shown to be specifically linked to *Co-4*^{2}. However, more robust molecular markers such as SSRs, CAPS and SNPs should be developed for future selection of French bean genotypes for resistance to *C. lindemuthianum* as the current set of SCAR markers were only useful to very few French bean genotypes in Kenya.

Conclusion

The current study has revealed that, the race composition of *C. lindemuthianum* in Kenya is continuously changing and highly variable, hence the need for continuous evaluation. The highest composition of the races was of the Andean origin that could probably be due to the large number of French beans used in this study that are predominantly Andean, evidence for co- evolution between *C. lindemuthianum* and the host within the two centers of origin. Local breeding lines in the MU series could offer a good basis of gene introgression in Kenya, a great advantage in reducing linkage drag common with introgression from dry beans. Identification of race zero (0) in this study is another evidence that there is a need to review the current set of differential cultivars to include a susceptible cultivar in the differential set that can distinguish this race. Use of molecular markers proved effective and can assist future breeding efforts focusing in identification of other possible resistance *loci* to buffer against resistance breakdown through gene pyramiding especially in combination with both field evaluation and race characterization.

ACKNOWLEDGMENT

The author acknowledges Kirkhouse Trust for financial support and Kenya Agricultural and Livestock Research Organization (KALRO), Kakamega for field experiments. While the research reported here was funded by Kirkhouse Trust, the design, execution and interpretation of the research remains wholly the responsibility of the authors.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Allaire H, Brady T (2010). Classification and botanical description of legumes. Retrieved from https://academics.hamilton.edu/foodforthought/our_research_files/beans_peas.pdf

Allen D, Edje O (1990). Common bean in African farming systems. Paper presented at the Progress in improvement of common bean in Eastern and Southern Africa. Network on Bean Research in Africa Workshop Series.

Alzate-Marin AL, Baía GS, De Paula TJ, De Carvalho GA, De Barros EG, Moreira MA (1997). Inheritance of anthracnose resistance in common bean differential cultivar AB 136. Plant Disease and Genetics 81(9):996-998.

Alzate-Marin AL, Menarim H, Chagas JM, Barros EGd, Moreira MA (2000). Identification of a RAPD marker linked to the *Co-6* anthracnose resistant gene in common bean cultivar AB 136. Genetics and Molecular Biology 23(3):633-637.

Aregbesola E, Ortega-Beltran A, Falade T, Jonathan G, Hearne S, Bandyopadhyay R (2020). A detached leaf assay to rapidly screen for resistance of maize to *Bipolaris maydis*, the causal agent of southern corn leaf blight. European Journal of Plant Pathology 156(1):133-145.

Arunga EE, Odikara OS (2020). Characterization of Kenyan French Bean genotypes into gene pool affiliations using allele specific markers. African Journal of Biotechnology 19(9):653-660.

Arunga EE, Kinyua M, Ochuodho J, Owuoche J, Chepkoech E (2015). Genetic diversity of determinate French beans grown in Kenya based on morpho-agronomic and simple sequence repeat variation. Plant Breeding and Crop Science 7:240–250.

Arunga EE, Ochuodho J, Kinyua M, Owuoche J (2012). Characterization of *Uromyces appendiculatus* isolates collected from snap bean growing areas in Kenya. African Journal of Agricultural Research 7(42):5685-5691.

Asfaw A, Blair MW, Almekinders C (2009). Genetic diversity and population structure of common bean (*Phaseolus vulgaris L.*) landraces from the East African highlands. Theoretical Applied Genetics 120(1):1-12.

Awale HE, Kelly JD (2001). Development of SCAR markers linked to Co-4^ 2 gene in common bean. Annual Report-Bean Improvement Cooperative 44:119-120.

Balardin R, Jarosz A, Kelly J (1997). Virulence and molecular diversity in *Colletotrichum lindemuthianum* from South, Central, and North America. Phytopathology 87(12):1184-1191.

Balardin RS, Kelly JD (1998). Interaction between *Colletotrichum lindemuthianum* races and gene pool diversity in *Phaseolus vulgaris*. Journal of the American Society for Horticultural Science 123(6):1038-1047.

Batureine MJ (2009). Diversity of Colletotrichum lindemuthianum and reaction of common bean germplasm to anthracnose disease. (B.Sc. AGR- Hons: Crop Option), Makerere University, Uganda.

Beraldo ALA, Colombo CA, Chiorato AF, Ito MF, Carbonell SM (2009). Use of SCAR markers in common bean breeding for anthracnose resistance. Bragantia 68(1):53-61.

Blair MW, Porch T, Cichy K, Galeano CH, Lariguet P, Pankhurst C, Broughton W (2007). Induced mutants in common bean (*Phaseolus vulgaris*), and their potential use in nutrition quality breeding and gene discovery. Israel Journal of Plant Sciences 55(2):191-200.

Broughton WJ, Hernandez G, Blair M, Beebe S, Gepts P, Vanderleyden

J (2003). Beans (*Phaseolus spp*.)–model food legumes. Plant Soil 252(1):55-128.
Browne R, Cooke B (2004). Development and evaluation of an in vitro detached leaf assay forc pre-screening resistance to Fusarium head blight in wheat. European Journal of Plant Pathology 110(1):91-102.
Burkett-Cadena M, Kokalis-Burelle N, Lawrence K, Van Santen E, Kloepper J (2008). Suppressiveness of root-knot nematodes mediated by rhizobacteria. Biological Control 47(1):55-59.
Campa A, Rodríguez-Suárez C, Giraldez R, Ferreira JJ (2014). Genetic analysis of the response to eleven *Colletotrichum lindemuthianum* races in a RIL population of common bean (*Phaseolus vulgaris L*.). BMC Plant Biology 14(1):115.
Chilagane LA (2017). Genotype–pathogen characterization and markers identification for angular leaf spot disease resistance in common bean in Tanzania. Sokoine University of Agriculture.
Coimbra-Gonçalves GK, Gonçalves-Vidigal MC, Coelho RT, Valentini G, Vidigal Filho PS, Lacanallo GF, Sousa LL, Elias HT (2016). Characterization and mapping of anthracnose resistance gene in Mesoamerican common bean cultivar Crioulo 159. Crop Science 56(6):2904-2915.
Conner RL, Gillard CL, Mcrae KB, Hwang SF, Chen YY, Hou A, Penner WC, Turnbull GD (2019). Survival of the bean anthracnose fungus (*Colletotrichum lindemuthianum*) on crop debris in Canada. Canadian Journal of Plant Pathology 41(2):209-217.
Costa M, Tanure J, Arruda K, Carneiro J, Moreira M, Barros E (2010). Development and characterization of common black bean lines resistant to anthracnose, rust and angular leaf spot in Brazil. Euphytica 176(2):149-156.
De Lima CSA, Gonçalves-Vidigal MC, TA, Santana Gilio, Lacanallo GF, Valentini G, Martins Vanusa dSR, Song Q, Galván Marta Z, Hurtado-Gonzales OP, Pastor-Corrales MA (2017). Genetics and mapping of a new anthracnose resistance locus in Andean common bean Paloma. BMC Genomics 18(1):306.
Deeksha J, Hooda K, Bhatt J, Mina B, Gupta H (2009). Suppressive effects of composts on soil-borne and foliar diseases of French bean in the field in the western Indian Himalayas. Crop Protection 28(7):608-615.
Drijfhout E, Davis J (1989). Selection of a new set of homogeneously reacting bean (*Phaseolus vulgaris*) differentials to differentiate races of *Colletotrichum lindemuthianum*. Plant Pathology 38(3):391-396.
Fernández MT, Fernandez M, Casares A, Rodriguez R, Fueyo M (2000). Bean germplasm evaluation for anthracnose resistance and characterization of agronomic traits: A new physiological strain of *Colletotrichum lindemuthianum* infecting *Phaseolus vulgaris L*. in Spain. Euphytica 114(2):143-149.
Ferreira JJ, Campa A, Kelly JD, Varshney R, Tuberosa RJ, Wiley (2013). Organization of genes conferring resistance to anthracnose in common bean. Translational Genomics for Crop Breeding. https://onlinelibrary.wiley.com/doi/pdf/10.1002/9781118728475.ch9
Garzón LN, Ligarreto GA, Blair MW (2008). Molecular marker-assisted backcrossing of anthracnose resistance into andean climbing beans (*Phaseolus vulgaris L*.). Crop Science 48(2):562-570.
Geffroy V, Sévignac M, Billant P, Dron M, Langin T (2008). Resistance to *Colletotrichum lindemuthianum* in *Phaseolus vulgaris*: a case study for mapping two independent genes. Theoretical Applied Genetics 116(3):407-415.
Gonçalves-Vidigal M, Cardoso AA, Vieira C, Saraiva LS (1997). Inheritance of anthracnose resistance in common bean genotypes PI 207262 and AB 136. Brazilian Journal of Genetics 20.
Gonçalves-Vidigal M, Pedro Filho SV, Medeiros A, & Pastor-Corrales M (2009). Common bean landrace Jalo Listras Pretas is the source of a new Andean anthracnose resistance gene. Crop Science 49(1):133-138.
Gonçalves-Vidigal MC, Cruz AS, Garcia A, Kami J, Vidigal Filho PS, Sousa LL, McClean P, Gepts P, Pastor-Corrales M (2011). Linkage mapping of the *Phg-1* and *Co-1*^{4} genes for resistance to angular leaf spot and anthracnose in the common bean cultivar and 277. Theoretical Applied Genetics 122(5):893-903.
Gonçalves-Vidigal MC, Kelly JD (2006). Inheritance of anthracnose resistance in the common bean cultivar Widusa. Euphytica 151(3):411-419.
Gonçalves-Vidigal MC, Sakiyama NS, Vidigal Filho PS, Júnior ATA, Poletine JP, Oliveira VR (2001). Resistance of common bean cultivar AB 136 to races 31 and 69 of *Colletotrichum lindemuthianum*: the *Co-6* locus. Crop Breeding Applied Biotechnology 1(2).
González AM, Yuste-Lisbona FJ, Rodiño AP, De Ron AM, Capel C, García-Alcázar M, Lozano R, Santalla M (2015). Uncovering the genetic architecture of *Colletotrichum lindemuthianum* resistance through QTL mapping and epistatic interaction analysis in common bean. Frontiers in Plant Science 6:141.
Kelly J, Gepts P, Miklas P, Coyne D (2003). Tagging and mapping of genes and QTL and molecular marker-assisted selection for traits of economic importance in bean and cowpea. Field Crops Research 82(2-3):135-154.
Kelly JD, Vallejo VA (2004). A comprehensive review of the major genes conditioning resistance to anthracnose in common bean. HortScience 39(6):1196-1207.
Kimno S, Kiplagat O, Arunga E, Chepkoech E (2016). Evaluation of Selected French Bean (*Phaseolus vulgaris L*.) Genotypes for Resistance to Angular Leaf Spot (*Pseudocercospora griseola*) in Western Kenya. American Journal of Experimental Agriculture 13(4):1-6.
Kiptoo GJ, Kinyua MG, Kiplagat OK, Matasyoh LG (2020). Incidence and severity of anthracnose (*Colletotrichum lindemuthianum*) on selected common bean (*Phaseolus vulgaris* L.) genotypes. Agricultural Research 5(84):1-12.
Lacanallo G, Gonçalves-Vidigal M, Vidigal Filho P, Kami J, Gonela A (2010). Mapping of an Andean gene for resistance to anthracnose in the landrace Jalo Listras Pretas. Paper presented at the Annual Report of the Bean Improvement Cooperative.
Mahuku G, Jara C, Cajiao C, Beebe (2002). Sources of resistance to *Colletotrichum lindemuthianum* in the secondary gene pool of *Phaseolus vulgaris* and in crosses of primary and secondary gene pools. Plant Disease 86(12):1383-1387.
Mahuku GS, Riascos JJ (2004). Virulence and molecular diversity within *Colletotrichum lindemuthianum* isolates from Andean and Mesoamerican bean varieties and regions. European Journal of Plant Pathology 110(3):253-263.
Melotto M, Kelly J (2000). An allelic series at the *Co-1* locus conditioning resistance to anthracnose in common bean of Andean origin. Euphytica 116(2):143-149.
Miklas P, Kelly J, Beebe S, Blair M (2006). Common bean breeding for resistance against biotic and abiotic stresses: from classical to MAS breeding. Euphytica 147(1-2):105-131.
Miklas P, Kelly J, Singh S (2003). Registration of anthracnose-resistant pinto bean germplasm line USPT-ANT-1. Crop Science 43(5):1889-1891.
Mogita W, Ochuodho J, Gohole L (2011a). Analysis of Western Kenya Isolates of *Colletotrichum lindemuthianum* Casual Agent of Bean Anthracnose using Pathogenicty and BOX-A1R Analysis. African Journal of Education, Science and Technology 1(3):155-162.
Mogita W, Ochuodho J, Gohole L, Arunga E, Billy M (2011b). Incidence of bean anthracnose in Western Kenya and its management using aqueous extract of Aloe vera. African Journal of Education, Science and Technology 3(3):6-12.
Mpeguzi M, Susan NM, Mabagala R, Chilagane LA (2020). Races of *Colletotrichum lindemuthianum* (Sacc. Magnus) Briosi Cavara in major bean growing regions in Tanzania. African Journal of Plant Science 14(8):308-314.
Musyimi AS (2014). Marker assisted gamete selection for multiple disease resistence in Andean Bean genotypes and characterization of Colletotrichum lindemuthianum in Kenya. (BSc Agriculture), University of Nairobi, 2014. Retrieved from http://hdl.handle.net/11295/73588
Mwaninki OK (2017). Determination of pre-harvest interval for carbendazim used for control of fungal diseases in French beans. University of Nairobi.
Njuguna SM (2014). Marker Assisted Gamete Selection For Multiple Disease Resistance in Mesoamerican Bean Genotypes and Race Typing of Angular Leaf Spot Pathogen In Kenya. University of

Nairobi, Retrieved from http://hdl.handle.net/11295/73617
Nkalubo S (2006). Study of anthracnose (Colletotrichum lindemuthianum) resistance and its inheritance in Ugandan dry bean germplasm. Retrieved from http://hdl.handle.net/10413/4501
Oblessuc P, Baroni R, da Silva Pereira G, Alisson Fernando, Carbonell S, Brinez B, Luciano Da Costa ES, Garcia AA, Luis EA, Kelly JD (2014). Quantitative analysis of race-specific resistance to *Colletotrichum lindemuthianum* in common bean. Molecular Breeding 34(3):1313-1329.
Ombiri J, Zinkernagel V, Gathuru E, Achwanya O (2002). First report of race 485 of *Colletotrichum lindemuthianum* in Kenya and its implication in bean resistance breeding. Gartenbauwissenschaft 67(3):81-85.
Otsyula R, Rachier G, Ambitsi N, Juma R, Ndiya C, Buruchara R, Sperling L (2004). The use of informal seed producer groups for diffusing root rot resistant varieties during period of acute stress. Paper presented at the Addressing seed security in disaster response: Linking relief with development.
Padder B, Sharma P, Awale H, Kelly J (2017). *Colletotrichum lindemuthianum*, the causal agent of bean anthracnose. Journal of Plant Pathology 99(2):317-330.
Pastor-Corrales M (1991). Standardization of differential varieties and race designation of *Colletotrichum lindemuthianum*. Phytopathology 81:694.
Pastor-Corrales MA, Jara C, Singh SP (1998). Pathogenic variation in, sources of, and breeding for resistance to *Phaeoisariopsis griseola* causing angular leaf spot in common bean. Euphytica *103*(2):161-171.
Queiroz V, de Sousa C, Costa M, Sanglad D, Arruda K, de Souza T, Ragagnin V, de Barros E, Moreira M (2004). *Development of SCAR markers linked to common bean anthracnose resistance genes Co-4 and Co-6* (0084-7747).
Ragagnin VA, Sanglard DA, De Souza T, Moreira MA, De Barros EG (2003). Simultaneous transfer of resistance genes for Rust, Anthracnose and Angular Leaf Spot to cultivar Perola assited by molecular markers. Paper presented at the Annual Report-Bean Improvement Cooperative.
Rezene Y, Tesfaye K, Mukankusi C, Arunga E, Gepts P (2018). Simple and rapid detached leaf technique for screening common beans (*Phaseolus vulgarise L*.) in vitro against angular leaf spot (*Pseudocercospora griseola*) disease. African Journal of Biotechnology 17(35):1076-1081.
Schwartz HF, Steadman JR, Hall R, Forster RL (2005). Compendium of bean diseases: American Phytopathological Society (APS Press).
Sharma P, Sharma O, Padder B, Kapil R (2008). Yield loss assessment in common bean due to anthracnose (*Colletotrichum lindemuthianum*) under sub temperate conditions of North-Western Himalayas. Indian Phytopathology 61(3):323.
Silvério L, Gonçalves-Vidigal M, Filho P, Barelli MA, Thomazella C, Nunes WM (2002). Genetic resistance to Collectotrichum lindemuthianum race 2047 in G2333. Paper presented at the Annual Report-Bean Improvement Cooperative.
Singh A, Singh AP, Ramaswamy HS (2015). Effect of processing conditions on quality of green beans subjected to reciprocating agitation thermal processing. Food Research International *78*:424-432.
Singh SP, Schwartz HF (2010). Breeding common bean for resistance to diseases: a review. Crop Science 50(6):2199-2223.
Sousa LL, Gonçalves AO, Gonçalves-Vidigal MC, Lacanallo GF, Fernandez AC, Awale H, Kelly JD (2015). Genetic characterization and mapping of anthracnose resistance of common bean landrace cultivar Corinthiano. Crop Science 55(5):1900-1910.
Sperling L (2001). The effect of the civil war on Rwanda's bean seed systems and unusual bean diversity. Biodiversity Conservation 10(6):989-1010.
Tu J (1986). A detached leaf technique for screening beans (*Phaseolus vulgaris L*.) in vitro against anthracnose (*Colletotrichum lindemuthianum*). Canadian Journal of Plant Science 66(3):805-809.
Twizeyimana M, Ojiambo P, Tenkouano A, Ikotun T, Bandyopadhyay R (2007). Rapid screening of Musa species for resistance to black leaf streak using in vitro plantlets in tubes and detached leaves. Plant Disease 91(3):308-314.
Valentini G, Gonçalves-Vidigal MC, Hurtado-Gonzales OP, de Lima Castro SA, Cregan PB, Song Q, Pastor-Corrales MA (2017). High-resolution mapping reveals linkage between genes in common bean cultivar Ouro Negro conferring resistance to the rust, anthracnose, and angular leaf spot diseases. Theoretical Applied Genetics 130(8):1705-1722.
Vallejo V, Kelly JD (2001). Development of a SCAR marker linked to Co-5 locus in common bean. Paper presented at the Annual Report-bean Improvement Cooperative.
Vallejo V, Kelly JD (2009). New insights into the anthracnose resistance of common bean landrace G 2333. Open Horticulture Journal 2(1):29-33.
Vaz Bisneta M, Gonçalves-Vidigal MC (2020). Integration of anthracnose resistance loci and RLK and NBS-LRR-encoding genes in the *Phaseolus vulgaris* L. genome. Crop Science. doi:https://doi.org/10.1002/csc2.20288
Vieira A, Almeida L, Rodrigues L, Costa J, Melo L, Pereira H, Sanglard D, Souza T (2018). Selection of resistance sources to common bean anthracnose by field phenotyping and DNA marker-assisted screening. Embrapa Arroz e Feijão-Artigo em periódico indexado. doi:http://dx.doi.org/10.4238/gmr18066
Wahome S, Kimani P, Muthomi J, Narla R, Buruchara R (2011). Multiple disease resistance in snap bean genotypes in Kenya. African Crop Science Journal 19(4):289-302.
Wortmann CS (1998). Atlas of common bean (Phaseolus vulgaris L.) production in Africa: CIAT.
Young R, Melotto M, Nodari R, Kelly J (1998). Marker-assisted dissection of the oligogenic anthracnose resistance in the common bean cultivar,'G2333'. Theoretical Applied Genetics *96*(1):87-94.
Young RA, Kelly JD (1997). RAPD markers linked to three major anthracnose resistance genes in common bean. Crop Science 37(3):940-946.
Zuiderveen GH, Padder BA, Kamfwa K, Song Q, Kelly JD (2016). Genome-wide association study of anthracnose resistance in Andean beans (*Phaseolus vulgaris*). PLoS ONE 11(6):e0156391.

Vol. 20(2), pp.66-76, February, 2021
DOI: 10.5897/AJB2020.17200
Article Number: 7095E9466066
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Analysis of the degradation of polyethylene, polystyrene and polyurethane mediated by three filamentous fungi isolated from the Antarctica

Rodrigo Oviedo-Anchundia[1], Daynet Sosa del Castillo[1], Jaime Naranjo-Morán[1], Nora Francois[2], José Álvarez-Barreto[3], Alejandro Alarcón[4], Jhonny Saulo Villafuerte[5] and Milton Barcos-Arias[1]*

[1]Centro de Investigaciones Biotecnológicas del Ecuador, CIBE, Facultad de Ciencias de la Vida, FCV, Escuela Superior Politécnica del Litoral, ESPOL, Campus Gustavo Galindo km. 30.5 Via Perimetral, P. O. Box 09-01-5863, Guayaquil, Ecuador.
[2]Grupo de Aplicaciones de Materiales Biocompatibles, Facultad de Ingeniería, Universidad de Buenos Aires, Av. Paseo Colón 850, 5º Piso, Buenos Aires, Argentina.
[3]Colegio de Ciencias e Ingenierías, Universidad San Francisco de Quito, Diego de Robles y Vía Interoceánica, Quito, Ecuador.
[4]Área de Microbiología, Postgrado de Edafología, Colegio de Postgraduados, km 36.5 Carretera México-Texcoco, C.P. 56230, Montecillo, Estado de México, México.
[5]Universidad Laica Eloy Alfaro de Manabí, Facultad Ciencias de la Educación. Ave.Circunvalación, C.P. 130802. Manta, Ecuador.

Received 4 July, 2020; Accepted 13 August, 2020

Plastic polymers are petroleum-derived synthetic materials that have multiple uses in everyday life, but their excessive production has led to the accumulation of approximately 1,000 million tons of residues, causing negative ecological impacts. This study analyzed the biological degradation in liquid medium of polyurethane, polystyrene, and polyethylene samples by filamentous fungi isolated from Antarctica. The plastic samples were used without pretreatment or pretreated with an artificial aging UV chamber according to ASTM G155 for 500 h, inoculated or not with the Antarctic fungi (*Penicillium*, *Geomyces*, *Mortierella* species). Samples were incubated at 18°C for 90 days to determine potential fungal biodegradation. The physical-chemical and biological degradation of plastics were evaluated by analyzing the weight loss in function of time, and by determining possible changes in the chemical structure, using the technique of Fourier Transform Infrared Spectroscopy (FTIR). The polymers exposed to the artificial aging chamber resulted in the oxidative degradation of plastics (detected by morphological and structural changes), which favored their biodegradation. Out of the three fungal strains, *Penicillium* spp. presented the highest degradation percentage in aged plastics corresponding to 28.3% in polyurethane, and to 8.39 and 3.53% in polystyrene and low-density polyethylene, respectively.

Key words: Plastic aging, polymers, filamentous fungi, fungal biodegradation, deterioration.

INTRODUCTION

Plastic polymers derived from fossil petroleum sources are used in the manufacture of short time disposable products which represent one of the main components of solid wastes. The United Nations Environment Program

(UNEP) reported around 13 million tons of plastics that are dumped into the oceans annually. America, Japan and the European Union are the largest producers of this type of wastes. Besides, only 9% of these plastic wastes produced worldwide is recycled (ONU, 2018).

The problem caused by plastic waste can be solved by adding pro-oxidants or biologically degradable polymers during manufacturing, then, allowing their deterioration in the environment in less time (Chiellini et al., 2007). Another alternative is related to the use of microorganisms able to biodegrade some plastic polymers effectively (Bonhomme et al., 2003; Hermann et al., 2011). Biodegradation is a natural decomposition process of a substance or product by the action of biological agents, achieving its elimination or transformation to less dangerous products for nature (Arutchelvi et al., 2008). Moreover, plastic deterioration is related to the conditions of exposition. Environmental factors such as light, heat and/or biological activity induce changes in the functional properties of polymers, then, causing the rupture of bonds and chemical transformations. These changes are observed due to the formation of cracks and discoloration. Furthermore, solar radiation is one of the most harmful abiotic factors for polymers (Aradilla et al., 2007; Paço et al., 2017). Scientific reports indicate that the biological degradation of polymers depends on the polymer characteristics, the nature of the applied pretreatments, the polymer surface area, the type and microbial activity, as well as temperature, humidity, and nutrient availability (Bonhomme et al., 2003; Wu et al., 2017).

Researchers from Yale University reported the microscopic endophytic fungus *Pestaloptiosis* species at the Yasuni National Park (Ecuador), and indicated that this fungus degrades polyurethane (PU) under aerobic and anaerobic conditions (Russell, 2011). Different polymer-degrading microorganisms have been described; for instance, Koutny et al. (2009) indicated that *Rhodococcus* species strains may form a biofilm on a low density polyethylene sheet (LDPE), suggesting potential assimilation into microorganisms. On the other hand, Roy et al. (2008) mentioned that LDPE films were biodegradable by bacteria of the genus *Bacillus*. Other studies were also reported about the deterioration of the surface of polyethylene films due to fungal activity. Moreover, Ojeda et al. (2009) and Zahra et al. (2010) agree that the genera *Aspergillus* and *Penicillium* species, are capable of degrading polyethylene.

Thus, both fungi and bacteria are the most utilized microbial groups for biodegrading synthetic polymers. However, many microbial species have not yet been evaluated for such environmental purposes, resulting in limited available information regarding plastic-degrading microorganisms, and the involved enzymes on it. For this reason, this work performed an experimental bioassay for determining the degradation process of polyurethane, polystyrene, and polyethylene, by inoculating three strains of filamentous fungi, isolated from Antarctica.

MATERIALS AND METHODS

Biological sampling site

Fungal strains were isolated from Antarctic soils by researchers from the Biotechnology Research Center of Ecuador (CIBE) in conjunction with the Instituto Antártico Ecuatoriano (INAE) in charge of the Pedro Vicente Maldonado Scientific Station, located on Greenwich-Antarctica Island (South Shetland Islands, Antarctic Peninsula, 62° 26' 57" S, 9° 44' 27" W). This project began with 40 fungal isolates that are deposited at the microorganism bank of CIBE-ESPOL. The isolates were reactivated on Papa Dextrose Agar (PDA) culture medium, at a temperature of 18°C. Once reactivated, a preliminary fungal screening was carried out, where the capacity of the microorganisms to grow in the presence of the polymers was evaluated. After this preliminary evaluation, three fungal strains were selected for further utilization in the present research (Table 1).

Fungal strain identification

Samples of mycelium of each fungal strain grown on Petri dishes were taken and placed in 1.5 mL microtubes added with 350 µL of extraction buffer (200 mM Tris, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, pH 8.5), and 150 µL of 3 M sodium acetate, pH 5.2 and placed at -20°C for 10 min. Microtubes were centrifuged at maximum speed (14000 rpm) for 10 min. Then, supernatant was carefully transferred in a new 1.5 mL microtube in which 500 µL of isopropanol was added and incubated for 5 min at room temperature, then, centrifuged for 2 min at maximum speed (14000 rpm). After centrifugation, the supernatant was removed and the DNA pellet was washed with 50 µL of 70% ethanol, followed by centrifugation for 2 min with the lid open to evaporate the ethanol. Finally, 30 µL of sterile ultrapure water was added to the pellet, and resuspended and stored at -20°C. The DNA quantification was carried out in a NANODROP 2000. The molecular identification was based on the amplification of the ITS1 and ITS4 rDNA sequences (Cenis, 1992), amplified by a polymerase chain reaction (PCR) in a thermocycler (Mastercycler Nexus Thermocycler) using oligonucleotides ITS1 and ITS4 (White et al., 1990).

Each PCR reaction was added with 0.5 µL of genomic DNA, 13 µL of GOTAQ, 0.5 µL of each oligonucleotide, and 11 µL of ultrapure water, resulting in a final volume reaction of 25.5 µL with the following parameters for PCR: 98°C for 1 min, 35 cycles of 98°C for 45 s, 59°C for 40 s, 72°C for 1 min, and a final cycle of 72°C for 3 min. The amplified products were applied in a 1.5% agarose gel loading 3 µL of the PCR product and 0.5 µL of the 6x loading buffer (invitrogen). A 1 kb DNA Ladder molecular weight marker was used. The gel was run at 95 V for 15 min and subsequently visualized in an ultraviolet (UV) light transilluminator. The amplified products of the different fungal isolates were purified by using the DNA Purification by Centrifugation kit according to the manufacturer's instructions. The purified and concentrated products

*Corresponding author. E-mail: mbarcos@espol.edu.ec. Tel: +593-042-269610. Fax: +(593) 2850747.

Table 1. Origin and collection site of Antarctic fungi samples.

Internal Code	Sample origin	Sample collection site	Fungal genus
CIBE-7-23a	Fort Williams I. Greenwich Point	Wide plant diversity	*Penicillium* spp.
CIBE-2-32a	Barrientos Island	Vegetation close to station	*Mortierella* spp.
CIBE-12.1-11	Fort Williams Point Greenwich Island	Vascular plants	*Geomyces* spp.

(30 ng/µL) were sent to the company Macrogen (Seoul, Korea).

Once the fungal sequences were obtained, these were analyzed with the free software program FinchTV. This allows cutting and cleaning DNA chains. After, the BLAST program was applied (NCBI, Basic Local Alignment Search Tool, 1988) for searching homologs of DNA or proteins based on the alignment of local type sequences, looking for identity (≥ 99%) with type strains reported. The sequences obtained with greater similarity were aligned with the method muscle for multiple alignments of the MEGA program. The Neighbor-Joining method was used to construct the phylogenetic tree (Saitou, 1987) with bootstrap values of 1000 iterations.

Polymeric materials

Samples of synthetic polymers were prepared by using 10, 20, and 40 mg, of low-density polyethylene, polystyrene and polyurethane, respectively. Part of the experiment was carried out by subjecting the polymer samples to a QUV accelerated aging treatment in a chamber with Xenon lamps, Q-SUN Xenon Test Chamber Model Xe-3-HBS following the ASTM G155 standard, “Standard Practice for Operating Xenon Arc Light Apparatus for Exposure of Non-Metallic Materials” for 500 h. Aged and non-aged samples were previously subjected to a disinfection process by soaking them successively for a minute in a solution of 10% sodium hypochlorite (v/v), sterile water heated to 60°C, and 70% ethanol (v/v).

In vitro polymer degradation

The degradation under *in vitro* conditions was carried out in 150 mL glass vials containing 50 mL of mineral liquid medium composed of: (g/L) KH_2PO_4, 1.0 g; $NaNO_3$, 2.0 g; $MgSO_4$ ($7H_2O$), 0.05 g; KCL, 0.007 g; $FeSO_4$ ($7H_2O$), 0.01 g; NH4CL, 0.01 g; and plysurf, 0.01 g (Ishii et al., 2008), and autoclaved at 120°C for 20 min. At laminar flow chamber, the disinfected polymers were placed in the flasks with the mineral medium and inoculated with 50 mg of the respective fungal strains. Subsequently, the flasks were placed in an orbital shaker at 110 rpm, 18°C, for 90 days.

Polymer FTIR characterization

A polymer degradation analysis was performed using a Fourier Transform Infrared Spectroscopy (FTIR). The analyzed region corresponded to an interval between 4000 and 1000 cm^{-1}, in where the presence of functional groups such as the amino (-NH), carbonyl (-C=O), nitrile (-C = N), amide (R-CO-N2), etc., can be determined. The structural changes that may occur in plastics as a result of a biodegradation process (Shah et al., 2008), and may be detected by the evolution of the bands corresponding to the mentioned functional groups involved in polymer degradation (Stuart, 2005). These tests were carried out on a Perkin-Elmer model Spectrum 100 infrared light spectrometer.

Both the dry weight of the polymer sample, and its degradation by Fourier transform infrared spectroscopy (FTIR) were determined every 30 days. Polymer mass loss was measured by the following equation:

$$\text{Mass loss (\%)} = [(W_o - W_t)/W_o] \times 100$$

where "Wo" is the initial weight of the polymer sample and "Wt" is the weight of the polymer after treatment and "t" indicates the number of days that the polymer remained in the mineral solution.

Absorption tests were performed to measure the percentage of water absorbed by each polymer for determining its hydrophilic character. The samples were dried in an oven for 30 min at 120°C. The percentage of water absorption was determined by the following equation:

$$\text{Water absorption percentage (\%)} = [(W_i - W_f)/W_o] \times 100$$

where "Wi" is the wet material weight and "Wf" is the weight of the dry material.

Statistical analysis

Statistical analyses were carried out with the variance analysis method (ANOVA) at a significance level of 5% ($\alpha = 0.05$). Data were subjected to the SPSS program where the Duncan procedure for the mean comparison test was applied at 5% of significance level (Montgomery and Hines, 1993).

RESULTS AND DISCUSSION

Sequence analysis and fungal identification

Results of the fungal identification are as shown in Figure 1 and Table 1. The CIBE-7-23a strain showed greater closeness to *Penicillium adametzioides* (99% identity), the strain CIBE-2-32a was phylogenetically related to two species of the genus *Mortierella*, but with the closest species of *Mortierella turficola* (98% identity). Likewise, the CIBE-12.1-11 strain is related to *Geomyces* species (98% identity). A fungus of *Glomus* species (Glomeromycota) was used as an external group.

In vitro polymer degradation

Polyethylene

Figure 2 shows the degradation percentages for the low-density polyethylene (LDPE), after three months; samples pretreated with aging process and without aging inoculated with *Penicillium* spp. had a degradation of 3.5 ± 0.26% and 1.31 ± 0.06%, respectively (Figure 2a and b).

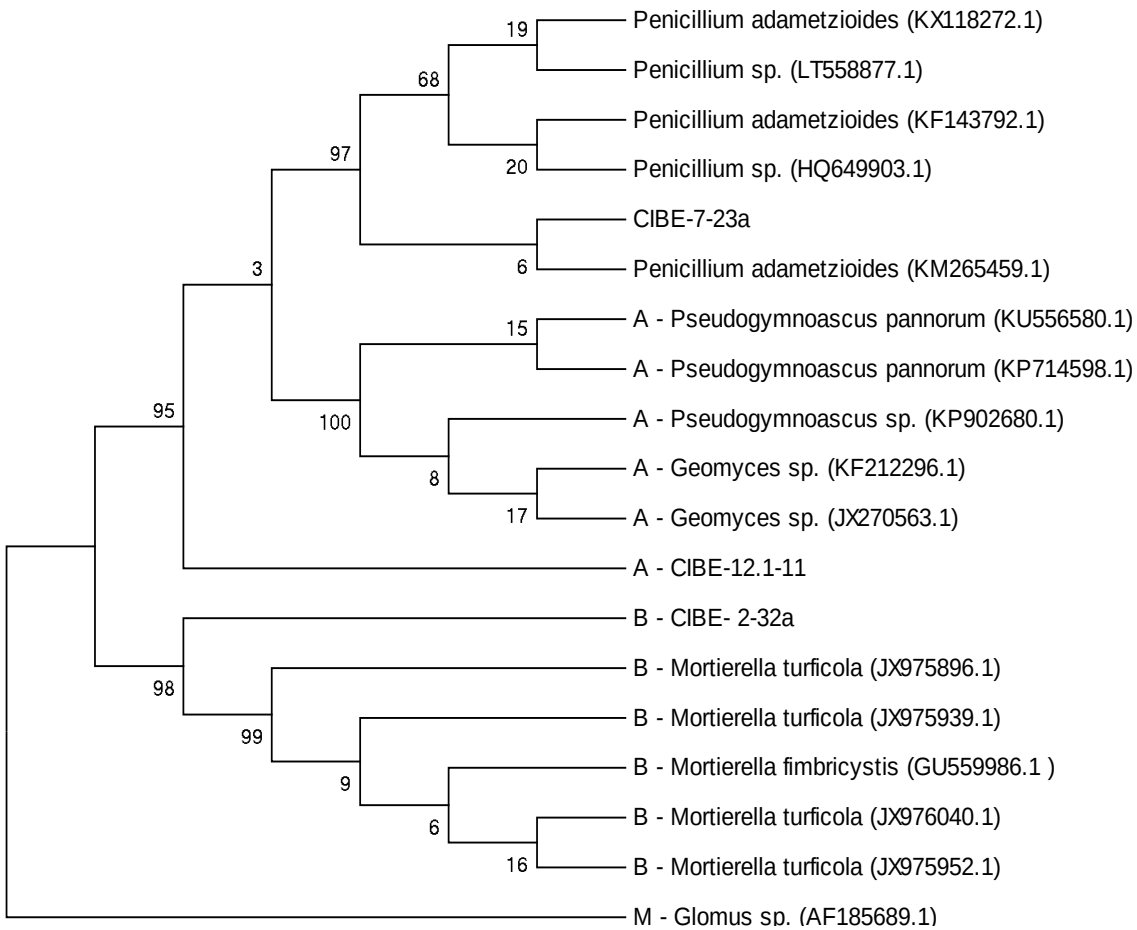

Figure 1. Phylogenetic tree for identification of three fungal strains generated by the maximum likelihood method.

In case of *Geomyces* spp. inoculation, the degradation of both LDEP conditions was 3.22 ± 0.32 and 1.55 ± 0.29%, respectively, whereas by inoculating *Mortierella* species, the degradation values were of 3.31 ± 0.06 and 0%, respectively. Statistical analysis shows significant differences between the aging treatments and the control, while in the treatments without aging there are no significant differences between the treatments and the control (Table 2). The recorded low degradation percentages could be associated with the properties of this polymer such as high resistance to be attacked or modified by chemical agents, and to its high temperature (95°C) for inducing thermal degradation (Coreno and Mendez, 2010). Thus, it seems that high temperatures (40°C) may take an important effect on the biodegradation process of polymers (Kharoufeh, 2003), but in our experimental conditions, the temperature oscillated from 16 and 18°C. Moreover, this work focused on utilizing Antarctic fungal strains that are adapted to a lower temperature rather than those high temperatures used in other experimental reports (Kumari et al., 2009). Thus, the adaptation process of our fungal strains may be related to the low effectiveness on polymer degradation when compared with results obtained from other microorganisms isolated from Ecuador or elsewhere under different temperature conditions (Villa et al., 2009; Gajendiran et al., 2016). In all cases, results about the percentage of biodegradation were significantly higher than those achieved in this research. On another hand, the efficiency of a single fungal strain on polymer degradation may be lower than that from a fungal consortium. In this regards, Uribe et al. (2011) achieved a polyethylene biodegradation of 4.7 to 5.4% by using microbial consortia isolated from a landfill soil, for 60 days at 20°C. Moreover, Gajendiran et al. (2016) identified a strain of *Aspergillus clavatus* from landfill sites, which was able to degrade polyethylene. One of the main strategies for LDPE degradation is that mediated for microorganisms which may use this polymer as the sole source of carbon (Roy et al., 2008). Then, filamentous fungi such as *Aspergillus* and *Penicillium* have been reported as biodegraders of aged polyethylene films (Ojeda et al., 2009; Motta et al., 2009; Corti et al., 2010).

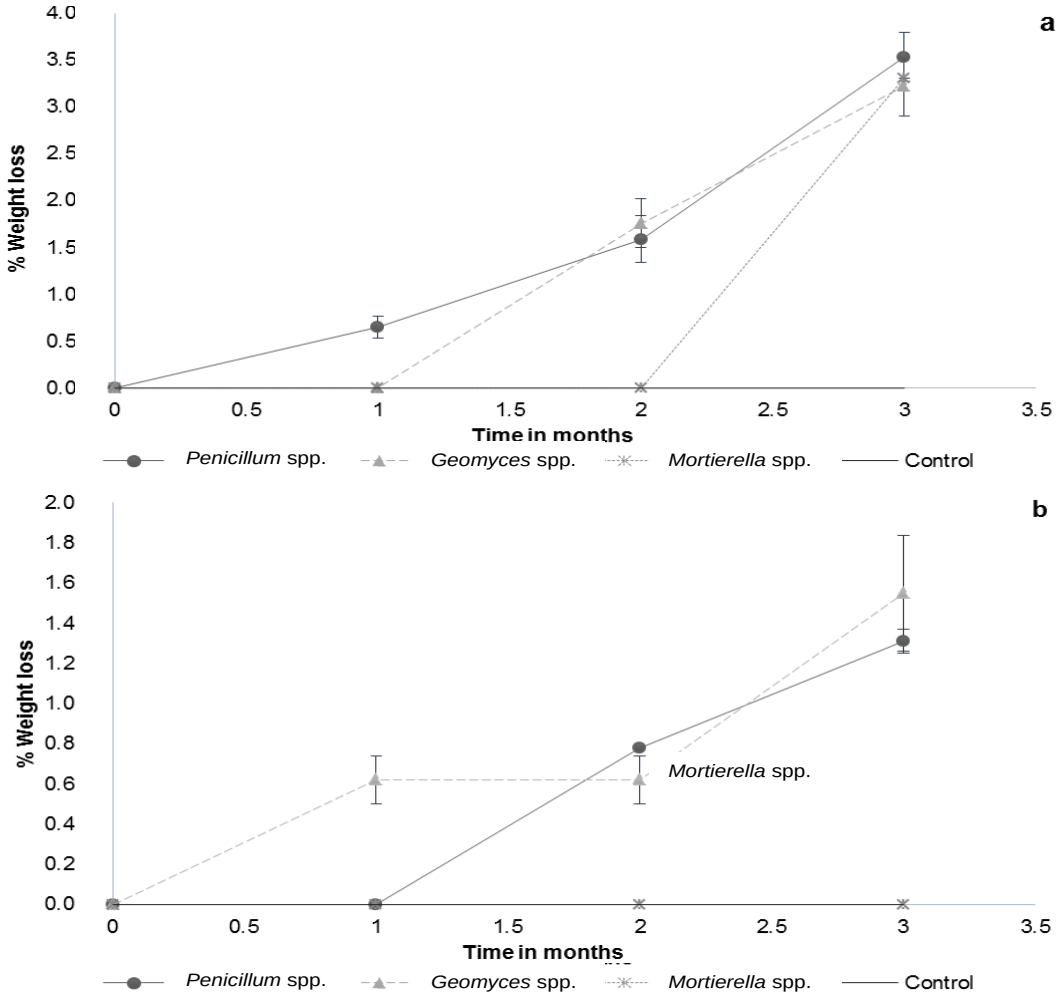

Figure 2. Weight loss percentage of polyethylene subjected to an aging pretreatment (a) or without pretreatment (b) and inoculated with three filamentous fungal strains isolated from Antarctica (*Penicillium* spp., *Geomyces* spp., and *Mortierella* spp. Means + Standard deviation, n=3.

Tabla 2. Degradation of polyethylene, polystyrene and polyurethane, with QUV accelerated aging for 500 h and without aging using three strains of filamentous fungi isolated from the antarctic, for 90 days.

Strains	Polymers degradation (%)		
	Polyethylene	Polystyrene	Polyurethane
	Aged treatment		
Geomices spp.	3.2 ±1.6^{b}	6.8 ±0.6^{b}	24.9 ±7.5^{bc}
Mortierella spp.	3.3 ±0.2^{b}	2.2 ±1.4^{a}	26.3 ±9.8^{bc}
Penicillium spp.	3.6 ±1.4^{b}	8.4 ±1.6^{b}	28.4 ±5.8^{c}
Control	0.0 ±0.0^{a}	0.0 ±0.0^{a}	12.3 ±1.4^{a}
	Unaged treatment		
Geomices spp.	1.5 ±0.8^{ab}	2.1 ±0.6^{a}	16.4 ±1.9^{ab}
Mortierella spp.	0.0 ±0.0^{a}	0.0 ±0.0^{a}	11.3 ±5.7^{a}
Penicillium spp.	1.3 ±0.3^{ab}	0.0 ±0.0^{a}	18.0 ±10.7^{abc}
Control	0.0 ±0.0^{a}	0.0 ±0.0^{a}	7.4 ±0.5^{a}

Different letters in the same column indicate significant statistical differences according to Duncan's test (α = 0.05); ± standard error; n = 3.

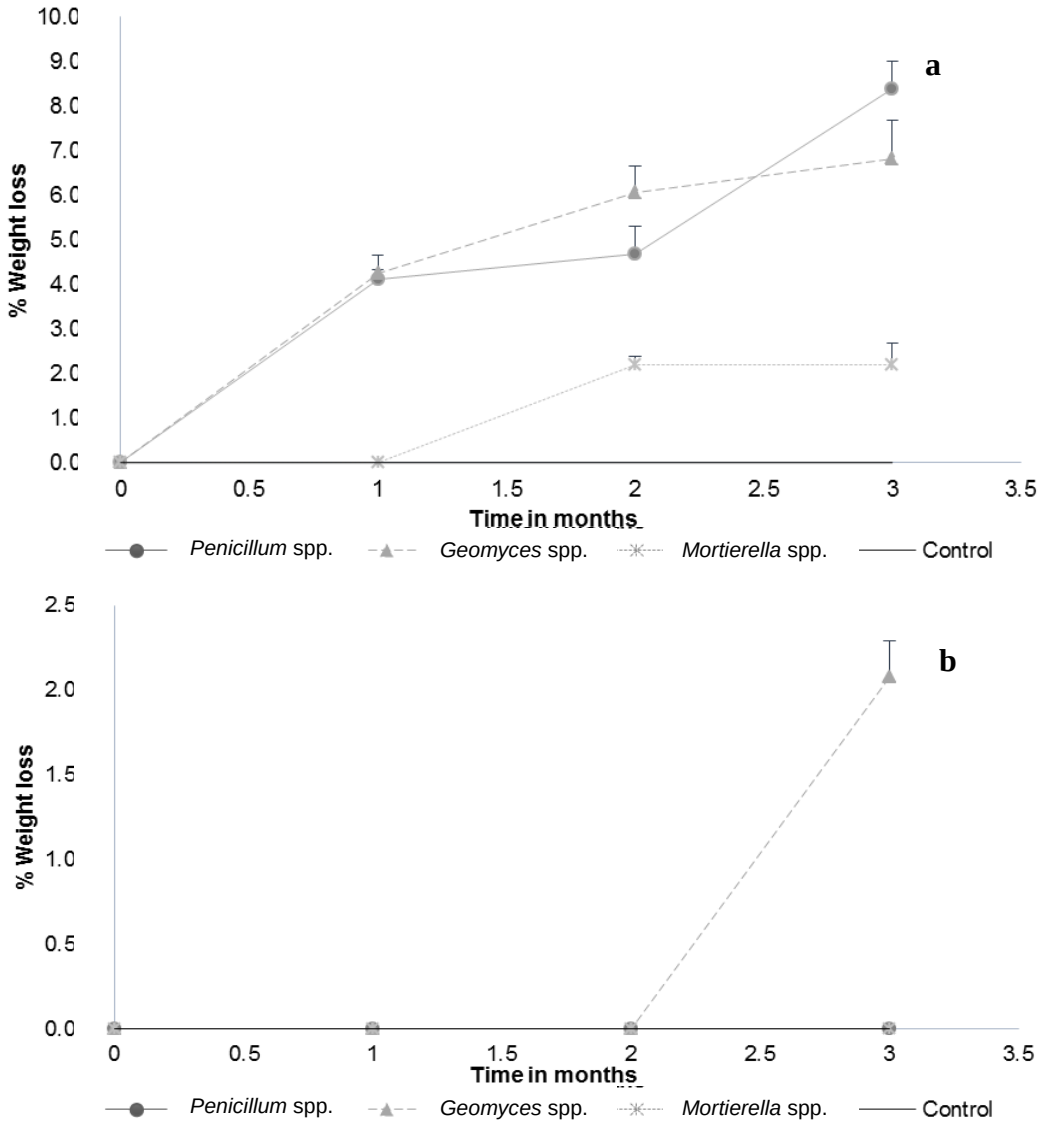

Figure 3. Weight loss percentage of polystyrene subjected to an aging pretreatment (a) or without pretreatment (b) and inoculated with three filamentous fungal strains isolated from Antarctica (*Penicillium* spp., *Geomyces* spp., and *Mortierella* spp.). Means + Standard deviation, n=3

Polystyrene

Percentages of polystyrene (PS) degradation by *Penicillium* spp., *Geomyces* spp., and *Mortierella* are presented in Figure 3a and b. It is inferred that the fungal degradation was more effective on aged polymer than without aging, possibly due to the molecular alterations induced by the action of pretreatment with UV energy applied during 500 h. There were significant differences among treatments. The strains *Penicillium* and *Geomyces* spp. contributed on the polymer degradation of 8.39% ± 0.61 and 6.82% ± 0.87, respectively, while *Mortierella* spp. degraded this polymeric material by 2.19% ± 0.5. There were significant differences between aged and control treatments (Figure 3a and Table 2). *Geomyces* spp., was the only fungus able of producing a significant loss of mass of polystyrene for those samples without aging pretreatment, showing a maximum biodegradation (2.08% ± 0.21) after the second month of experimentation (Figure 3b). As accounted for polyethylene, the aging pretreatment made the polymer samples more susceptible for fungal attack and therefore, inducing a biodeterioration process. Some research reported different types of microorganisms able to deteriorate polystyrene, such as the worldwide distributed fungus *Aureobasidium pullulans* (Castiglia and Kuhar, 2015), known as "black yeast", that produces a large number of hydrolytic enzymes (Ma et al., 2007; Singh at al., 2008).

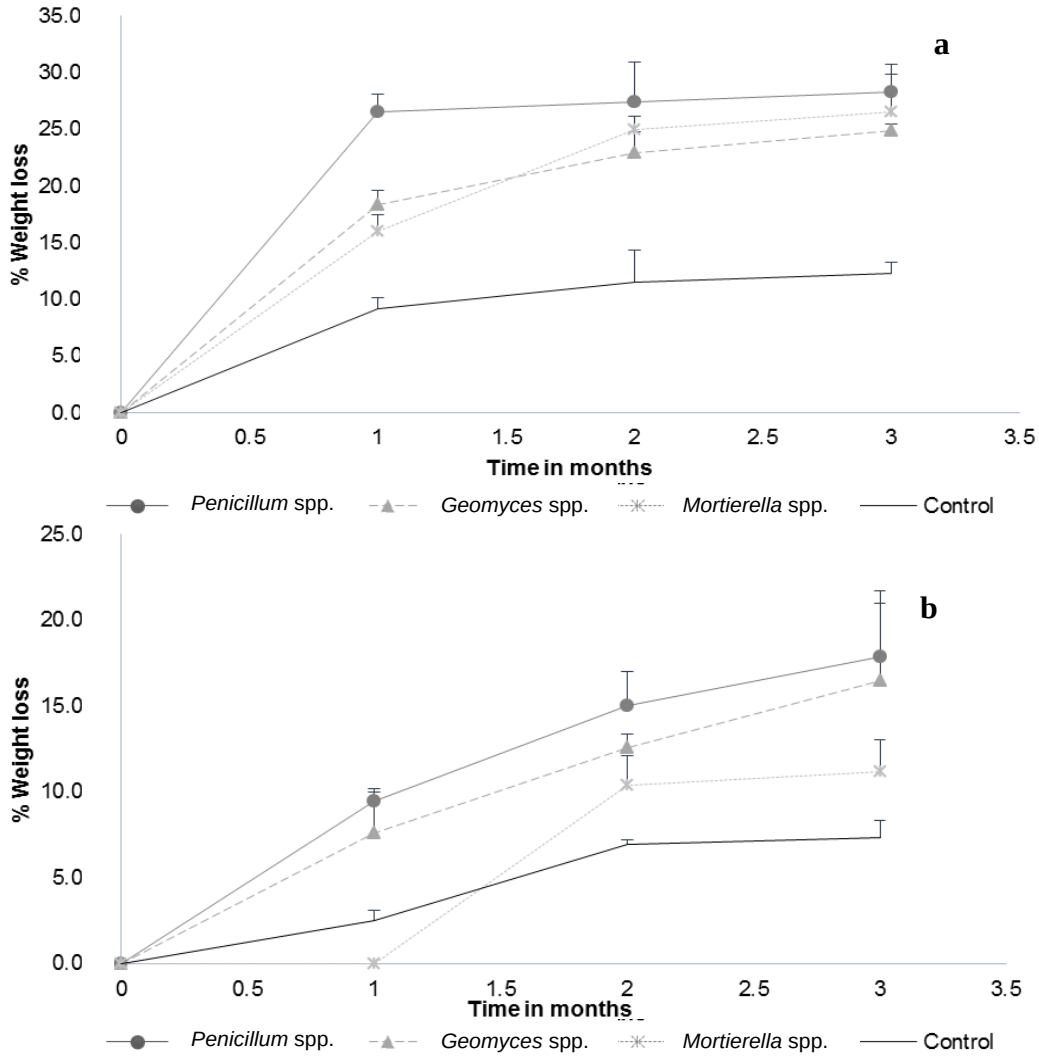

Figure 4. Weight loss percentage of polyurethane subjected to an aging pretreatment (a) or without pretreatment (b) and inoculated with three filamentous fungal strains isolated from Antarctica (*Penicillium* spp., *Geomyces* spp., and *Mortierella* spp.). Means + Standard deviation, n=3.

According to Castiglia and Kuhar (2015), *A. pullulans* is involved in the early stages of biodeterioration of plasticized materials like vinyl chloride and polystyrene. This fungus can be compared with our fungal strains for inducing the degradation of pretreated polymer since these fungi causes damage by mechanical action in where hyphae penetrate substrates that underwent an oxidation process (Webb et al., 2000). Furthermore, these results correlate with those obtained by FTIR, as described subsequently.

Polyurethane

Results of the polyurethane (PU) weight loss (%) at different sampling times are as shown in Figure 4a and b. The initial degradation rate of aged polyurethane was high due to the oxidation of water soluble oligomers (polymers of lower molecular weight that are hydrolyzed) present in the polymer matrix. On the other hand, the non-aging polyurethane has a monthly increase in the biodegradation process, but always smaller than that obtained for pretreated samples. Initially, the biodegradation occurs on the surface, since it is in contact with microorganisms, and the fungus spreads into the PU matrix (swelling process) (Amaral et al., 2012). The degradation mechanism of PU is achieved by fractions that are easy to transform and to hydrolyze since ester bonds are the first to break because they are more sensitive than urethane bonds. Moreover, the biodeterioration rate decreases because degradation of urethane bonds occurs at a slower rate (Zhang et al., 2009; Zhou et al., 2011).

Results from Figure 4 show that there are significant

statistical differences among treatments ($p < 0.05$). Pretreatment for aging polyurethane with the fungi after 3 months, produced a biodegradation around 26.5%; the most effective fungal strain was *Penicillium* spp. (28.34% ± 2.39). Taking into account the weight loss of the control sample (un-aged), the weight loss caused by fungal action was 16.09% (Figure 4a). All the aged treatments show significant differences with respect to the control (Tabla 2). In polyurethane without aging after 3 months, produced a biodegradation around 15.0%. The strain that degraded most effectively the polyurethane unaged was *Penicillium* spp. obtaining a 18.00% ± 10.7, but considering the results of the control sample, the effective loss per action of the fungi would be 10.7% (Figure 4b). In the case of the control sample, the polymer weight loss recorded with and without pretreatment was of 12.25% ± 1.40 and 7.35% ± 0.5, respectively. There are no statistically significant differences between the non-aging treatments and the control.

Scientific reports indicate that both bacteria and filamentous fungi are capable of degrading polyurethane; for instance, the fungal species like *Geomyces pannorum*, *Nectria* species, *Penicillium inflatum*, *Plectosphaerella*, *Penicillium venetum*, *Neonectria ramulariae*, and *Penicillium viridicatum* (Cosgrove et al., 2007); in all cases, the loss of mass would be related to the utilization of PU as a source of carbon or nitrogen (Urgun-Demirtas et al., 2007), and to the release of enzymes responsible of such degradation like proteases, esterases, and ureases (Ruiz et al., 1999). Most of the related experiments on PU degradation are focused on studies regarding bacteria. However, recent research has focused on the use of fungi (Amaral et al., 2012; Khan et al., 2017). Fungi are only capable of using organic carbon sources; thus, PU can be used as a potential carbon source for fungal growth (Loredo et al., 2017). Cosgrove et al. (2007) demonstrated in a soil microcosms that the sole application of yeast extract or its combination with Impranil, resulted in increased PU degradation up to 62%. Moreover, Loredo et al. (2017) applied three fungi (*Trichoderma* species, *Aspergillus ustus*, and *Paecelomyces* species) obtaining a 40% weight loss of rigid PU, after 30 days.

Analysis of the FTIR spectra of polymers

Polyethylene

The FTIR spectra obtained for LDPE films during the biodegradation process confirmed the existence of a possible microbial attack on polymers. The reduction of peaks corresponding to the C-H, C-O, and C = C groups would be related to polymer degradation. Figure 5a and b shows the spectra of the polyethylene samples used as control that, has not been subjected to the action of fungi. In these spectra, a series of characteristic peaks corresponding to the structure of the polyethylene can be observed. For pretreated polymer (Figure 5a), a wide absorption band appears between 1725 and 1700 cm^{-1} (centered around 1715 cm^{-1}), indicating the modification of the polyethylene structure. The application of UV radiation, which has more energetic than visible radiation (> 298 kJ/mol), an oxidative polymer degradation occurred, generating the appearance of a band corresponding to the group C = O (Gulmine et al., 2003). Other reports indicate a decrease in the intensity of bands located at 719 cm^{-1}, to the flexion of the CH group and 1472 cm^{-1}, corresponding to the carbonyl group (-C = O) when the LDPE was in contact with bacteria (Das and Kumar, 2015). However, Figure 5a shows no considerable differences among spectra, by which it is difficult to assure biological action on this polymer related to the biodegradation. Nevertheless, the loss of weight achieved in the polymer could be due to the chemical action of either salts contained in the mineral medium or excretion or secretion of acids and pigments by fungi, which may modify the chemical properties and cause biodeterioration of polymers (Abrusci et al., 2009; Scott and Wiles, 2001).

Polystyrene

The spectra analysis showed that the material did not suffer a biodegradation process since no significant variations in the FTIRR spectra were observed in both control sample and samples subjected to fungal action. However, by analyzing the obtained spectra (Figure 5b and c) and the weight loss recorded for the different samples, it can be concluded that the material underwent a biodeterioration process due to the interaction of mycelium with the polymer. This interaction may exert a chemical action resulted for excretion or secretion of acids and pigments by fungi which are deposited on the support modifying its chemical properties (Gu, 2007; Abrusci et al., 2009).

Polyurethane

The UV aging treatment caused significant morphological and structural changes on polyurethane. This physical process generated an oxidative degradation of the polymer, which increased its susceptibility to microbial attack. Bands between 3500 and 3200 cm^{-1} are attributed to the overlap of the signal corresponding to the stretching and bending vibrations of bonds O-H, respectively (Jagtap et al., 2011; Spontón et al., 2013). The spectra of the control samples subjected or not to abiotic pretreatment (Figure 5e) show that aged samples had a widening of the band centered at 3300 cm^{-1}, corresponding to the overlapping of the stretching of NH and OH groups. This widening would be related to the

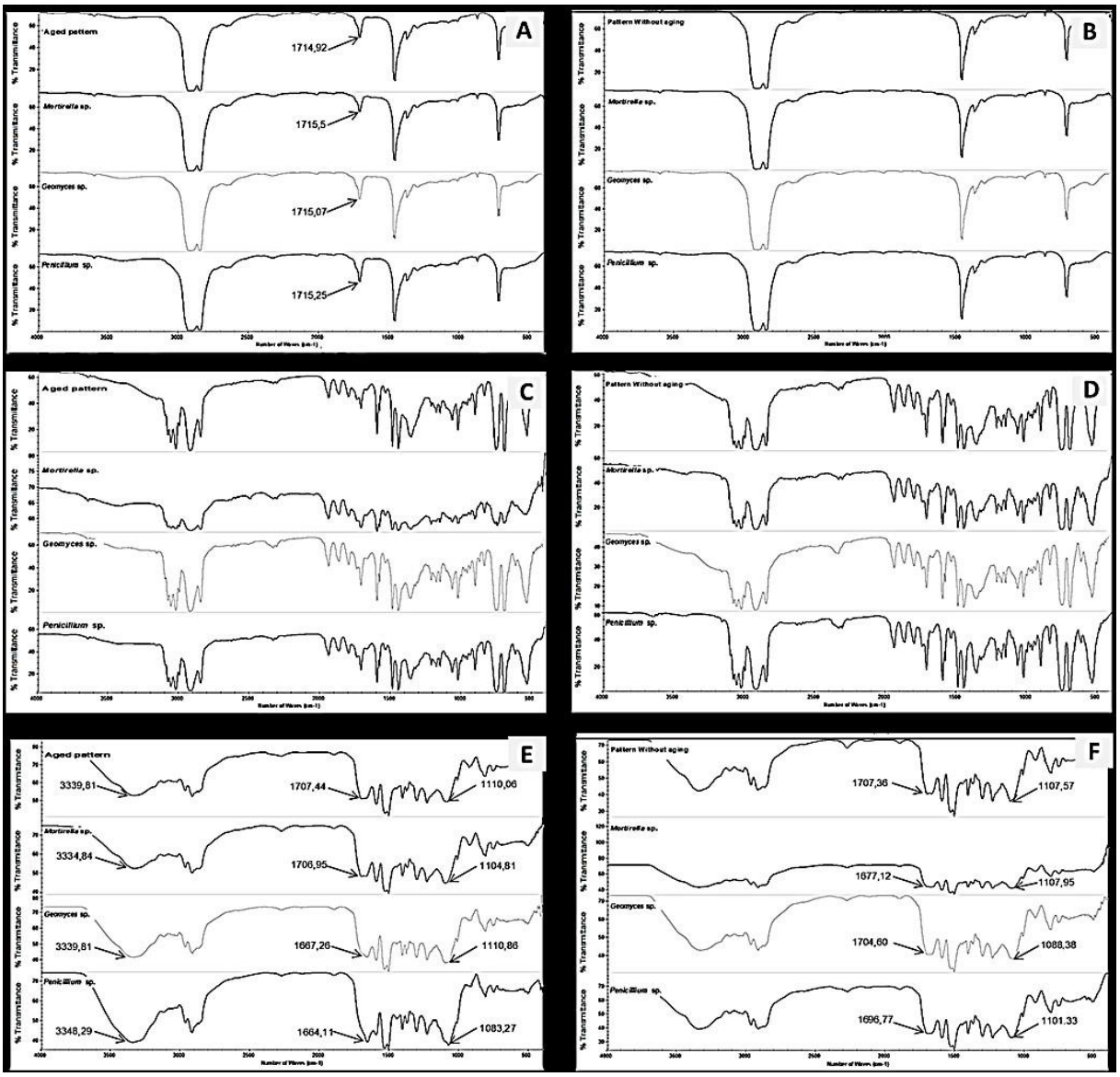

Figure 5. FTIR spectrum. (A-B) Polyethylene, (C-D) Polystyrene, (E-F) Polyurethane subjected to an aging pretreatment or without pretreatmente respectively, and inoculated with *Mortierella* spp., *Geomyces* spp., or *Penicillium* spp., after three months of incubation.

appearance of primary NH groups formed during the hydrolysis of urethane group that is benefited by the aforementioned pretreatment (Oprea and Oprea, 2016; Tavares and Schleder, 2016). The hydrolysis equation produced is as follows:

$$R-NH-\underset{\underset{O}{\|}}{C}-O-R' \xrightarrow[H_2O]{\text{papain}} R\,NH_2 + CO_2 + R'OH$$

The FTIR spectra obtained for PU samples subjected to aging treatment, show differences in the area between 1720 and 1500 cm^{-1} that allow the comparison of the effect caused by the action of the three fungal strains and the sample used as control. *Penicillium* and *Geomyces* were the two strains that produced the greatest weight loss, the spectra show a signal around 1664 cm^{-1} corresponding to the amide group, and this signal is related to a hydrolysis process (Oprea and Oprea, 2016). The increase of the signal located at 1541 cm^{-1} in those samples subjected to the pretreatment in contact with the two mentioned fungi, corresponded to the vibration of the NH, which is probably a consequence of the aforementioned hydrolysis.

On the otherhand, by comparing the spectra of both graphs (Figure 5e and f) some displacements were found

towards smaller numbers of waves of the peaks located in 1707.44 cm^{-1} for the aged control sample, and in 1707.36 cm^{-1} for the control sample without aging. These peaks are related to hydrogen bond interactions between NH groups and carbonyl groups (Loredo et al., 2017), as a consequence of a biodegradation action. In both spectra there were no variations in the area between 1000 and 600 cm^{-1} that corresponds to the polymer fingerprint (Vaghani et al., 2012).

Based on the evidence in this work, we can see that there are microorganisms in nature that have the ability to degrade certain wastes that are dangerous for the environment. A biotechnological approach based on the identification of the enzymes responsible for the degradation of these residues could make the degradation processes more efficient.

Conclusions

The present study evaluated the ability of three fungal strains to use LDPE, PS, and PU as the only carbon source. It was possible to identify that the aged materials are more susceptible to fungal attack in comparison to those without treatment. However, not all polymers were biodegraded as occurred for LDPE and PS that only accounted in a biodeterioration process. Unlike, the PU was susceptible to a biodegradation action, as demonstrated in the FTIR spectra. Thus, PU in the aging treatment was the most degraded polymer by the activity of the three fungal strains. The fungal strain that produced the highest degradation of polyurethane (28.34%), was *Penicillium* spp.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abrusci C, Marquina D, Santos A, Del Amo A, Corrales T, Catalina F (2009). Biodeterioration of cinematographic cellulose triacetate by *Sphingomonas paucimobilis* using indirect impedance and chemiluminescence techniques. International Biodeterioration Biodegradation 63(6):759-764.

Amaral JS, Sepúlveda M, Cateto CA, Fernandes IP, Rodrigues AE, Belgacem MN, Barreiro MF (2012). Fungal degradation of lignin-based rigid polyurethane foams. Polymer Degradation and Stability 97(10):2069-2076.

Aradilla D, Estrany F, Oliver R (2007). Degradación de residuos de materiales plásticos. Revista Ingeniería Química 448(1):186-190.

Arutchelvi J, Sudhakar M, Arkatkar A, Doble M, Bhaduri S, Uppara PV (2008). Biodegradation of polyethylene and polypropylene. Indian Journal of Biotechnology 7(1):9-22.

Bonhomme S, Cuer A, Delort A. M, Lemaire J, Sancelme M, Scott G (2003). Environmental biodegradation of polyethylene. Polymer Degradation and Stability 81(3):441-452.

Castiglia VC, Kuhar F (2015). Deterioration of expanded polystyrene caused by *Aureobasidium pullulans* var. melanogenum. Revista Argentina de Microbiologia 47(3):256-260.

Coreno AJ, Méndez M (2010). Relación estructura-propiedades de polímeros. Revista Educación química de UNAM 21(4):291-299.

Corti A, Muniyasamy S, Vitali M, Imam SH, Chiellini E (2010). Oxidation and biodegradation of polyethylene films containing pro-oxidant additives: Synergistic effects of sunlight exposure, thermal aging and fungal biodegradation. Polymer Degradation and Stability 95(6):1106-1114.

Cosgrove L, McGeechan PL, Robson GD, Handley PS (2007). Fungal communities associated with degradation of polyester polyurethane in soil. Applied and Environmental Microbiology 73(18):5817-5824.

Cenis JL (1992). Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Research 20(9):2380.

Chiellini E, Corti A, D'Antone S (2007). Oxo-biodegradable full carbon backbone polymers – biodegradation behaviour of thermally oxidized polyethylene in an aqueous medium. Polymer Degradation and Stability 92(7):1378-1383.

Das MP, Kumar S (2015). An approach to low-density polyethylene biodegradation by *Bacillus amyloliquefaciens*. 3 Biotech 5(1):81-86.

Gajendiran A, Krishnamoorthy S, Abraham J (2016). Microbial degradation of low-density polyethylene (LDPE) by *Aspergillus clavatus* strain JASK1 isolated from landfill soil. 3 Biotech 6(1):52.

Gu JD (2007). Microbial colonization of polymeric materials for space applications and mechanisms of biodeterioration: A review. International Biodeterioration Biodegradation 59(3):170-179.

Gulmine JV, Janissek PR, Heise HM, Akcelrud L (2003). Degradation profile of polyethylene after artificial accelerated weathering. Polymer Degradation and Stability 79(3):385-397.

Hermann B, Debeer L, Wilde B, Blok K, Patel M (2011). "To compost or no to compost: Carbon and energy footprints of biodegradable materials' waste treatment. Polymer Degradation and Stability 96:1159-1171.

Ishii N, Inoue Y, Tagaya T, Mitomo H, Nagai D, Kasuya K (2008). Isolation and characterization of poly (butylene succinate)-degrading fungi. Polymer Degradation and Stability 93(5):883-888.

Jagtap S, Yenkie MK, Das S, Rayalu S (2011). Synthesis and characterization of lanthanum impregnated chitosan flakes for fluoride removal in water. Desalination 273(2-3):267-275.

Koutny M, Amato P, Muchova M, Ruzicka J, Delort AM (2009). Soil bacterial strains able to grow on the surface of oxidized polyethylene film containing prooxidant additives. International Biodeterioration Biodegradation 63(3):354-357.

Khan S, Nadir S, Shah ZU, Shah AA, Karunarathna SC, Xu J, Hasan F (2017). Biodegradation of polyester polyurethane by *Aspergillus tubingensis*. Environmental Pollution 225:469-480.

Kharoufeh JP (2003). Explicit results for wear processes in a Markovian environment. Operations Research Letters 31(3):237-244.

Kumari K, Aanad RC, Narula N (2009). Microbial degradation of polyethylene (PE). The South Pacific Journal of Natural and Applied Sciences 27(1):66-70.

Loredo A, Argüello A, Rodríguez-Herrera R, Gutiérrez-Sánchez G, Escamilla A, Aguilar C (2017). Biodegradacion fungica de poliuretano rígido. Química Nova 40(8):885-889.

Ma C, Ni X, Chi Z, Ma L, Gao L (2007). Purification and characterization of an alkaline protease from the marine yeast *Aureobasidium pullulans* for bioactive peptide production from different sources. Marine Biotechnology 9(3):343-351.

Montgomery D, Hines W (1993). Probabilidad y estadística para ingeniería y administración. Management Science. Facultad de ciencias de la UNAM. Compañía Editorial: Continental S.A. México. [accessed 2019 Sep 10]. https://www.academia.edu/40410016/Probabilidad_y_estadistica_para_ingenieros_Hines_y_Montgomery

Motta O, Proto A, De Carlo F, De Caro F, Santoro E, Brunetti L, Capunzo M (2009). Utilization of chemically oxidized polystyrene as co-substrate by filamentous fungi. International Journal of Hygiene and Environmental Health 212(1):61-66.

Paço A, Duarte K, da Costa JP, Santos P, Pereira R, Pereira ME, Freitas A, Duarte A, Rocha-Santos T (2017). Biodegradation of polyethylene microplastics by the marine fungus Zalerion maritimum. Science of the Total Environment 586:10-15.

Ojeda TF, Dalmolin E, Forte MM, Jacques RJ, Tima FM, Vio FA (2009). Abiotic and biotic degradation of oxo-biodegradable polyethylenes.

Polymer Degradation and Stability 94:965-970.
Organización de las Naciones Unidas (ONU) (2018). O nos divorciamos del plástico, o nos olvidamos del planeta. (Blog) ONU Medio Ambiente/Shawn Heinrichs. ONU Noticias (30.01.2019). [accessed 2019 Sep 10]. https://news.un.org/es/story/2018/06/1435111.
Oprea S, Oprea V (2016). Biodegradation of crosslinked polyurethane acrylates/guar gum composites under natural soil burial conditions. e-Polymers 16(4):277-286.
Roy PK, Titus S, Surekha P, Tulsi E, Deshmukh C, Rajagopal C (2008). Degradation of abiotically aged LDPE films containing pro-oxidant by bacterial consortium. Polymer Degradation and Stability 93(10):1917-1922.
Ruiz C, Main T, Hilliard NP, Howard GT (1999). Purification and characterization of twopolyurethanase enzymes from *Pseudomonas chlororaphis*. International Biodeterioration Biodegradation 43(1-2):43-47.
Russell J (2011). Biodegradation of Polyester Polyurethane by Endophytic Fungi. Applied and Environmental Microbiology 6076-6084.
Saitou N (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4):406-425.
Scott G, Wiles DM (2001). Programmed-Life Plastics from Polyolefins: A New Look at Sustainability †. Biomacromolecules 2(3):615-622.
Shah AA, Hasan F, Hameed A, Ahmed S (2008). Biological degradation of plastics: A comprehensive review. In Biotechnology Advances 26(3):246-265.
Singh RS, Saini GK, Kennedy JF (2008). Pullulan: Microbial sources, production and applications. Carbohydrate Polymers 73(4):515-531.
Spontón M, Casis N, Mazo P, Raud B, Simonetta A, Ríos L, Estenoz D (2013). Biodegradation study by *Pseudomonas* sp. of flexible polyurethane foams derived from castor oil. International Biodeterioration Biodegradation 85(5):85-94.
Stuart B (2005). Infrared Spectroscopy. In Kirk-Othmer Encyclopedia of Chemical Technology (19). John Wiley & Sons, Inc. [accessed 2019 Sep 10]. doi.org/10.1002/0471238961.0914061810151405.a01.pub2
Tavares L, Schleder GR (2016). Bio-based polyurethane prepared from Kraft lignin and modified castor oil. Express Polymers Letter 10(11):927-940.
Urgun-Demirtas M, Singh D, Pagilla K (2007). Laboratory investigation of biodegradability of a polyurethane foam under anaerobic conditions. Polymer Degradation and Stability 92(8):1599-1610.
Uribe D, Giraldo D, Gutiérrez S, Merino F, Merino F (2011). Biodegradación de polietileno de baja densidad por acción de un consorcio microbiano aislado de un relleno sanitario, Lima, Perú. Revista Peruana de Biología 17(1):133-136.
Vaghani SS, Patel MM, Satish CS (2012). Synthesis and characterization of pH-sensitive hydrogel composed of carboxymethyl chitosan for colon targeted delivery of ornidazole. Carbohydrate Research 347(1):76-82.
Villa M, Rivera JD, Capilla V, Gardé JA (2009). Degradación biológica de polímeros mediante la selección y producción de potenciales cultivos iniciadores. Revista Técnica de Medio Ambiente 22(136):80-83.
Webb JS, Nixon M, Eastwood IM, Greenhalgh M, Robson GD, Handley PS (2000). Fungal colonization and biodeterioration of plasticized polyvinyl chloride. Applied and Environmental Microbiology 66(8):3194-3200.
White TJ, Bruns T, Lee S, Taylor JW (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, pp. 315-322. Academic Pres Inn: Massachusetts. doi: 10.1016/B978-0-12-372180-8.50042-1.
Wu W, Yang J, Criddle CS (2017). Estrategias de reducción y contaminación de microplásticos. Frontiers of Environmental Science and Engineering 11(6).
Zhang Y, Xia Z, Huang H, Chen H (2009). A degradation study of waterborne polyurethane based on TDI. Polymer Testing 28(3):264-269.
Zhou L, Yu L, Ding M, Li J, Tan H, Wang Z, Fu Q (2011). Synthesis and Characterization of pH-Sensitive Biodegradable Polyurethane for Potential Drug Delivery Applications. Macromolecules 44(4):857-864.
Zahra S, Abbas SS, Mahsa MT, Mohsen N (2010). Biodegradation of low-density polyethylene (LDPE) by isolated fungi in solid waste medium. Waste Management 30(3):396-401.

Vol. 20(2), pp.77-84, February, 2021
DOI: 10.5897/AJB2020.17229
Article Number: 207635866092
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Expression, purification and testing of zinc metalloproteinase aureolysin as potential vaccine candidate against *Staphylococcus aureus*

Samar Solyman[1], Mohamed Reda[1, 2] and Amro Hanora[1*]

[1]Department of Microbiology and Immunology, Faculty of Pharmacy, Suez Canal University, 41511 Ismailia, Egypt.
[2]Department of Microbiology and Immunology, Faculty of Pharmacy, Egyptian Russian University, Badr City, Egypt.

Received 21 August, 2020; Accepted 27 October, 2020

***Staphylococcus aureus* (*S. aureus*) is a versatile bacterium which exhibits multiple antibiotic resistances. To ameliorate the undesirable diseases causing potential, there is a need to design a protective vaccine capable of stimulating immune response against this pathogen. In a similar study in our laboratory, reverse vaccinology approach was used to nominate potential vaccine candidate genes against *S. aureus*. Zinc Metalloproteinase Aureolysin (aur) gene was one of the nominated genes based on that previously published in-silico study. The objective of this study is the cloning, expression, purification of aur gene and testing the aur protein reactivity with serum antibodies collected from groups of human patients with confirmed Staphylococcal disease. Cloning was done in pH6HTN His6HaloTag® vector and it was expressed in *E. coli* BL21 (*DE3*) using these conditions; 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37°C. Purification was carried out by Immobilized Metal Affinity Chromatography (IMAC). The his-tag aur protein was detected at ~86 KDa as a single band after western blot assay and was successfully reacted with antibodies obtained from humans infected with *S. aureus*. The results encourage further testing of aur protein as a potential vaccine candidate for *S. aureus*.**

Key words: *Staphylococcus aureus,* Zinc Metalloproteinase Aureolysin (*aur),* cloning, expression.

INTRODUCTION

Staphylococcus aureus has been indicated as a causative microorganism a lot of diseases, including osteomyelitis, septic arthritis and Necrotizing pneumonia. Furthermore, Olaniyi et al. (2017) reported that *S. aureus* is responsible for most of the skin and soft tissue infections. Methicillin-Resistant *S. aureus* (MRSA) has been implicated as the cause of most nosocomial infections and is reported with high prevalence especially in the hospitals with significant mortality and morbidity as reported by Toleman et al., (2019), Marlieke et al.,(2011). In a related development, it was also reported that multidrug resistant *S. aureus* was increasingly detected globally with fewer antibiotics remaining for effective treatment as reported by Harik et al. (2016), Chong et al. (2015) and Bendary et al. (2016).

Currently, there is no effective vaccine against S. *aureus,* despite many trials have been done for example,

*Corresponding author. E-mail: ahanora@yahoo.com. Tel: +201000323406.

during clinical developmental stage (phase III) StaphVAX (capsule glycol-conjugated vaccine) was stopped at phase III due to its low efficacy compared with placebo in patients with end-stage kidney failure as reported by Fattom et al. *(*2015)**.** A second vaccine trail termination was reported by McNeely et al. (2014) for the Merck V710 vaccine during Phase III (iron-regulated surface determinant B (IsdB)), the termination was due significant increase in mortality rate following post-cardiothoracic surgeries infections. Suggested opinions for why these vaccines failed was because they were limited to B cells and opsonic antibody initiation steps and not including T cell stimulation (Redi et al., 2018). Another opinion is that, over-reliance on rodent models and a focus on targeting cell surface components have been major contributing factors to this failure as reported by Salgado-Pabón et al. (2014).

For effective vaccines against *S. aureus* to be designed, humoral and cellular immunity should be stimulated, the vaccine should be multi-components because of the numerous *S. aureus* virulence mechanisms and the heterogeneous nature of the genome (Proctor, 2015). Conventional approaches for vaccine design which is based on pathogen culture and testing only the expressed antigens during culture are extremely time consuming, costly and they are not appropriated for non-culturable pathogens as reported by Bruno et al. (2015). Reverse vaccinology is a computer-based technique, for selection of candidate genes with potential for use as vaccines, developed by Rappuoli (2001). Reverse vaccinology is based on the analysis of whole genomes sequence data of pathogens. There are many successful trails and researches based on reverse vaccinology approach for developing vaccine against many pathogens as *Streptococcus agalactiae, Streptococcus pyogenes,* pathogenic *Escherichia coli*, *helicobacter pylori* and serogroup B *Neisseria meningitides* (Seib et al., 2012; Naz et al., 2015). In 2013, Novartis launched Bexsero® as the first vaccine based on reverse vaccinology approach against meningococcal serotype B disease as reported by Carter (2013)**.** It has been licensed in Canada, Australia, United States and United Kingdom as stated by Heinson et al. (2015).

As reported by Soltan et al. (2020), reverse vaccinology approach was used for the selection of potential vaccine gene candidates for *S. aureus*. Candidate genes were selected based on antigenicity score (antigenicity score > 0.45 were selected) and cellular localization (Only extracellular proteins and cell wall proteins were selected). The selected genes were subjected for screening in clinical isolates. The ones which are present in almost all isolates are selected for further cloning, expressing, purification and in-vitro and in-vivo vaccine testing. The selected aur gene shown to have high score of antigenicity and it was present in 96% of the tested clinical isolates. The aims of the current study are cloning, expression, purification of aur and demonstration of the reactivity of the purified aur against antibodies obtained from human infected with *S. aureus*.

MATERIAL AND METHODS

In silico studies

An *in-silico* study Soltan et al. (2020) was carried out in the laboratory (College of Pharmacy, Suez Canal University, Egypt), nominated aur gene to be tested for its potential use as vaccine candidate based on surface location and antigenicity score (0.7). Aur gene was tested for presence in a large panel of *S. aureus* isolates.

Isolates collection and DNA extraction

Seventy-five of S. *aureus* isolates were previously isolated by Bendary et al. (2016) were used. These isolates were cultured on Mannitol salt agar (Lab M®, UK) and their genomic DNA were extracted by QIAamp DNA Mini Kit (Qiagen, Germany) according to manufacturer's recommendation. The extracted DNA was stored at -80°C for downstream application.

Primer design for PCR assay

Primers in the current study was designed manually and the specificity of the selected primers was confirmed by blasting in nucleotide blast tool of NCBI website https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.The designed forward primer: 5-GAGGTGACTCAAAAGAGTGAT-3 and reverse primer; 5-TGTGTAGACATCTTCACCCAT-3 were used to amplify *aur* by PCR.

The amplification was performed in 50 µl containing 1 µl of Forward primer (10 µM), 1 µl of reverse primer (10 µM), 25 µl of one PCR master mix (GenedireX, Germany), 1 µl of DNA template (50 ng/µl) and the final volume was adjusted to 50 µl PCR water (Qiagen, Germany) under the following conditions; Initial denaturation at 94°C for 5 min, followed by 35 cycle of (denaturation at 94°C for 40 seconds, annealing at 52^{o}C for 45 s and extension at 72°C for 2 min) and final extension at 72°C for 5 min. The amplified PCR products were detected by 1.5% agarose and were visualized after staining with ethidium bromide under ultraviolet trans illuminator (IFM-20 UVP, upland, USA).

Cloning primer design

Forward primer contain the started nucleotides of the inserted gene and reverse primer contain the last nucleotide sequences of the inserted gene were designed. The open reading frame (ORF) design was confirmed by EMBOSS Transeq software for sequence analysis (http://www.bioinformatics.nl/cgi-bin/emboss/transeq). The restriction sites of the selected endonuclease enzymes were added at the beginning of the primers. The selection of the endonuclease enzymes was based on their ability to cut both vector and insert at their specific sites and not able to cut inside the insert that was checked by using NEBcutter V2.0 http://nc2.neb.com/NEBcutter2/. The restriction site of *XbaI* (TCTAGA) was added at the beginning of the forward primer. While, the restriction site of *NotI* (GCGGCCGC) was added at the beginning of the reverse primer. To increase the cleavage efficiency of restriction enzymes, 5'-nucleotide extension was added at the beginning of both XbaI and NotI sites, GC nucleotides were added at the 5' of XbaI site and

TGTATC nucleotides were added at 5' of *NotI* sites.

Cloning and expression of zinc metalloproteinase aureolysin

Cloning

The aur cloning vector was PH6HTN His6HaloTag® plasmid (Promega, USA). At first, *aur* was amplified with the same conditions that were performed in screening of genes with 58°C annealing temperature and using the designed cloning primers. The forward primer was 5-GCTCTAGAATGGCAGCATTAACCTTGTTG-3 and the reverse primer was 5-TGTATCGCCGGCGTTACTCCACGCCTACTTCAT-3. After detection of *aur* band on gel electrophoresis, it was cut and purified by PCR clean-up and gel purification kit (GenedirX, Germany) according to manufacturer specifications. The purified *aur* and PH6HTN His6HaloTag® plasmid were double digested with Fast Digest *XbaI* and *NotI* restriction enzymes (Thermo- Scientific, USA) based on the manufacturer specifications. Ligation step was followed by the action of T4-DNA ligase enzyme (New England Biolabs (NEB), USA) with 3:1 ratio of insert and plasmid respectively. The transformation of the cloned plasmid into *E. coli (DH5α)* competent cells were accomplished by heat shock at 42°C for 30 s followed by immediately transferring to ice for 2 min as mentioned in Nakata et al. (1997) protocol followed by streaking the transformed cells over Luria agar (LB) (Lab M®, UK) containing 100 µg/ ml of ampicillin and incubated overnight at 37°C. The positive colonies harbored the recombinant plasmid and the negative colonies harbored the empty plasmid. These were then screened by colony PCR using confirmatory primers with forward primer: 5-GGTCTGAATCTGCTGCAAGAA-3 and reverse primer: 5-GGTTATGCTAGT TATTGCTCAG -3. The amplification condition was the same condition for screening the genes except when the annealing temperature was 52°C for 45 s. The positive colonies were determined and preserved at -80°C for further application.

Expression

Expression of aur was accomplished under the influence of T7 promoter. First, the plasmid of the positive colonies was extracted using "PureYield™ Plasmid Miniprep System" (Promega, USA) according to manufacture specifications. The extracted plasmid was transformed into *BL21* (*DE3*) Competent *E. Coli* by heat shock followed by streaking over Luria Broth (LB) (Lab M®, UK) containing 100 $\mu g.ml^{-1}$ of ampicillin and incubated overnight at 37°C. Single colony from the previous culture was inoculated in 5 ml LB medium containing 100 µg/ml ampicillin and was then incubated in a shaker incubator with 250 rpm at overnight. After incubation period, 1 ml of the previous culture was diluted in 100 ml fresh LB medium then the diluted culture was incubated on shaker incubator with 150 rpm at 37°C to an optical density 600 nm (OD_{600}) of 0.5 to 0.6. These concentrations of IPTG 0.1, 0.2, 0.5 and 1 mM were added for induction of proteins expression. Expression was accomplished under different condition for every concentration of IPTG. The applied different conditions included different temperature (20, 30 and 37°C), different incubation time intervals (3, 4, 5 and overnight) on shaker incubator at 150 rpm. The pellet of these different conditions was obtained by centrifugation at 15,000 rpm for 20 min at 4°C.

Protein analysis by SDS-PAGE

To liberate the proteins from the cultured cells, the pellets were washed with lysis buffer containing (5 mM Imidazole, 300 mM NaCl50 mM, Na2HPO4, pH at 7.4) then re-suspended in 1:10 W/V lysis buffer. Five micro liter triton-x-100, 250 µl 100 mM PMSF and 5 µl lysozyme (100mg/ml) were added to each 5 ml of lysis buffer and this suspension was incubated in refrigerator (4°C) for 15 min. Freeze-thaw technique was used to facilitate cell lysis where the previous suspension was exposed to 4 cycles of freezing (-20°C) and thawing (37°C). The cell lysate was centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant was collected. To prepare the samples for SDS-PAGE, the supernatant was mixed with Laemmli sample buffer (BIO-RAD, USA) and then the mixture was boiled at 95°C for 10 min. Protein samples were separated by 12% SDS-PAGE according to Laemmli SDS-PAGE protocol mentioned in He (2011). The gel was stained by Coomassie Brilliant Blue R-250 dye (Thermofisher, USA) for 8 h followed by destaining by using mixture of 40% methanol, 20% glacial acetic acid and 40% distilled water.

Protein purification

Protein purification was performed based on IMAC technique that a profanity nickel metal charged resin (Bio-Rad, USA) was used. First, the 50% (v/v) profanity IMAC (Bio-Rad, USA) slurry was suspended by gently swirling with plastic rod. The slurry was applied to the column (2.5 cm ID x 15 cm). Then, the column was equilibrated with 5 CV of equilibrated column buffer containing 300 mM NaCl, 50 mM Na_2HPO_4, and 5 mM imidazole, pH at 7.0 followed by application of 3 to 5 column volume charging metal solution (300 mM Nickel sulphate at pH of 4). The column was washed with 5 CV equilibrated column buffer and 10 CV deionized water to remove unbound metal ions. Then 5 CV of washing buffer (50 mM Na_2HPO_4, 300 mM NaCl and 20 mM Imidazole) was added to the column. The supernatant was loaded onto column with swirl at 4°C. The 5 CV of wash buffer was used to remove unbound sample and the resulted fractures were collected for further analysis. Finally, 5 CV of elution buffer (50 mM Na_2HPO_4, 300 mM NaCl and 250 mM Imidazole) was applied to elute the protein and protein containing fractions were collected and protein was analysed using SDS-PAGE.

Demonstration of reactivity of aur with serum antibodies of *S. aureus*

Western blot technique was used for protein identification of the purified aur. Ten sera samples were collected from patients who reside at Suez Canal University Educational Hospital and suffered from pneumonia and septicemia caused by *S. aureus.* The sera were collected after the approval from ethics committee of faculty of pharmacy, Suez Canal University-Egypt (Reference number of 20166H1). After separation of protein by 12% SDS-PAGE, the gel was transferred onto 0.45 µm.30 cm×3.5 m nitrocellulose membrane (Thermo fisher, USA) (Catalog number 88018) using mini trans-blot© electrophoretic transfer cell (Bio rad, USA) under constant 300 mA for 15 min using transferring buffer contained 25 mM tris-HCL, 192 mM glycine and 20% methanol pH 8.3. The transferred nitrocellulose membrane was blocked by 3% Blocker Bovine Serum Albumin BSA (10X) (Thermo fisher, USA) diluted in PBS overnight 0.05% Tween-20 (TBST) at room temperature followed by two time washing with 0.05% Tween-20 (TBST) for 10 min. The membrane was incubated with sera (1/10 dilution) with gentle agitation on rocking shaker at 4°C overnight followed by four time washing with 0.05% Tween-20 (TBST) for 10 min to remove any residuals of primary antibodies (Sera). After washing steps, the membrane was incubated with the secondary antibody (goat antihuman IgG(H+L) conjugated with HRP) (Invitrogen, USA) (Cat number 31410) with the dilution 1:10,000 with gentle agitation on rocking shaker at room temperature for 1 h followed by three time

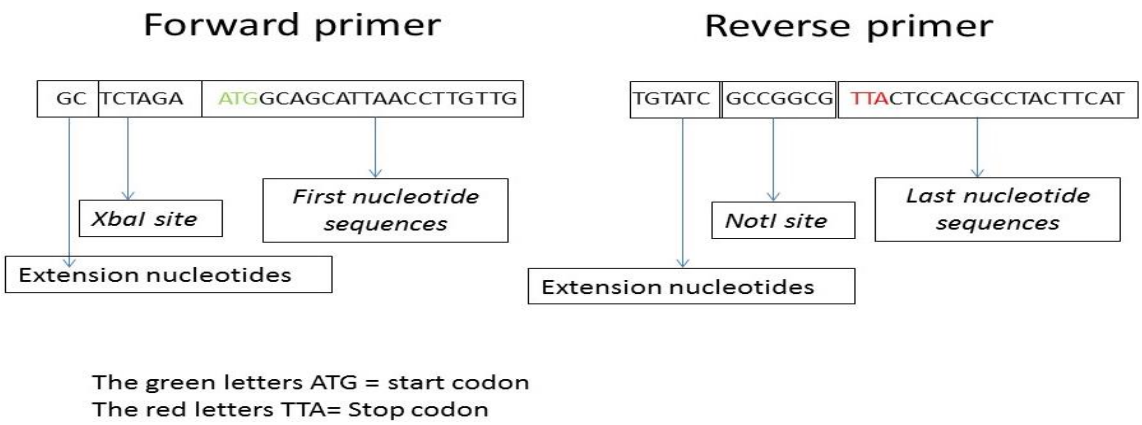

Figure 1. Cloning primer analysis.

washing with 0.05% Tween-20 (TBST) for 10 min. Finally, Super Signal West Pico PLUS substrate (Thermo scientific™, USA) was applied to the membrane and incubated at room temperature (30°C) for 5 min. The signal was captured.

RESULTS

Primer design

All the designed primers (screening and cloning primers) in this study were specific to their templates with low undesired secondary structure annealing. The structure of the designed cloning primers was illustrated in Figure1.

Cloning and screening of the correctly inserted *aur* in MCS of plasmid

The insert was cloned in PH6HTN His6HaloTag® plasmid between *XbaI* site and *NotI* site of multiple cloning sites (Figure 2). After screening of positive colonies that harbored the recombinant plasmid and negative colonies that harbored the empty plasmid by colony PCR technique using confirmatory primers previously mentioned, the amplicon size of positive colonies was detected at ~ 1500 bp (Figure 3). While, negative colonies were detected at ~245 bp.

Expression and purification of aur

After application of different expression conditions (different concentration of IPTG, incubation times and temperatures) and analysis of these conditions by SDS-PAGE, the optimum condition for Aur expression was 0.5 mM IPTG at 37°C after 4 h that showed heavy band at ~86 KDa (the molecular weight of the expressed Aur + His6 tag) (Figure 4). The supernatant of this condition was purified by IMAC and analyzed by SDS-PAGE showed single band at the expected molecular weight (86 KDa).

Demonstration of reactivity with serum antibodies

The reactivity of aur was demonstrated by using western blot assay. Positive reaction of the purified aur band was showed at ~86 KDa that was the molecular weight of the purified aur protein (his6halo tagged). Each human serum sample was tested individually and the positive reaction of aur against patient's serum is illustrated in Figure 5.

DISCUSSION

The increasing rate of prevalence of antibiotic resistance in *S. aureus* necessitated the research into the development of a protective vaccine against the organism, but unfortunately all the clinical trials that were carried out to date failed as reported by Proctor (2015).

The new vaccines will be targeted at preventing infections by reducing biofilm formation and / or adhesion to the foreign body. This criterion has already been tested in some clinical trials where anti-capsular polysaccharide (types 5 and 8), anti-SdrG and anti-Clamping factor A (ClfA) were tested either for passive or active immunity (Rupp et al., 2007) but, all of them have failed in human clinical trials.

Clinical trial failures can be attributed to many reasons, some of which includes: Many of the trial targeted B-cell activation rather than T-cell activation especially Th17 as suggested by Proctor (2012). These are essential for activation and mobilization of neutrophils as reported by Lin et al (2009). Furthermore, *S. aureus* causes variety of diseases therefore there is a need for more than a single vaccine that will be potent against many of the strains as stated by Anderson et al. (2012). Another

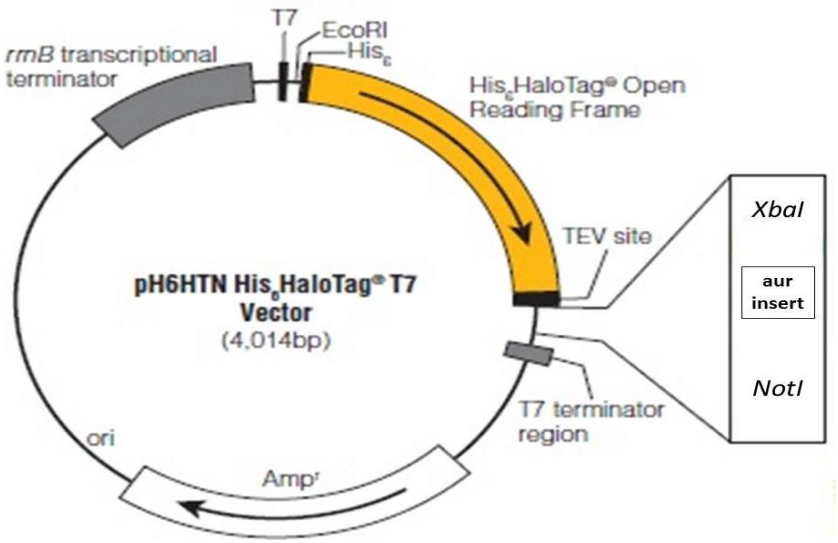

Figure 2. Recombinant PH6HTN His6HaloTag®-*aur* plasmid structure. *aur* was cloned between *XbaI* and *NotI* sites of plasmid. The PH6HTN His6HaloTag® plasmid contains many features such as T7 promoter that promote the expression of the recombinant plasmid in *E. coli* strains having T7 RNA polymerase, The N-terminal His6HaloTag® region that facilitate simple purification of protein via 6 histidine tag. A TEV protease site that cleaves the expressed protein form His6HaloTag® region by the action of Halo TEV protease and ampicillin resistant gene (Ampr) that facilitates the selection of the transformed colonies. This figure was modified from the Promega pH6HTN His6HaloTag®T7 Vector Certificate analysis part no. 9PIG797, printed 2/2017.

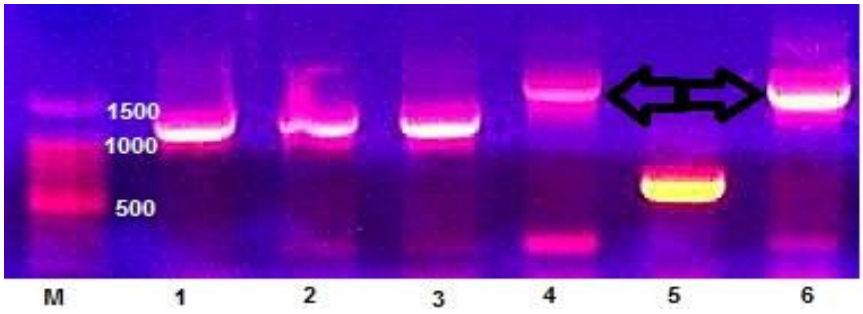

Figure 3. Gel electrophoresis of the amplified positive colonies after transformation of recombinant PH6HTN His6HaloTag®-aur plasmid into *E. coli* (*DH5α*) strain. M was 100 bp DNA Ladder RTU (GendireX, Germany). Lane 1, 2 and 3 was the amplified recombinant PH6HTN His6HaloTag®-*plc* plasmid that detected at 1232 bp while lane 5 was non-specific amplification. Lane 4 and 6 was the amplified recombinant PH6HTN His6HaloTag®-aur plasmid that detected at 1500 bp.

important factor is the variability between isolates which may give rise to triggering of inappropriate immune response, and may cause organs failure as reported by Lloyd et al. (2020). Finally, *S. aureus* is one of the normal flora and has the ability to develop several mechanisms for escaping from host immunity particularly opsonophagocytic processes (Fowler et al., 2013; Van Kessel et al., 2014).

Soltan et al. (2020) reported the use of reverse vaccinology to nominate genes that are surface localized and showed with high antigenicity scores, the nominated genes (16 genes) were screened for presence in a large panel of *S. aureus* clinical isolates. The genes present in almost all the tested isolated were used further to test their vaccine potential. Phosphatidylinositol phosphodiesterase (PI) was selected for cloning, expression and B cell and T cell epitope mapping. PI was shown to be highly reactive with antibodies obtained from

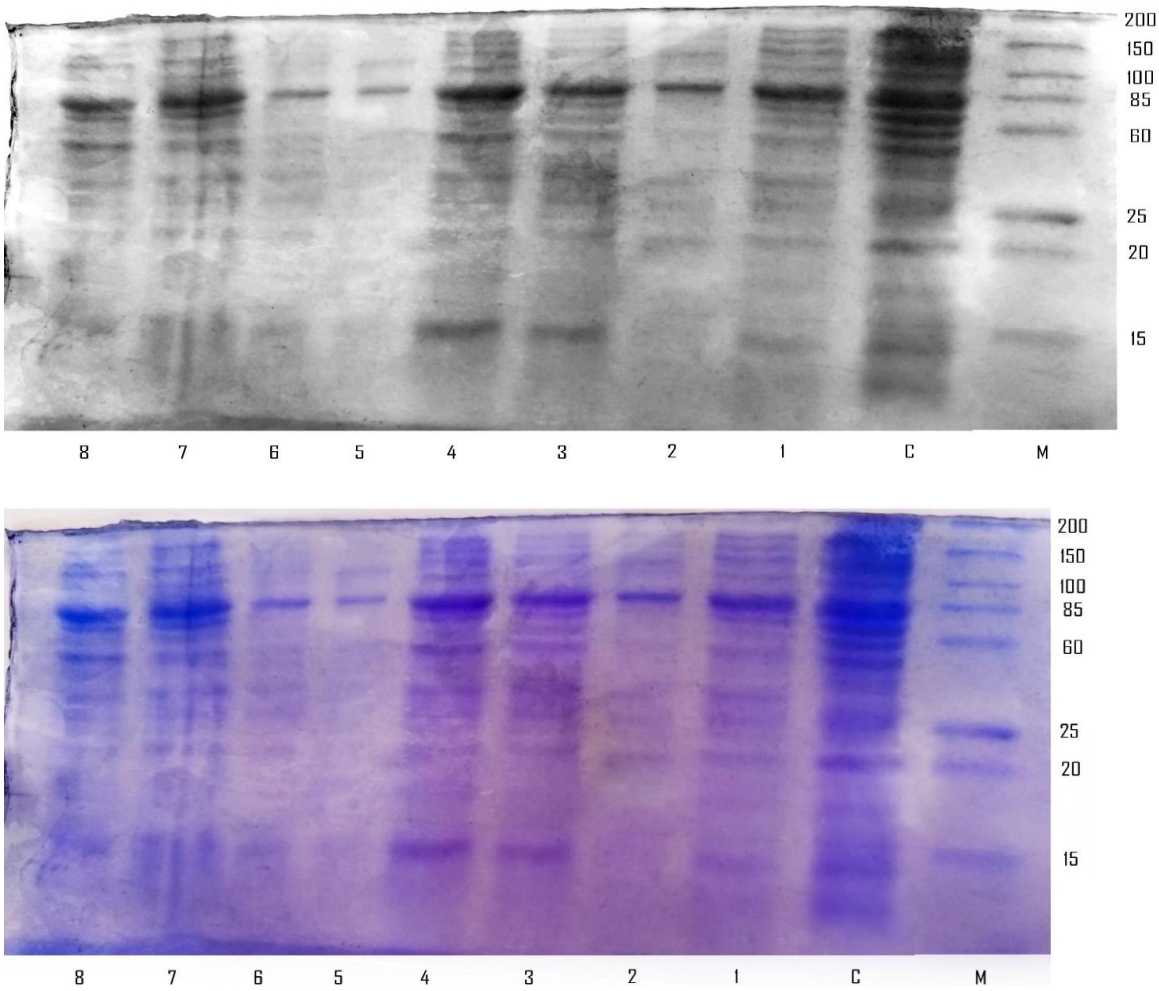

Figure 4. SDS-PAGE analysis of the optimum condition of His6HaloTag®-aur expression under the induction of IPTG. Lane M: Unstained Protein Standard (10-200 kDa) (NEB, USA). Lane C: The culture condition without the addition of IPTG. Lane 1-4: Culture growth under 0.1, 0.2, 0.5 and 1 mM IPTG respectively at 37°C after 3 h. Lane 5-8: Culture growth under 0.1, 0.2, 0.5 and 1 mM IPTG respectively at 37°C after 4 hours. Lane 7: The best condition of recombinant His6HaloTag®-Aur expression.

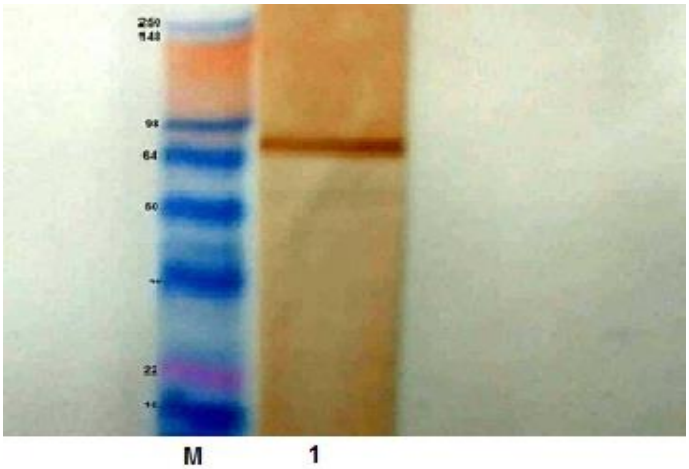

Figure 5. Western blot analysis of the purified His6HaloTag®-Aur. Lane M: Protein marker; lane1: Purified recombinant Aur at 86 KDa.

serum of humans infected with *S. aureus*. The in-vivo induction of immune response against PI in a bacteremia mice model resulted in protection of the mice injected with B cell and T cell epitope mixture.

The second nominated gene by Soltan et al. (2020) was aur gene; it was detected in large number of *S. aureus* isolated (96%). In addition to its critical role in *S. aureus* pathogenesis, *aur* has a role in *S. aureus* resistance to innate immunity because it degrades the antimicrobial peptide dermicidin and cathelicidin LL-37 (Beaufort et al., 2008). Also aur has a role in transition of *S. aureus* form adherent to invasive stage by cleavage of staphylococcal surface- associated proteins (Stach et al., 2018). Therefore, immunization against aur antigen can affect *S. aureus* invasiveness.

For proper cloning and expression of different proteins of *S. aureus*, many researches use two vectors (one for cloning and other for expression) as mentioned in Das and Biswas (2019) who use pGEM-T Easy cloning vector and pET28a expression vector for Phi11 *gp07,* however, this approach is expensive. On the other hand, other studies employ one vector for cloning and expression as mentioned by Chen et al (2019), in which pJET1.2 vector in serine acetyltransferase cloning was used. In this study, *aur* gene was cloned and expressed in pH6HTN His6HaloTag® T7.

As reported in some of the previous research works, different IPTG concentrations ranged from 0.1 to 1 mM and different induction periods (2 to overnight) at different temperature (20 to 37°C) were used for optimization of the protein expression. But in this study, the optimal IPTG concentration was 0.5 mM. In contrast, Nickerson et al. (2008) study has induced aur by using 1 mM IPTG.

There was a little increment in the yield of aur expression between overnight induction and after 4 h. Therefore, induction after 4 h is economical compared with overnight as it saves energy that was been consumed by the shaker incubator. This 4 h conditioning was consistent with Nickerson et al (2008) where they induced aur after 4 h.

Many affinity tags can be used to purify the expressed protein and in this study, the His-tag was selected as it is highly specific in target protein purification and highly effective more than 80% of pure protein can be obtained in one chromatographic step as stated by Kimple et al (2013). His-tag was also used by Nickerson et al. (2008) study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This study was funded by Science and Technology Development Fund (STDF). The authors are greatly thankful for assistant lecturer, Mohamed A. Elkhodary for his support during the study.

REFERENCES

Anderson AS, Miller AA, Donald RG, Scully IL, Nanra JS, Cooper D, Jansen KU (2012). Development of a multicomponent Staphylococcus aureus vaccine designed to counter multiple bacterial virulence factors. Journal of Human vaccines and immunotherapeutics 8:1585-1594.

Beaufort N, Wojciechowski P, Sommerhoff CP, Szmyd G, Dubin G, Eick S, Kellermann J, Schmitt M, Potempa J, Magdolen V (2008). The human fibrinolytic system is a target for the staphylococcal metalloprotease aureolysin. Biochemical Journal 410:157-165.

Bendary MM, Solyman SM, Azab MM, Mahmoud NF, Hanora AM (2016). Characterization of Methicillin Resistant Staphylococcus aureus isolated from human and animal samples in Egypt. Journal of Cellular and Molecular Biology 62:94-100.

Bruno L, Cortese M, Rappuoli R, Merola M (2015). Lessons from Reverse Vaccinology for viral vaccine design. Journal of Current Opinions Virololgy 11:89-97.

Carter NJ (2013). Multicomponent meningococcal serogroup B vaccine (4CMenB; Bexsero®): a review of its use in primary and booster vaccination (2013). Journal of BioDrugs 27:263-274.

Chen C, Yan Q, Tao M, Shi H Han, X, Jia L, Huang Y, Zhao L, Wang C, Ma X, Ma Y (2019). Characterization of serine acetyltransferase (CysE) from methicillin-resistant *Staphylococcus aureus* and inhibitory effect of two natural products on CysE. Journal of Microbial pathogenesis 131(1):218-26.

Chong YP, Park KH, Kim ES, Kim MN, Kim SH, Lee SO, Choi SH, Jeong JY, Woo JH, Kim YS (2015). Clinical and microbiologic analysis of the risk factors for mortality in patients with heterogeneous vancomycin-intermediate *Staphylococcus aureus* bacteremia. Journal of Antimicrobial agents and chemotherapy 59:3541-3547.

Das A, Biswas M (2019). Cloning, overexpression and purification of a novel two-domain protein of *Staphylococcus aureus* phage Phi11. Journal of Protein expression and purification 1(154):104-111.

Fattom A, Matalon A, Buerkert J, Taylor K, Damaso S, Boutriau D (2015). Efficacy profile of a bivalent *Staphylococcus aureus* glycoconjugated vaccine in adults on hemodialysis: Phase III randomized study. Journal of Human vaccines & immunotherapeutics 116:32-41.

Fowler VG, Allen KB, Moreira ED, Moustafa M, Isgro F, Boucher HW, Corey GR, Carmeli Y, Betts R, Hartzel J, Chan I, McNeely T, Kartsonis NA, Guris D, Onorato MT, Smugar SS, DiNubile MJ, Meulen AS (2013). Effect of an investigational vaccine for preventing *Staphylococcus aureus* infections after cardiothoracic surgery: a randomized trial. JAMA The Journal of the American Medical Association 309(13):1368-1378.

Harik NS, Com G, Tang X, Castro MM, Stemper ME, Carroll JL (2016). Clinical characteristics and epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) in children with cystic fibrosis from a center with a high MRSA prevalence. American journal of infection control 44(4):409-415.

He F (2011). Laemmli-SDS-PAGE. Bio-protocol Bio101:e80. DOI: 10.21769/BioProtoc.80.

Heinson AI, Woelk CH, Newell M-L (2015). The promise of reverse vaccinology. Journal of International health 7:85-89.

Kimple ME, Brill AL, Pasker RL (2013). Overview of affinity tags for protein purification. Current protocols in protein science 73(1):9-17.

Lin L, Ibrahim AS, Xu X, Farber JM, Avanesian V, Baquir B, Fu Y, French SW, Edwards JE, Spellberg B (2009). Th1-Th17 cells mediate protective adaptive immunity against Staphylococcus aureus and Candida albicans infection in mice. Journal of PLoS pathogens 5:e1000703.

Lloyd SM, Vance GFJr, Sanjay KS, Warren ER, Richard AP (2020). Development of a vaccine against *Staphylococcus aureus* invasive infections: Evidence based on human immunity, genetics and bacterial evasion mechanisms, FEMS. Journal of Microbiology Reviews 44(1):123-153.

Marlieke EA de Kraker, Martin W, Peter GD, Walter K, Jutta B, Jan N, Claudine I, Smilja K, Jasminka H, Harald S, Achim JK, Olga P, Athina A, Maria B, Edmond S, Mairead S, Annibale R, Uga D, Agita MK, Michael B, Deborah X, Mihaela CG, Michelle N, Jana K, Stanko G, David T, Louise L, Hajo G (2011). Clinical Impact of Antimicrobial Resistance in European Hospitals: Excess Mortality and Length of Hospital Stay Related to Methicillin-Resistant *Staphylococcus aureus* Bloodstream Infections. Journal of Antimicrobial Agents and Chemotherapy 55(4):1598-1605.

McNeely TB, Shah NA, Fridman A, Joshi A, Hartzel JS, Keshari I, Florea Lupu RS, DiNubile MJ (2014). Mortality among recipients of the Merck V710 *Staphylococcus aureus* vaccine after postoperative *S. aureus* infections: an analysis of possible contributing host factors. Journal ofHuman vaccines & immunotherapeutics 10(3):513-516.

Nakata Y, Tang X, Yokoyama KK (1997). Preparation of competent cells for high-efficiency plasmid transformation of *Escherichia coli*. cDNA Library Protocols: Springer pp. 129-137.

Naz A, Awan FM, Obaid A, Muhammad SA, Paracha RZ, Ahmad J, Ali J (2015). Identification of putative vaccine candidates against *Helicobacter pylori* exploiting exoproteome and secretome: a reverse vaccinology-based approach. Journal of Infection, Genetics and Evolution 32:280-291.

Nickerson NN, Joag V, McGavin MJ (2008). Rapid autocatalytic activation of the M4 metalloprotease aureolysin is controlled by a conserved N-terminal fungalysin-thermolysin-propeptide domain. Journal of Molecular microbiology 69(6):1530-1543.

Olaniyi R, Pozzi C, Grimaldi L, Bagnoli F (2017). *Staphylococcus aureus*-Associated Skin and Soft Tissue Infections: Anatomical Localization, Epidemiology, Therapy and Potential Prophylaxis. Current Topics in Microbiology and Immunology 409:199-227.

Proctor RA. (2012). Is there a future for a *Staphylococcus aureus* vaccine? Vaccine 30:2921-2927.

Proctor RA (2015). Recent developments for *Staphylococcus aureus* vaccines: clinical and basic science challenges. Journal of European Cell and Material 30(734):315-326.

Rappuoli R (2001). Reverse vaccinology, a genome-based approach to vaccine development. Journal of Vaccine 19:2688-2691.

Redi D, Raffaelli CS, Rossetti B, Luca AD, Montagnani F (2018). *Staphylococcus aureus* vaccine preclinical and clinical development: current state of the art. Journal of New Microbiology 41:208-213.

Rupp ME, Holley HP, Lutz J, Dicpinigaitis PV, Christopher W W, Levine DP, Veney N, Fowler VG (2007). Phase II, randomized, multicenter, double-blind, placebo-controlled trial of a polyclonal anti-*Staphylococcus aureus* capsular polysaccharide immune globulin in treatment of *Staphylococcus aureus* bacteremia. Antimicrobial Agents and Chemotherapy 51(12):4249-4254.

Salgado-Pabón W, Schlievert PM. Models matter (2014). The search for an effective *Staphylococcus aureus* vaccine. Journal of Nature Review in Microbiology 12(8):585-591.

Seib KL, Zhao X, Rappuoli R (2012). Developing vaccines in the era of genomics: a decade of reverse vaccinology. Journal of Clinical Microbiology and Infection 18:109-116.

Soltan MA, Magdy D, Solyman SM, Hanora A (2020). Design of *Staphylococcus aureus* New Vaccine Candidates with B and T Cell Epitope Mapping, Reverse Vaccinology, and Immunoinformatics. OMICS A Journal of Integrative Biology 24(4):195-204.

Stach N, Kaszycki P, Władyka B (2018). Extracellular Proteases of *Staphylococcus spp.* Pet-To-Man Travelling Staphylococci. Elsevier Journal pp. 135-145.

Toleman MS, Sandra R, Dorota J, Hayley JW, Beth B, Ewan MH, Francesc C, Russell JH, Angela K, Julian P, Sharon JP, Török ME (2019). Prospective genomic surveillance of methicillin-resistant *Staphylococcus aureus* (MRSA) associated with bloodstream infection, England, 1 October 2012 to 30 September 2013 https://doi.org/10.2807/1560-7917.ES.2019.24.4.1800215

Van Kessel KP, Bestebroer J, van Strijp JA (2014). Neutrophil-mediated phagocytosis of *Staphylococcus aureus*. Journal of Frontiers in Immunology 5:467.

Vol. 20(2), pp.85-91, February, 2021
DOI: 10.5897/AJB2020.17232
Article Number: 1CDD9A366119
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

A novel genotype-independent technique for successful induction of somatic embryogenesis of adult plants of *Jatropha curcas* L. using petiole transverse Thin Cell Layer (TCL)

Cristian Nicolás Mendoza-Peña and Anne Kathrine (Trine) Hvoslef-Eide*

Department of Plant Sciences, Faculty of Biosciences, Norwegian University of Life Sciences – NMBU, Universitetstunet 3, 1433 Aas, Norway.

Received 25 August, 2020; Accepted 1 February, 2021

Developing a protocol for somatic embryogenesis (SE) for adult mother plants of *Jatropha curcas*, aids to overcome problems such as harvesting time and an uneven yield, providing the opportunity to propagate proven elite genotypes. Until now, several authors have achieved SE in *J. curcas* using different explants, however, in none of these protocols adult plants have been used as mother plant material. Furthermore, the challenge of overcoming the morphogenesis limitations successfully and further regeneration of adult plants of *J. curcas* has been great. The main objective of this study was to develop a somatic embryogenesis protocol for adult plants of the oil producing *J. curcas.* transverse thin cell layers (tTCL) of young petioles were used as explants. Half strength Y3 and MS media were used containing three different concentrations of 2,4-dichloropherioxyacetic acid and 6-benzil-aminopurine in a factorial trial. Nine accessions of *J. curcas* of various ages (3-12 years.) were tested using this protocol. All accessions responded positively to callus induction and to the induction of somatic embryos, demonstrating the protocol to be genotype-independent. This study enables micropropagation of adult of proven elite plants of *J. curcas* via somatic embryogenesis.

Key words: *Jatropha curcas*, transverse thin cell layers (tTCL), somatic embryos, half strength media, regeneration.

INTRODUCTION

In a world of an increasing demand for energy, fossil fuels are becoming more scarce and less favourable due to their CO_2 emissions and impact on global warming (Rehman et al., 2019). The focus on renewable energies has identified *Jatropha curcas* L. as a promising plant to contribute to a more sustainable alternative. This is due to the yield and quality of the oil extracted from the seeds, having a relatively high oil content, ranging from ~30% to ~35% (Barros et al., 2015; de Oliveira et al., 2009).

*Corresponding author. E-mail: trine.hvoslef-eide@nmbu.no. Tel: +47 934 33 775.

Nevertheless, even though *J. curcas* is highly promising as a renewable energy source, some challenges have been encountered whilst attempting to exploit the crop (Mulpuri et al., 2019). The plant is still a wild type plant, that is not domesticated yet and this presents high heterogeneity between individuals within cultivars (Bahadur et al., 2012). Consequently, agronomical and harvesting processes are challenging (Mulpuri et al., 2019). In order to minimize and overcome these challenges, the selection and mass propagation of elite genotypes has become an encouraging solution (Mulpuri et al., 2019).

To achieve mass propagation, the somatic embryogenesis (SE) technique was chosen due to its high amount of plants obtained per explant, considering that, the plants regenerated from this technique are mostly non-chimeric and the populations derived are true clones (Egertsdotter et al., 2019). However, adult plants are highly recalcitrant to tissue culture (Jyoti et al., 1998). To achieve SE in *J. curcas*, different explants have been used: embryo axis and cotyledons (Nindita et al., 2014); root and hypocotyl (Galaz-Ávalos et al., 2012); leaf and shoot tips (Medipally et al., 2014); young leaves from young plants (Baran Jha et al., 2007); and immature zygotic embryos (Cai et al., 2011). However, there are no reports on SE protocols using transverse Thin Cell Layers (tTCL) in adult plants of *J. curcas*. TCL explants are small pieces of somatic tissue that usually consist of different types of cells (parenchyma, epidermal, cambium, vascular and medullar tissues) (Van, 1980). As explained by Nhut et al. (2003), TCLs are very responsive and the morphogenic responses are dependent on different factors, also involving growth conditions (PGRs, light quality, temperature, photoperiod).

The aim of this paper was to develop an efficient protocol to induce SE in adult plants of *J. curcas*, to obtain successful micropropagation of elite genotypes, using transverse Thin Cell Layers of young petioles.

MATERIALS AND METHODS

Nine accessions of *J. curcas* L. were used, with an age range of 4 to 12 years from different countries (Table 1) where the plant is widely cultivated and economically important. The reason for the wide location range and age span was to develop a robust genotype-independent micropropagation protocol, which could be applied to elite material.

Mother plants growing in two different conditions, greenhouse and field, were used. Second and third leaves were excised from apical buds in active growth (Figure 1). The leaves were rinsed with Milli-Q water, and subsequently, the petioles were separated. The excised petioles were washed in 70% alcohol for 1 min. For the plants derived from the field, 2 g of disinfectant soap was used for 2 min, as an extra step. The petioles were washed with Milli-Q water for 1 min and placed into a laminar flow chamber. Petioles were surface sterilized with a solution of 0.5% sodium hypochlorite (Sigma-Aldrich 10-14% W/W) with 0.02 % of Tween® 20 for 7 min. The field plants were additionally treated with a fungicide solution (Carbendazim 2% (Sigma-Aldrich 97%) + 2 g Tween® 20), before surface sterilization with sodium hypochlorite. The petioles were then rinsed three times (at 5, 10 and 20 min) with autoclaved double distilled water. Both ends of the petioles were removed and the middle part was sectioned using a sterile scalpel to obtain the tTCLs. Subsequently, the tTCLs were used as explants to induce somatic embryogenesis (Figure 1). In addition, different types of explants were used, that is, leaf discs, petiole segments, longitudinal and transverse TCL of leaf and petiole.

Two basal media, Y3 salts and vitamins (Eeuwens, 1976) and MS salts and vitamins (Murashige and Skoog, 1962) at full and half strength, were tested. Both media were supplemented with 30 $g.L^{-1}$ sucrose, 100 $mg.L^{-1}$ Myo-inositol, 100 $mg.L^{-1}$ arginine, 100 $mg.L^{-1}$ asparagine, 100 $mg.L^{-1}$ L-glutamine and jellified with 3.8 $g.L^{-1}$ of Gelrite™ (DUCHEFA Biochemie B.V, Netherlands). The pH was adjusted to 5.7 ± 0.1, prior to autoclaving for 15 min at 121°C. The growth chamber conditions for all the experiments were darkness and temperature 26 ± 1°C. Light conditions were only used for the maturation of embryos.

The two basal media, were supplemented with different concentrations of the auxins 2,4-D, indole-3-acetic acid (IAA), Picloram, the cytokines 6-Benzyl-aminopurine (BAP) and Kinetin, alone and in combinations. When alone, the hormones were added in a decreasing gradient of 0.5 from 5 to 0 $mg.L^{-1}$, and when in combination, in a decreasing gradient of 0.5 from 2.5 to 0 $mg.L^{-1}$. 30 ml of culture medium was poured post-autoclaving into polystyrene Petri dishes (9 cm). Seven tTCLs from petioles were inoculated per Petri dish, and the Petri dish was sealed with Parafilm before being placed in a growth chamber.

After 4 weeks of initial inoculation, the explants with developed callus were transferred onto callus multiplication medium consisting of the two basal media supplemented with 0.05 $mg.L^{-1}$ of 2,4-D and 2 $mg.L^{-1}$ BAP. The Petri dishes were sealed and placed in the growth chamber.

Two weeks after multiplication, to induce SE, calli were transferred onto regeneration media. The regeneration media consisted of the two basal media, supplemented with 2 $mg.L^{-1}$ of BAP. Petri dishes containing the callus were sealed with Parafilm and kept in the growth chamber.

After 2 weeks in the induction phase, the calli showing embryo structures were transferred to both basal media containing 2 $mg.L^{-1}$ of BAP. The Petri dishes were kept in the growth chamber for further two weeks.

For the maturation of the embryos, they were transferred to both basal media, containing 2 $mg.L^{-1}$ of BAP, and the Petri dishes were moved to a growth chamber with 26 ± 1°C with a photoperiod of 16 h light/8 h darkness and an irradiance of ± 40 $\mu Mol.m^{-2}\ s^{-1}$.

RESULTS AND DISCUSSION

In order to overcome the recalcitrance, characteristic of adult plants of *J. curcas* for micropropagation (Jyoti et al., 1998; Nhut et al., 2013), it was sought to develop a protocol to induce SE using different types of explants from adult mother plants. Several types of explants were tested, and it was found that tTCLs of young petioles were the most suitable explants (Figure 2B). As shown in Figure 2A, tTCL of the petiole contains a small number of cells of different types, which means that this type of explant includes a new and greater number of patterns for morphogenesis compared to those present in other somatic cell systems (Gendy et al., 1996).

Contamination was not observed in the plant material from the greenhouse and in less than 10% of the plant material coming from the field. Thus, the methodology used for partial surface sterilization and disinfestation on

Table 1. Nine different accessions of *Jatropha curcas* L. used for the induction of somatic embryogenesis of adult plants.

Accession	Age (year)	Location	Condition
Ghana 1	12	Norwegian University of Life Sciences - NMBU	Greenhouse
Ghana 2	12	Norwegian University of Life Sciences – NMBU	Greenhouse
Cape Verde	11	Norwegian University of Life Sciences – NMBU	Greenhouse
Tanzania	12	Norwegian University of Life Sciences – NMBU	Greenhouse
Ethiopia	4	Norwegian University of Life Sciences – NMBU	Greenhouse
Indonesia 1	10	Norwegian University of Life Sciences – NMBU	Greenhouse
Indonesia 2	10	Norwegian University of Life Sciences – NMBU	Greenhouse
Colombia	6	Antioquia University – Medellín, Colombia	Field
Brazil	5	Antioquia University – Medellín, Colombia	Field

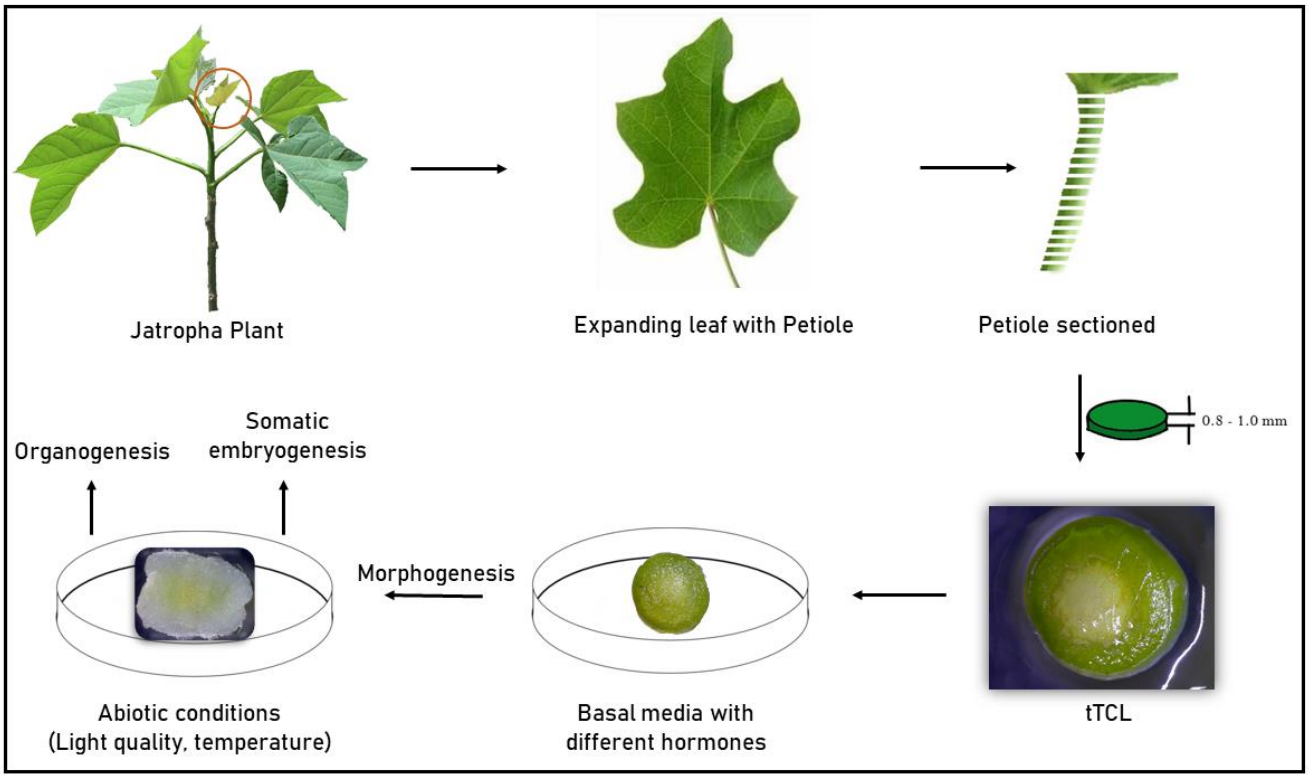

Figure 1. System used for obtaining somatic embryos from adult plants of *J. curcas* using young petioles tTCL and two different basal media, 5 different hormones and 85 different hormones combinations and concentrations.
Source: Nhut et al., (2005).

both plant materials was efficient, allowing the *in vitro* establishment of the selected accessions.

In this study, the results indicate that half-strength Y3 medium supplemented with 0.5 mg.L^{-1} of 2,4-D and 0.5 mg.L^{-1} of BAP gave the best results for callus induction (100%) in all accessions and more embryogenic cells compared to the other treatments (Figure 3). The other explants either died or the cells were hyper hydrated. To induce somatic embryos, Medipally et al. (2014) used the same concentration and type of hormones as were used in this paper, but with very young plants (9-months-old) and using leaf lamina and shoot tips as explant. For this protocol, responses were obtained from adult plants using petioles. Calli showed granular/nodular aspect, were friable and of a light green/yellow colouration. The cells were rounded and obtained seven days after first inoculation (Figure 2D).

It could be observed that callus started developing on

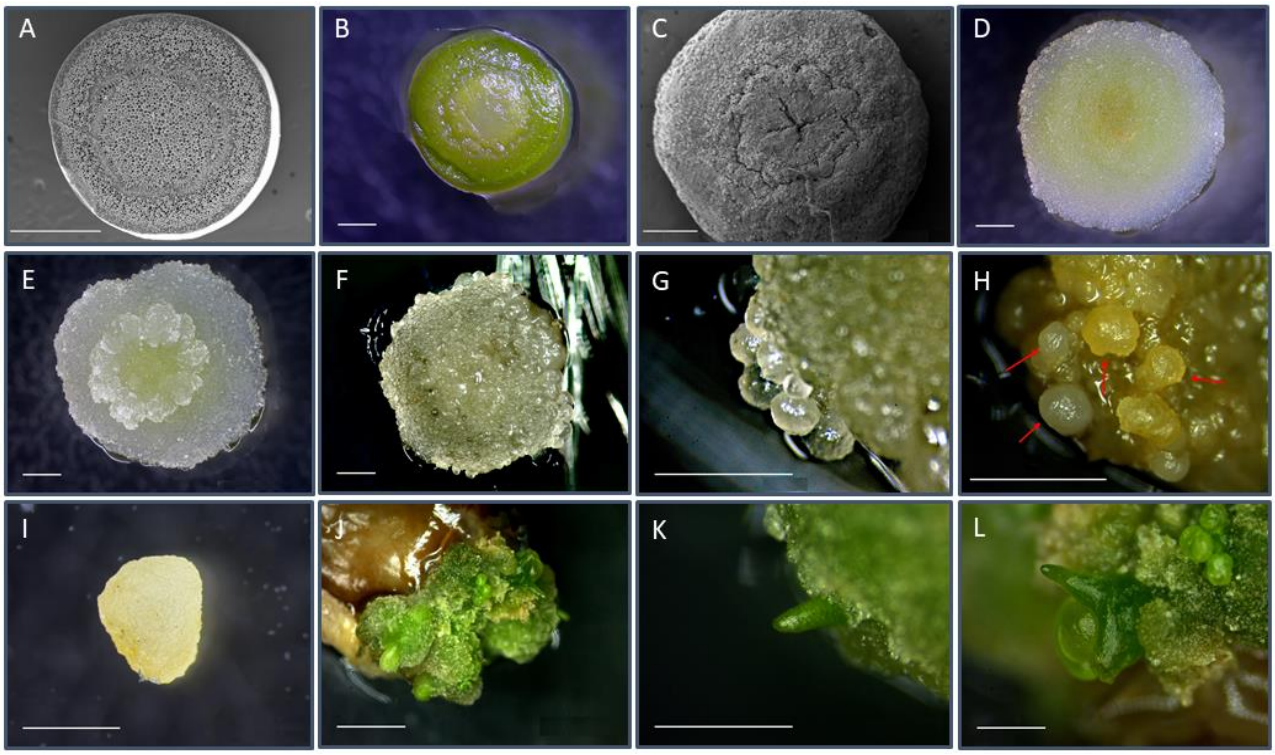

Figure 2. Somatic embryogenesis development and maturation from young petioles tTCL of *J. curcas* (A-D) Embryogenic callus obtained using half-strength Y3 medium supplemented with 0.5 mg.L^{-1} of 2,4-D and 0.5 mg.L^{-1} of BAP. (E-G) Callus multiplication and formation of pro-embryo structures using half-strength Y3 supplemented with 0.05 mg.L^{-1} of 2,4-D and 2 mg.L^{-1} of BAP. (H-L) Embryo formation and maturation using half-strength MS medium supplemented with 2 mg.L^{-1} of BAP and under light conditions. Red arrow shows formation of somatic embryos. Bar = 1 mm.

the central part of the petiole TCL and continued all over the explant (Figure 2C). Moreover, Nhut et al. (2013) described a protocol for propagating *J. curcas* through direct and indirect SE and Organogenesis using tTCL. However, they also performed it on very young plants and used leaves lamina, while in this study, adult plants were used given the advantage that the protocol can be used for elite genotypes. Even though in a more recent study (Galaz-Ávalos et al., 2018), SE was obtained using relatively low concentrations of exogenous hormones. The time for each stage (seed germination, plantlet preparation, individualisation of explant, embryo induction, etc.) was extremely long (≥100 days before first response was seen), whilst in this study, somatic embryos were obtained before 45 days after first inoculation and from *in vivo* explants.

The four-week-old calli were then transferred to multiplication media containing 0.05 mg. L^{-1} of 2,4-D and 2 mg.L^{-1} of BAP. Shortly after, callus cells started to multiply rapidly and form small clumps, mostly on the edges of the callus as well as on the central part (Figure 2E). It could be observed that the cells started growing and forming globular structures on the edges of the callus 45 days after first inoculation (Figure 2F-G), which was also recorded in carrot (Masuda et al., 1995), *J. curcas* (Kalimuthu et al., 2007), *Coffea arabica* (Bartos et al., 2018) and macaw palm (Meira et al., 2019).

For this experiment, the embryogenic cells were obtained only when both 2,4-D and BAP were present in the culture media. The importance of the interaction between auxins and cytokinins has been well studied and described previously (Litz and Gray, 1995) to improve cell organisation in morphogenetic processes (Fujimura and Komamine, 1980). Baran Jha et al. (2007) obtained embryos when the embryogenic calli were transferred onto MS supplemented with 0.5 mg.L^{-1} Kinetin and 0.25 mg.L^{-1} IBA, also proving the importance of auxin-cytokinin interaction.

Once the exogenous auxin 2,4-D was completely removed, and 2 mg.L^{-1} of BAP was kept in the medium, embryos started to form 55 days after inoculation (Figure 2H). Medium free of added auxins induces a reduction in the level of endogenous auxins in the explant, which has triggered somatic cells in the callus to start differentiation processes and also leads to the maturation of the embryos (Ribnicky et al., 1996). The somatic embryos in this protocol presented a white/yellow colouration and could be easily separated. (Figure 2I). Several authors have shown that callus growing in medium with 2,4-D, and thereafter, complete removal of the hormone has

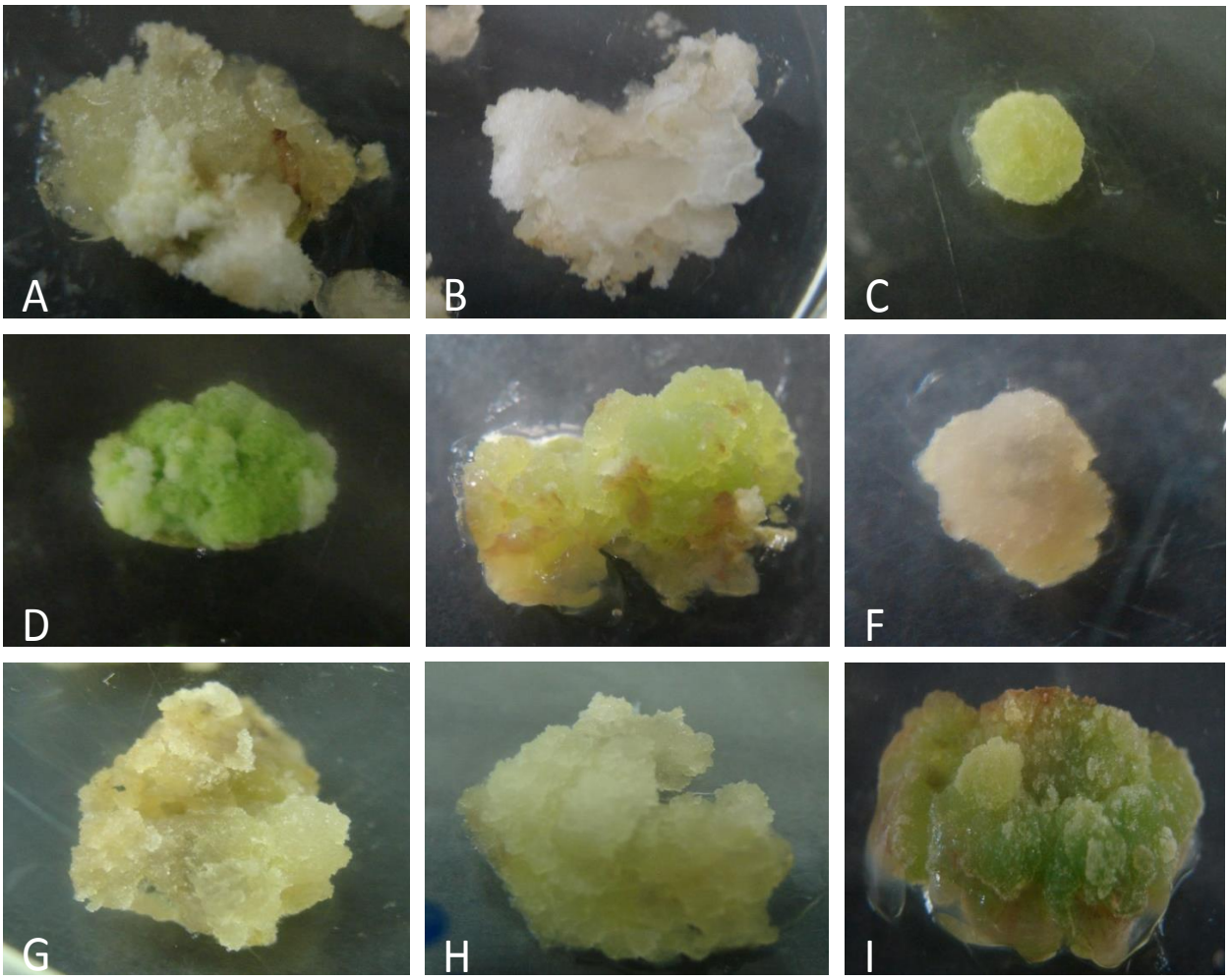

Figure 3. Type of calli of *J. curcas* obtained with the different PGR concentrations. (A) 1 mg.L^{-1} of 2,4-D and 0,5 mg.L^{-1} of Kin. (B) 2 mg.L^{-1} of 2,4-D. (C) 1 mg.L^{-1} of 2,4-D and 1 mg.L^{-1} of BAP. (D) 2,5 mg.L^{-1} of Kin. (E) 1 mg.L^{-1} of IAA and 1 mg.L^{-1} of BAP. (F) 2 mg.L^{-1} of Picloram (G) 1 mg.L^{-1} of 2,4-D and 2 mg.L^{-1} of BAP. (H) 1,5 mg.L^{-1} of IAA and 2 mg.L^{-1} of BAP. (I) 2,5 mg.L^{-1} of BAP.

induced SE (Cheema, 1989; Godbole et al., 2002; Seldimirova et al., 2019). Kalimuthu et al. (2007) managed to induce direct somatic embryos from green cotyledons using 2 mg.L^{-1} of BAP. In this study, when the embryos were moved onto half-strength MS medium, supplemented with 2 mg.L^{-1} of BAP under a photoperiod of 16h/8h, the embryos started to mature and advance into further stages after 85 days after inoculation (Figure 2J-L). In accordance with previous publications, BAP showed higher responsiveness on the somatic embryos, for maturation (Agarwal and Kamal, 2004; Cai et al., 2011; Kumar et al., 1994) and germination (Payghamzadeh and Kazemitabar, 2012; Raemakers et al., 1993; Siang et al., 2012).

Up to the date of submission of this study, there has not been a report on the importance of shifting between different basal media for inducing somatic embryo-genesis. However, there are many reports describing the importance of media composition on stimulating subsequent stages of SE, as well as for the optimisation of the morphogenetic response (Kumar et al., 2008; Pinto et al., 2008; Rodríguez-Sahagún et al., 2011; Walker and Sato, 1981).

As far as we are aware, this is the first report on the successful induction of somatic embryogenesis from several accessions of adult plants of *J. curcas* L., using tTCL of petioles. The fast and high response from the type of explant and the developmental stage of the mother plants make this protocol a great tool for inducing somatic embryogenesis from elite genotypes of *J. curcas.* This protocol also provides a great opportunity for plant breeders to develop improved varieties.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by the Norwegian Agency for Development Cooperation (NORAD) funded project "*Research and Capacity Building in Clean and Renewable Bioenergy in Ethiopia*" under the Norwegian Programme for Capacity Development in Higher Education and Research for Development (NORHED/ EnPe) Program, coordinated by Dr. Meseret Tesema of Hawassa University, Ethiopia and Norwegian University of Life Sciences (NMBU), Norway. The authors acknowledge a short stay in Prof. Lucia Atehortua's lab in Colombia.

ABBREVIATIONS

2,4-D, 2,4-dichlorophenoxyacetic acid; **BAP,** 6-Benzyl-aminopurine; **IAA,** indole-3-acetic acid; **Kinetin,** 6-furfuryl aminopurine; **MS,** Murashige and Skoog medium; **Picloram,** 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; **TCL,** thin cell layer; **Y3,** Y3 minerals medium.

REFERENCES

Agarwal M, Kamal R (2004). In vitro clonal propagation of *Momordica charantia* L. Indian Journal of Biotechnology 3:426-430.

Bahadur B, Sujatha M, Carels N (2012). Jatropha, Challenges for a New Energy Crop . Genetic Improvement and Biotechnology 2:139-142.

Baran Jha T, Mukherjee P, Datta MM (2007). Somatic embryogenesis in *Jatropha curcas* Linn., an important biofuel plant. Plant Biotechnology Reports 1:135-140.https://doi.org/10.1007/s11816-007-0027-2.

Barros TFS, Arriel NHC, Queiroz MF, Fernandes PD, Mendonça S, Ribeiro JAA, Medeiros EP (2015). Fatty acid profiles of species of *Jatropha curcas* L., *Jatropha mollissima* (Pohl) Baill. and *Jatropha gossypiifolia* L. Industrial Crops and Products 73:106-108.

Bartos PMC, Gomes HT, Gomes SM, Vasconcelos Filho SC, Teixeira JB, Scherwinski-Pereira JE. (2018). Histology of somatic embryogenesis in *Coffea arabica* L. Biologia 73:1255-1265.https://doi.org/10.2478/s11756-018-0131-5

Cai L, Fu L, Ji L (2011). Regeneration of *Jatropha curcas* through efficient somatic embryogenesis and suspension culture. GM Crops 2(2):110-117.https://doi.org/10.4161/gmcr.2.2.16126

Cheema GS (1989). Somatic embryogenesis and plant regeneration from cell suspension and tissue cultures of mature himalayan popular (*Populus ciliata*). Plant Cell Reports 8(2):124-127. https://doi.org/10.1007/BF00716855

de Oliveira JS, Leite PM, de Souza LB, Mello VM., Silva EC, Rubim JC, Suarez PA (2009). Characteristics and composition of *Jatropha gossypiifoliaand* and *Jatropha curcas* L. oils and application for biodiesel production. Biomass and Bioenergy 33(3):449-453.

Eeuwens C (1976). Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured in vitro. Physiologia Plantarum 36(1):23-28.https://doi.org/10.1111/j.1399-3054.1976.tb05022.x

Egertsdotter U, Ahmad I, Clapham D (2019). Automation and Scale Up of Somatic Embryogenesis for Commercial Plant Production, With Emphasis on Conifers. Frontiers in Plant Science 10(109):109. https://doi.org/10.3389/fpls.2019.00109

Fujimura T, Komamine A (1980). Mode of action of 2, 4-D and zeatin on somatic embryogenesis in a carrot cell suspension culture. Zeitschrift für Pflanzenphysiologie 99(1):1-8.https://doi.org/10.1016/s0044-328x(80)80106-1

Galaz-Ávalos RM, Avilez-Montalvo RN, Ucan-Uc C M, Chan-López JA, Loyola-Vargas VM (2012). *Jatropha curcas* una alternativa para la obtención de biodiésel sin afectar al sector alimentario. Biotecnología 16:94-114.

Galaz-Ávalos RM, Martínez-Sánchez HG, Loyola-Vargas VM (2018). Induction of Somatic Embryogenesis in *Jatropha curcas*. In: Plant Cell Culture Protocols pp. 207-214.

Gendy C, Sene M, Van Le B, Vidal J,Thanh KVT (1996). Somatic embryogenesis and plant regeneration in *Sorghum bicolor* (L.) Moench. Plant Cell Reports 15(12):900-904.

Godbole S, Sood A, Thakur R, Sharma M, Ahuja PS (2002). Somatic embryogenesis and its conversion into plantlets in a multipurpose bamboo, *Dendrocalamus hamiltonii* Nees et Arn. Ex Munro. Current Science pp. 885-889.

Jyoti S, Amla B, Renu S (1998). In vitro plantlet formation and micropropagation of *Jatropha curcas* (L.). Advances in Plant Sciences 11(2):167-169.

Kalimuthu K, Paulsamy S, Senthilkumar R, Sathya M (2007). In vitro propagation of the biodiesel plant *Jatropha curcas* L. Plant Tissue Culture and Biotechnology 17(2):137-147.

Kumar JV, Kumari BR, Sujatha G, Castano E (2008). Production of plants resistant to *Alternaria carthami* via organogenesis and somatic embryogenesis of safflower cv. NARI-6 treated with fungal culture filtrates. Plant Cell, Tissue and Organ Culture 93(1):85-96. https://doi.org/10.1007/s11240-008-9346-4

Kumar VD, Kirti PB, Sachan JKS, Chopra VL (1994). Plant regeneration via somatic embryogenesis in chickpea (*Cicer arietinum* L.). Plant Cell Reports 13(8):468-472. https://doi.org/10.1007/BF00231969

Litz RE, Gray DJ (1995). Somatic embryogenesis for agricultural improvement. World Journal of Microbiology and Biotechnology 11(4):416-425. https://doi.org/10.1007/BF00364617

Masuda H, Oohashi SI., Tokuji Y, Mizue Y (1995). Direct embryo formation from epidermal cells of carrot hypocotyls. Journal of Plant Physiology 145(4):531-534. https://doi.org/10.1016/s0176-1617(11)81783-9

Medipally SR, Naresh B, Manoj Kumar S, Yusoff FM, Devi P (2014). Somatic Embryogenesis from leaf and shoot tip explants of *Jatropha curcas* L.. Indian Journal of Science and Technology 7:1842-1846.

Meira FS, Luis ZG, de Araújo Silva-Cardoso IM,Scherwinski-Pereira JE (2019). Developmental pathway of somatic embryogenesis from leaf tissues of macaw palm (*Acrocomia aculeata*) revealed by histological events. Flora 250:59-67 https://doi.org/10.1016/j.flora.2018.11.011

Mulpuri S, Carels N, Bahadur B (2019). Jatropha, Challenges for a New Energy Crop: A Sustainable Multipurpose Crop 3:4-23.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15(3):473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nhut DT, da Silva JAT, Van Le B, Van KTT (2003). Thin cell layer (TCL) morphogenesis as a powerful tool in woody plant and fruit crop micropropagation and biotechnology, floral genetics and genetic transformation. Micropropagation of Woody Trees and Fruits pp. 783-814.

Nhut DT, Hai NT, Huyen PX, Huong DTQ, Hang NTT, da Silva JAT (2005). Thidiazuron induces high frequency shoot bud formation from Begonia petiole transverse thin cell layer culture. Propagation of Ornamental Plants 5(3):149-155.

Nhut DT, Loan NTK, Huy NP, Hai NT, Chien HX, Tuan TT, DuTX(2013). Thin Cell Layer Technology in Micropropagation of *Jatropha curcas* L. In Biotechnology of Neglected and Underutilized Crops pp. 33-42.

Nindita A, Purwoko BS, Efendi D, Dewi IS (2014). Somatic embryogenesis media optimization study of physic nut (*Jatropha curcas*) as biodiesel feedstock. Energy Procedia 47:21-28. https://doi.org/10.1016/j.egypro.2014.01.192

Payghamzadeh K, Kazemitabar S (2012). The effects of BAP, IBA and genotypes on in vitro germination of immature walnut embryos. International Journal of Plant Production 4(4):309-322.

Pinto G, Silva S, Park YS, Neves L, Araújo C, Santos C (2008). Factors influencing somatic embryogenesis induction in *Eucalyptus globulus* Labill.: basal medium and anti-browning agents. Plant Cell, Tissue and Organ Culture 95(1):79-88. https://doi.org/10.1007/s11240-008-9418-5

Raemakers C, Bessembinder J, Staritsky G, Jacobsen E, Visser R (1993). Induction, germination and shoot development of somatic embryos in cassava. Plant Cell, Tissue and Organ Culture 33(2):151-

156. https://doi.org/10.1007/bf01983228
Rehman A, Rauf A, Ahmad M, Chandio AA, Deyuan Z (2019). The effect of carbon dioxide emission and the consumption of electrical energy, fossil fuel energy, and renewable energy, on economic performance: evidence from Pakistan. Environmental Science and Pollution Research 26(21):21760-21773. https://doi.org/10.1007/s11356-019-05550-y
Ribnicky DM, Ilic N, Cohen JD, Cooke TJ (1996). The Effects of Exogenous Auxins on Endogenous Indole-3-Acetic Acid Metabolism (The Implications for Carrot Somatic Embryogenesis). Plant Physiology 112(2):549-558. https://doi.org/10.1104/pp.112.2.549
Rodríguez-Sahagún A, Acevedo-Hernández G, Rodríguez-Domínguez JM, Rodríguez-Garay B, Cervantes-Martínez J, Castellanos-Hernández OA (2011). Effect of light quality and culture medium on somatic embryogenesis of *Agave tequilana* Weber var. Azul. Plant Cell, Tissue and Organ Culture 104(2):271-275. https://doi.org/10.1007/s11240-010-9815-4
Seldimirova O, Kudoyarova G, Kruglova N, Galin I, Veselov D (2019). Somatic Embryogenesis in Wheat and Barley Calli in vitro Is Determined by the Level of Indoleacetic and Abscisic Acids. Russian Journal of Developmental Biology 50(3):124-135. https://doi.org/10.1134/s1062360419030056
Siang TC, Soong ST,Yien ATS (2012). Plant regeneration studies of *Jatropha curcas* using induced embryogenic callus from cotyledon explants. African Journal of Biotechnology 11(31):8022-8031.
Van KTT (1980). Control of morphogenesis by inherent and exogenously applied factors. in thin cell layers [Plants, de novo buds, roots]. International Review of Cytology 11:175-194.
Walker K, Sato S (1981). Morphogenesis in callus tissue of *Medicago sativa*: the role of ammonium ion in somatic embryogenesis. Plant Cell, Tissue and Organ Culture 1(1):109-121. https://doi.org/10.1007/bf02318910

Vol. 20(2), pp.92-99, February, 2021
DOI: 10.5897/AJB2020.17237
Article Number: C4A94DB66155
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Identification of zygotic and nucellar seedling of Harumanis mango through molecular markers and morphological approach

Zul Helmey Mohamad Sabdin[1*], Muhammad Najib Othman Ghani[2], Shahril Ab Razak[3], Mashitah Jusoh[4], Muhamad Hafiz Muhamad Hassan[1] and Ahmad Hafiz Buniamin[1]

[1]Horticulture Research Centre, Malaysian Agricultural Research and Development Institute (MARDI) Sintok, 06050 Bukit Kayu Hitam, Kedah, Malaysia.
[2]Industrial Crop Research Centre, Persiaran MARDI-UPM, Malaysian Agricultural Research and Development Institute (MARDI) Headquarters, 43400, Serdang, Selangor, Malaysia.
[3]Biotechnology and Nanotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI) Headquarters, Persiaran MARDI-UPM 43400 Serdang, Selangor, Malaysia.
[4]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia.

Received 1 September 2020; Accepted 8 February 2021

Identification of zygotic and nucellar seedlings of Harumanis mango by morphological approach is impossible or hard to be performed. Therefore, this research were to identify zygotic or nucellar seedlings from Harumanis mango polyembryonic seeds through simple sequence repeat (SSR) molecular markers in relation with germination sequence and vigour of seedlings for source of true-to-type cutting material. The results showed that there were 3 seedlings per seed on average. The result from the molecular analysis of 136 seedlings (45 seeds) showed four zygotic seedlings in seeds 1, 15, 29 and 43 representing 8.9% of seeds evaluated. Most of the zygotic seedlings were found towards the end of germination sequence except for seed number 15 and the rest were considered as nucellar seedlings. Based on the fitted logistic regression, the predicted sequence to obtain 90% nucellar seedlings is 5.47. This means that, the germination sequence of less than 6 has 90% chance of getting nucellar seedlings compared to zygotic seedlings. Morphological characters such as number of leaves, stem diameter and leaf area could also be used as references with germination sequence. The results showed that there were significant ($p<0.01$) relationships between germination sequence and all the growth variables. All growth variables were negatively correlated with germination sequence. This suggested that in order to have 90% chances of getting nucellar seedling (germination sequence below 6), the seedling needs to exhibit several morphology characters; big stem girth, tall plant, high leaf number and large leaf area. Therefore, choosing vigour seedling will increase chances of getting nucellar seedlings, which can be used as cutting source for true-to-type planting material or for breeding purposes.

Key words: Harumanis, molecular, morphology, nucellar, SSR markers, zygotic.

INTRODUCTION

Harumanis mango (*Mangifera indica*) is a mango variety that is economically important and classified as one of the sought-after mango variety in Malaysia (Farook et al., 2013). The demand for Harumanis mango is increasing

yearly due to the exquisite taste and aroma of the fruit (Khalid et al., 2017). However, the plant is only cultivated in the Northern region of Peninsular Malaysia such as in Perlis and Kedah since the weather is suitable for the growth of the Harumanis mango (Muhamad Hafiz et al., 2019; Rosidah et al., 2010). As recorded in 2019, there was about 6,373 ha of mango cultivation in Malaysia with 15,766 metric tons production (Department of Agriculture, 2019). This valuable crop is generally propagated by the means of grafting rather than via seedlings to ensure true-to-type planting material (Ahmad Hafiz et al., et al., 2020). This method of propagation however is laborious, costly, requires skilled worker, time-consuming and dependable on availability of seeds for rootstock; which explain the deficit in supply of Harumanis mango planting materials in Malaysia. An alternative method of mass production of true-to- type mango planting material is via cutting, which is reported to be more cost effective, efficient and uniform planting materials (Deependra et al., 2018). This method however is hindered in mango due to the polyembryonic attributes of the mango seed.

Harumanis mango is one of the several mango fruit trees that possess polyembryonic genotype (Mohd Asrul et al., 2018). Generally, cultivars originating from Southeast Asia, as well as tropical Latin America are polyembryonic, while those originating from Florida and India are largely monoembryonic (Vasanthaiah et al., 2007). Polyembryony is defined by the development of more than one seedling in a single seed, and one may be zygotic and the rest are nucellar (Simon et al., 2010; Ravishankar et al., 2004), in some reports all could be nucellar (Degani et al., 1993). This trait is genetically controlled, and in mangos, it is linked to a single dominant gene (Aron et al., 1998). The number of seedlings per seed varies with the cultivar and environmental conditions (Aline et al., 2014). The nucellar embryos in mango trees are developed in the nucellar tissue that covers the embryo sac, and the seedlings derived from these embryos are genetically identical to the parent plant (Aron et al., 1998). In contrast, the zygotic embryo is derived from fertilization by self-pollination or by cross-pollination. It is the objective in breeding programs for the selection of superior genotypes and variability achievement (Aline et al., 2014).

The identification of nucellar seedlings by morphological criteria is impossible or hard to be performed (Desai, 2004). Different morphological and biochemical markers had been used to distinguish nucellar from zygotic seedlings, but none was as efficient as molecular marker (Elisa Del et al., 2012). Thus, the use of molecular or isoenzymatic markers is necessary to observe the differences. Since it was difficult to select nucellar seedlings in seeds of polyembryonic mangos using morphological characteristics, several researchers had reported on the usage of genetic markers to identify zygotic and nucellar embryos. Some of the genetic markers used were Amplified Fragment Length Polymorphism (AFLP) (Eiadthong et al., 2000), Random Amplified Polymorphic DNA (RAPD) (Ravishankar et al., 2000; Elisa Del et al., 2012) and Inter Simple Sequence Repeat (ISSR) polymorphism (Aline et al., 2014). However, seedling industries find difficulties in identifying these markers and continuously choosing plants according to their morphological characteristics. To make identification of nucellar seedlings an easy task in selecting of cutting source, germination sequence and vigour of Harumanis seedlings were investigated in this study, to determine whether these characteristics exhibit relationship to their genetic origin. The objectives of this work were to identify the genetic origin, zygotic or nucellar of seedlings from Harumanis mango polyembryonic seeds by using SSR molecular markers and thereafter relating it to the seedling germination sequence and vigour.

MATERIALS AND METHODS

Plant and growth conditions

Seeds for the experiment were collected from Harumanis mango fruits obtained from Malaysian Agriculture Research and Development Institute (MARDI) Station, Sintok, Kedah on May 2019. Forty-five mature fruits of Harumanis mango were chosen. The flesh and seed coat were removed and washed with clean water and soaked in 0.2% Benomyl before the seeds were sown in the sandy seedbed. The seedbed was shaded with black netting (70%) and watered daily.

Germination sequence and seedling vigour determination

Seedlings that germinated from each seed were colour-tagged according to their germination sequence until 30 days of germination period. At 30 days after germination, plant height, stem diameter, number of leaves and leaf area of each seedling were determined. Measurement of plant height was taken from the soil surface to the highest shoot tip using a measuring tape. Stem diameter was measured at the lowest part of stem using Electronic Digital Caliper (Model SCM DIGV-6) while the leaf number was manually counted based on fully expanded leaves. Leaves areas were measured using an automatic leaf area meter (MODEL LI-300, LI-COR) and recorded as a total leaf-area per plant (Figure 1).

DNA extraction

Genomic DNA was extracted following the method described by

*Corresponding author. E-mail: zulhelmey@mardi.gov.my. Tel: +6049222601.

Figure 1. Seedlings of Harumanis mango at 30 days after germination in sandy seedbed.

Mace et al. (2003) with some modifications in term of incubation time. Leaf samples of each seedling at 30 days after germination was ground using the Tissue Lyser (Qiagen, Netherlands) before incubated with extraction buffer (2% CTAB, pH 8, 100 mMTris-HCl, 20 mM EDTA, 1.4 M NaCl, 0.05% *β*-mercaptoethanol) at 65°C for 1 h. Then, an equal volume of cold isopropanol was used to precipitate the DNA before being washed with 70% ethanol. The DNA pellet was air-dried before being eluted in 50 µl of TE-RNase buffer. The DNA concentration and integrity was measured using Epoch Biotek (Thermo Scientific, USA), and a 0.8% agarose gel, respectively.

SSR genotyping

The SSR markers were selected from Ravishankar et al., (2011) for the assessment of nucellar and zygotic seedlings presented in Table 1. The genotyping process followed the protocol as suggested by Schuelke (2000) by concatenating each of the forward primer with M13 sequence. The PCR was conducted in final volume of 10 µl reaction mixtures containing 10 × Invitrogen PCR Buffer, 2.5 mM $MgCl_2$, 1 µl genomic DNA (20 ng/µl), 2 µM dNTPs (0.2 µM/µl), 10 µM of each primer pair (0.5 µM/µl), 5 µM of fluorescent dye (FAM/NED/PET/VIC) (0.5 µM/µl) and 1 Unit of Taq polymerase (Invitrogen, California). The amplification of the target region was performed using the Applied Biosystem Gene Amp (Thermo Fischer Scientific, California). The PCR profile was set with an initial denaturation for 2 min at 95°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45 to 60°C for 30 s and extension at 70°C for 45 s before being terminated with a final extension for 5 min at 70°C. Then, the PCR products were resolved using an ABI3730xl DNA Analyzer (Thermo Fischer Scientific, California) with Gene Scan TM 500 LIZ (Applied Biosystems, California) used as the DNA ladder.

Scoring and data analyses

The output files (fsa. file) from ABI3730 DNA Analyzer were analyzed using Gene Mapper 5.0 (Applied Biosystems). The allele peaks were identified and scored as suggested by Arif et al., (2010). The seedlings alleles were compared with the parental alleles. POWERMARKER (Liu and Muse, 2005) was used to calculate the genetic distance. Number of seedlings germinated in for each seed was analyzed using descriptive analysis while the responses (zygotic or nucellar) was analyzed using logistic regression. The logistic regression model was used to estimate the sequence of germination for a certain odd ratio of nucellar: zygotic seedlings. Pearson correlation coefficient (r) was determined between the sequence of germination and all the vegetative parameters at $p \leq 0.05\%$.

RESULTS AND DISCUSSION

Polyembryony in Harumanis mango

The results in this study revealed that there were 3.07 seedlings per Harumanis mango seed on average; ranging from 1 to 7 seedlings. Most of the seeds, 51.1% had 2 or 3 seedlings per seed while only 4.4% had 7 seedlings (Figure 2). The results in this study are in accordance with Zakaria et al. (2002) that reported 2 to 6 seedlings per seed in *M. indica* (cultivar Sala and Tangkai Panjang) while 1 to 2 seedlings per seed in *Mangifera foetida* and *Mangifera caesia*. The difference between the numbers of seedlings in species of *Mangifera* has been reported by Cordeiro et al. (2005)

Table 1. List of SSR markers used in this study based on Ravishankar et al.,(2011).

SSR ID	Forward	Reverse	Motif	Size (bp)
MiIIHR01	GGATGCACAACAACAAGCAC	TCAGCAAGCAATCCCTTCTT	(GAA) 4 CAG(CAA) 2 (TA) 2	237-261
MiIIHR02	CCCCAACATTTCATAAACACA	CCTCCTTACATGCCTCCTTG	(CA) 2 A(CA) 7 AG(CA) 5	165-221
MiIIHR03	GTCGATGCCTGGAATGAAGT	AAGCATCGAACAGCTCCAAT	(CTT) 6 (CA) 2	227-235
MiIIHR06	CGCCGAGCCTATAACCTCTA	ATCATGCCCTAAACGACGAC	(CA) 7 CG(CA) 5	93-122
MiIIHR07	GCCACTCAGCTAAATAGCCTCT	TGCAGTCGGTAAAGTGATGG	(GA) 11	159-185
MiIIHR09	GTTGTGACCGAGGCCTTAAA	CTTTGACATCGCTGATCTGG	(CT) 3 TTGC(CT) 2 GT(CT) 4 TC(GT) 2 (CT) 2	273-291
MiIIHR10	CGATTCAAGACGGAAAGGAA	TTCAAGCACAGACGACCAAC	(GTT) 6	161-184
MiIIHR11	CAGTGAAACCACCAGGTCAA	TGGCCAGCTGATACCTTCTT	(CT) 2 TT(CTT) 5	203-213
MiIIHR12	GCCCCATCAATACGATTGTC	ATTTCCCACCATTGTCGTTG	(GA) 11	154-188

and is due to the genetic differences between the species.

Molecular analysis

The analysis of 136 seedlings which were derived from 45 seeds using microsatellite markers showed a total of four zygotic seedlings and they were identified in seeds 1, 15, 29 and 43 representing 8.9% of seeds evaluated (Table 2). Most of the zygotic seedlings were found towards the end of germination sequence except for seed number 15. This was in agreement with the study conducted by Aline et al. (2014) on Uba cultivars. However, details and depth study are required to understand the sequence order and the occurrence of the zygotic seedling in Harumanis seed. Meanwhile, the remaining seedlings were considered as nucellar seedlings as the SSR based DNA profile match with the parent DNA profile of Harumanis mango. In the present study, seedlings that showed polymorphism at least by one primer were also considered as zygotic since this study was using codominant marker system. However, in this study, zygotic seedling from seed 15 showed the least polymorphic across all nine. As we were using codominant marker, we can directly and perfectly identified the heterozygote seedling which happened due to the pollination with the external pollen source. Hence, the seedlings with the presence of heterozygote allele form even at one marker were considered as zygotic seedling. Figure 3 describes the allelic segregation of nucellar and zygotic seedling on locus. The number of polymorphic marker was different between the zygotic seedlings which might be caused by receiving a different pollen donor. There is the successful study on identification of pollen donor in European Plum using microsatellite markers (Meland et al., 2020). Unlike previous studies which were using dominant marker system such as RAPD and ISSR, the researcher was required to set at least three polymorphic primers in order to consider the seedling is zygotic (Aline et al., 2014). Since the dominant marker system such RAPD and ISSR is unable to differentiate between homozygote and heterozygote of allele which lead to numerous number of marker is needed in order to identify the occurrence of zygotic and nucellar seedlings (Miah et al., 2013). The present study also showed there was no occurrence of double zygotic seedling in a single seed (Policaulismo).

Relationship of genetic material in accordance with germination sequence and vigour

In computing the probability of getting nucellar seedling for source of true-to-type cutting material based on germination sequence, the logistic regression model fitted from the data of germination sequence and genetic material (nucellar or zygotic) is:

$$Y = 5.29 - 0.57x$$

where Y is the outcome of nucellar or zygotic (nucellar (1) or zygotic (0)) and X is the germination sequence. Based on the parameter estimate in Table 3, the estimated odd ratio of

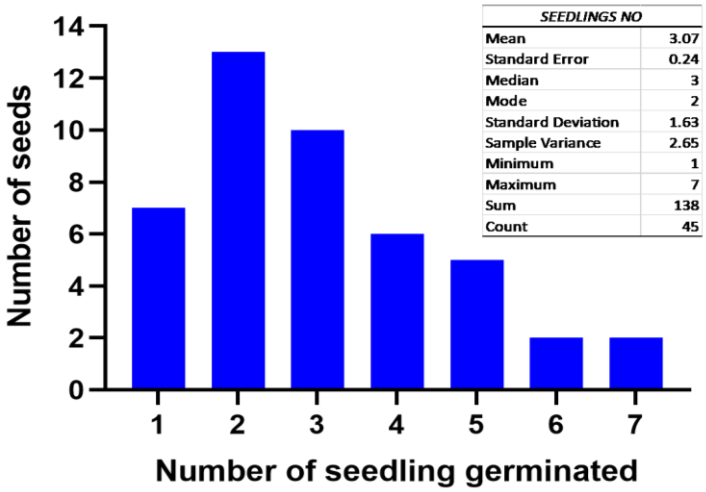

Figure 2. Number of seeds with different number of seedlings germinated

Table 2. Seedlings evaluated from 45 seeds of Harumanis mango.

Seed	Germination sequence	Seed	Germination sequence	Seed	Germination sequence
1	1, 2, 3, 4, 5, **6**, 7	16	1	31	1, 2, 3, 4, 5
2	1, 2, 3, 4, 5	17	1, 2, 3	32	1, 2, 3, 4
3	1	18	1, 2	33	1, 2, 3, 4, 5
4	1, 2, 3	19	1	34	1, 2, 3
5	1, 2, 3	20	1, 2	35	1
6	1	21	1, 2	36	1, 2
7	1, 2, 3, 4	22	1, 2	37	1, 2
8	1, 2, 3, 4, 5	23	1, 2, 3	38	1, 2, 3
9	1, 2, 3	24	1, 2, 3	39	1, 2
10	1, 2, 3	25	1	40	1, 2, 3, 4
11	1, 2, 3	26	1, 2	41	1, 2
12	1, 2	27	1	42	1, 2, 3, 4, 5, 6, 7
13	1, 2, 3	28	1, 2, 3, 4	43	1, 2, 3, 4, **5**
14	1, 2, 3, 4, 5, 6	29	1, 2, 3, **4**	44	1, 2
15	**1**, 2, 3, 4	30	1, 2	45	1, 2, 3, 4, 5, 6

*Numbers underlined on the germination sequence column are zygotic seedlings; not underlined are nucellar

getting a nucellar seedling is $\widehat{OR} = e^{-0.5676} = 0.57$. This means that as the germination sequence increases by one unit, the odds of getting nucellar seedlings reduces by 57%.

Based on the fitted logistic regression, the predicted sequence to obtain 90% nucellar seedlings is 5.47. This means that, the germination sequence of less than6 has 90% chances of getting nucellar seedlings compared to zygotic seedlings. Figure 4 shows the logistic regression plot for the germination sequence vs. predicted probability of getting a nucellar seedling.

Using germination sequence as a reference in getting nucellar seedlings for source of cutting materials serve some challenges as the nurserymen have to closely monitor germination and tag the seedlings. Alternatively, morphological characteristics such as leaf number, stem diameter and leaf area could also be used as reference with germination sequence. The results showed that there were significant (p<0.01) relationships between germination sequence and all the growth variables (Figure 5). All growth variables were negatively correlated with germination sequence, with the strongest relationship

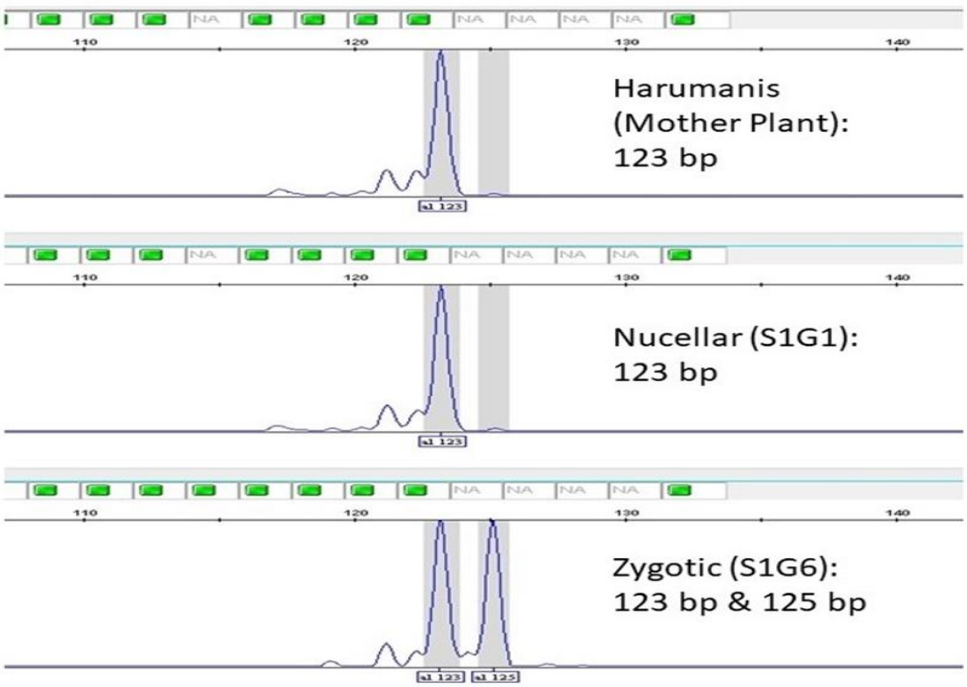

Figure 3. Allelic size of nucellar and zygotic seedlings with their respective parent (Harumanis) on locus MiIIHR06. Zygotic seedling received an extra allele (125 bp) from external pollen source. S1G1 (seed 1, germination sequence 1), S1G6 (Seed 1, germination sequence 6).

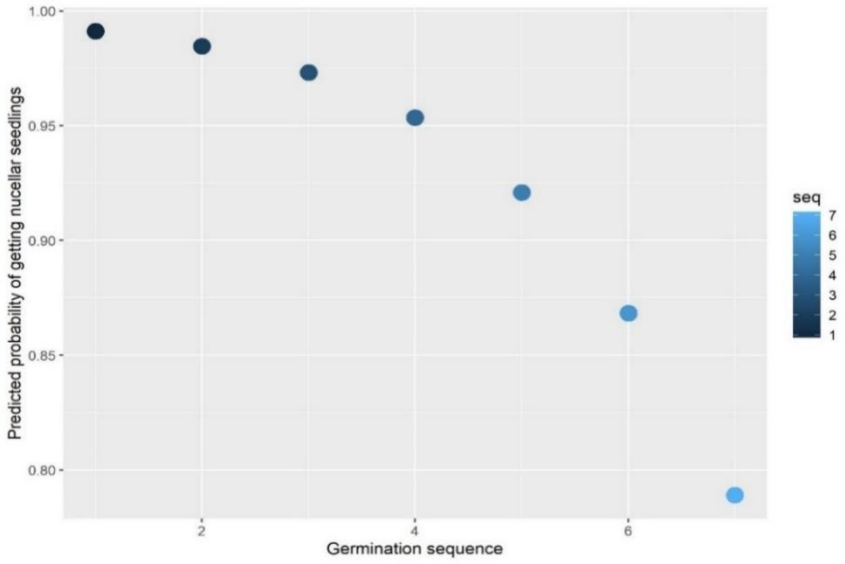

Figure 4. Predicted probability plot of getting nucellar seedlings (Pr (X=1))

(r= -0.39; $p \leq 0.01$) was recorded between germination sequence and stem diameter. This was followed by plant height (r= -0.38; $p \leq 0.01$), leaf number (r = -0.29; $p \leq 0.01$) and lastly leaf area (r = -0.27; $p \leq 0.01$) (Figure 3). However, leaf number showed positive correlation with plant height (r = 0.49; $p \leq 0.01$), stem diameter (r = 0.43; $p \leq 0.01$) and leaf area (r = 0.43; $p \leq 0.01$). These results showed that there was an increase in leaf number with

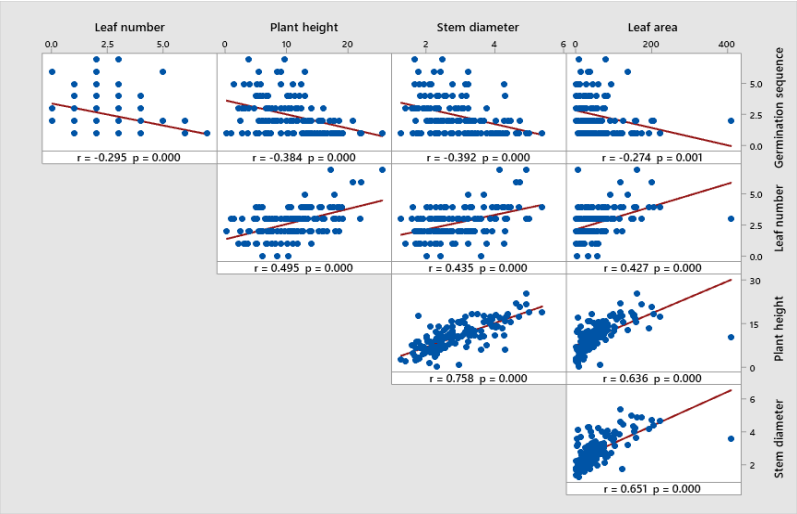

Figure 5. Matrix plot of germination sequence with vegetative variables.

the increase of plant height, stem diameter and leaf area. The same result was obtained by Shaban (2010) who found that leaf number was correlated positively with leaf area and plant height and leaf area for Zebda mango seedlings from Egypt. In term of plant height, there was a strong positive correlation with stem diameter ($r = 0.76$; $p \leq 0.01$) and leaf area ($r = 0.64$; $p \leq 0.01$). The stem diameter also had strong positive correlation with leaf area ($r = 0.65$; $p \leq 0.01$). Zakaria et al. (2002) found that the seedlings from different variety of *Mangifera* species seeds differ in terms of vigour, plant size or height depending on whether they are nucellar or zygotic in origin. Zakaria et al. (2002) and Muralidhara et al. (2015) suggested that the removal of seed coat might had given a superior response in all initiation of plant height, stem diameter, number of leaves per plant and leaf area. At the same time, the different response growth of seedlings produced after germination and emergence that may be caused by competition between seedlings for nutrient uptake, light and space.

This suggested that in order to have 90% chances of getting nucellar seedling (germination sequence below 6), the seedling needs to exhibit several morphological characteristics; big stem girth, tall plant, high leaf number and large leaf area. These are morphological characteristics of vigor seedlings. Generally, the most vigorous seedling from each seed are used by the nurserymen for the production of rootstock however the nucellar seedling is not always the most vigorous, which results in uneven orchards (Simon et al., 2010). In 'Uba' mangos, 60% of the seedlings tested were discovered as zygotic, and not correlated with the vigorous character tested (Aline et al., 2014). In addition, 90% of the most vigorous seedlings from seeds of 'Rosinha' mangos collected in 2002 and 2003 were of zygotic origin, while seedlings from seeds harvested in 2004 were mostly identified as nucellar, indicating no relationship between the type of embryo and seedling size (Cordeiro et al., 2005).

Conclusions

Harumanis is a polyembryonic mango with average 3 seedlings per seed. Based on SSR molecular markers, zygotic seedlings were found towards the end of germination sequence with 8.9% of total seeds evaluated. DNA marker system was proven to be the ideal approach in identifying zygotic and nucellar seedlings as the identification is not influenced by environmental factor and agronomic practices. Choosing vigour seedling will increase the chances of getting nucellar seedlings, which can be used as cutting source for true-to-type planting material or for breeding purposes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This research work was supported by MARDI and Malaysian Government under 11th Development Fund Project (P-RH405). The authors also thank Mr. Muhammad Faris Ali for assisting in samples preparation and data collection.

REFERENCES

Aron Y, Czosnek H, Gazit S, Degani C (1998). Polyembryony in mango (*Mangifera indica* L.) is controlled by a single dominant gene. Horticultural Science 33:1241-1242.

Arif IA, Khan HA, Shobrak M, Al Homaidan AA, Al Sadoon M, Al Farhan AH, Bahkali AH (2010). Interpretation of electrophoretograms of seven microsatellite loci to determine the genetic diversity of the Arabian Oryx. Genetics and Molecular Research 9(1):259-265.

Aline R, Fernandes S, TâniaM, Lopes SD, Damião CC, Chamhum SC (2014). Identification of 'Ubá' mango tree zygotic and nucellar seedlings using ISSR markers. Revista Ceres 61(5):597-604.

Ahmad Hafiz B, Mahmad Noor J, Mohd Asrul S, Hartinee A (2020). Growth performance of different mango (*Mangifera indica* L.) varieties as rootstock for Harumanis planting material production. Journal of Tropical Plant Physiology 12(1):49-56.

Cordeiro MCR, Ramos VHV, Pinto ACQ, Fraga LMS, Dias JN, Lopes GKB (2005). Molecular identification of the zygotic or nucellar origin of polyembryonic mango seed 'Rosinha' seedlings based on RAPD. In: 3rd Brazilian Congress of Plant Breeding, Gramado. Anais, Embrapa Wheat. CD-ROM.

Degani C, Cohen M, Reuveni O, El-Bastri R, Gazit S (1993). Frequency and characteristics of zygotic seedlings from polyembryonic mango cultivars, determined using isozymes as genetic markers. Acta Horticulturae 341:78-85.

Desai BB (2004). Seeds handbook: biology, production, processing and storage. 2nd ed. New York, CRC Press, p. 787.

Yadav D, Pal AK, Singh SP (2018). Vegetative methods of plant propagation: II- grafting, cutting, layering and budding in mango. International Journal of Pure and Applied Bioscience 6(3): 575-586.

Department of Agriculture (DOA) (2019). Crops Statistic (Sub-Sector Food Crops) 2019. Putrajaya, Malaysia.http://www.doa.gov.my Accessed August 13, 2020.

Eiadthong W, Yonemori K, Kanzaki S, Sugiura A, Utsunomiya N, Sabhadrabandhu S (2000). Amplified fragment length polymorphism analysis for studying genetic relationships among *Mangifera* species in Thailand. Journal of the American Society of Horticultural Science 125(2):160-164.

Elisa Del CMO, Andrade‐Rodríguez M, Mario RR, Angel VM (2012). Identification of zygotic and nucellar seedling in polyembryonic mango cultivars. Pesquisa Agropecuária Brasileira 47(11):1629-1636.

Farook RSM, Ali H, Harun A, Ndzi DL, Shakaff AYM, Jaafar MN, Husin Z, Aziz AHA (2013). Harumanis mango flowering stem prediction using machine learning techniques. Research Notes in Information Science 13:46-51.

Khalid NS, Abdullah AH, Shukor SAA, Fathinulsyahir AS, Chau SC, Nor Dalila ND, Mansor H (2017). Image processing techniques for Harumanis disease severity and weighting estimation for automatic grading system application. Journal of Telecommunication, Electronic and Computer Engineering 10:1-15.

Liu K, Muse SV (2005). Power Marker: an integrated analysis environment for genetic marker analysis. Bioinformatics 21(9):2128-2129.

Mace ES, Buhariwalla KK, Buhariwalla HK, Crouch JH (2003). A high-throughput DNA extraction protocol for tropical molecular breeding programs. Plant Molecular Biology Report 21(4):459-460.

Meland M, Frøynes O, Fotiric Akšić M, Pojskić N, Kalamujić Stroil BK, Lasic L, Gasi F (2020). Identifying pollen donors and success rate of individual pollinizers in European plum (*Prunus domestica* L.) using microsatellite markers. Agronomy 10(2):1-16.

Miah G, Rafii MY, Ismail MR, Puteh AB, Rahim HA, Islam K, Latif MA (2013). A review of microsatellite markers and their applications in rice breeding programs to improve blast disease resistance. International journal of molecular sciences 14(11):22499-22528.

Muralidhara BM, Reddy YTN, Srilatha V, Akshitha HJ (2015). Effect of seed coat removal treatments on seed germination and seedling attributes in mango varieties. International Journal of Fruit Science 16:1-9.

Mohd Asrul S, Hartinee A, Mahmad Nor J, Mohamad Bahagia AG (2018). Morphological characterisation of Harumanis mango (*Mangifera indica* Linn.) in Malaysia. International Journal of Environmental and Agriculture Research 4(1):36-42.

Muhamad Hafiz MH, Hartinee A, Nor Dalila ND, Zul Helmey MS, Razali M, Ab Kahar S, Siti Aisyah A, Wan Mohd Reza IWH, Shaidatul Azdawiyah AT (2019). Effect of multilocation production on the reproductive growth, yield and quality of Harumanis mango. International Journal of Current Advanced Research 08(04):18175-18180.

Ravishankar KV, Lalitha A, Dinesh MR (2000). Assessment of genetic relatedness among mango cultivars of India using RAPD markers. The Journal of Horticultural Science and Biotechnology 75(2):198-201.

Ravishankar KV, Chandrashekara P, Sreedhara SA, Dinesh MR, Lalitha A, Saiprasad GVS (2004). Diverse genetic bases of Indian polyembryonic and monoembryonic mango (*Mangifera indica* L) cultivars. Current Science 87:870-871.

Rosidah M, Faridah H, Jamaliah MY, Norzaidi MD (2010). Examining market accessibility of Malaysia's Harumanis mango in Japan: challenges and potentials. Business Strategy Series 11(1):3-12.

Ravishankar KV, Mani BH, Anand L, Dinesh MR (2011). Development of new microsatellite markers from Mango (*Mangifera indica*) and cross-species amplification. American Journal of Botany 98(4):96-99.

Schuelke M (2000). An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology 18(2):233-234.

Simon AM, Festus KA, Gudeta S, Oluyede CA (2010). Rootstock growth and development for increased graft success of mango (*Mangifera indica*) in the nursery. African Journal of Biotechnology 9(9):1317-1324.

Shaban AEA (2010). Comparative study on some polyembryonic mango rootstocks. American-Eurasian Journal Agriculture and Environmental Science 7(5):527-534.

Vasanthaiah HKN, Ravishankar KV, Mukunda GK (2007). Mango. In: Kole. C. (ed.) Genome mapping and molecular breeding in plants. Springer-Verlag, Berlin Heidelberg 4:303-323.

Zakaria W, Tengku Ab. Malik TM, Masri M (2002). Germination pattern of three *Mangifera* species. Journal of Tropical Agriculture and Food Science 30(2):163-171.

Vol. 20(2), pp.100-107, February, 2021
DOI: 10.5897/AJB2020.17265
Article Number: 5C9A0D866176
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Review

Aspects of mushroom cultivation to obtain polysaccharides in submerged cultivation

L. H. Campestrini* and Salles-Campos C.

Laboratorio de cultivo de fungos comestíveis, Instituto Nacional de Pesquisas da Amazônia, 13418-900, Manaus, AM, Brazil.

Received 22 October, 2020, Accepted 27 November, 2020

Medicinal and edible mushrooms have biological properties that are important in promoting health. In the last decades, an increasing number of studies have explored various aspects of the cultivation and metabolism of various species. Many isolated substances have potential for use in medicine and among them are polysaccharides, which have been shown to have physiological properties such as anti-tumor, immunomodulatory, anti-hypercholesterolemia, antiviral and antiinflammatory. The most common way of obtaining products from mushrooms is through cultivation in a solid medium. However, in this type of cultivation there is a difficulty in controlling the physical and chemical parameters and, consequently, the quality of the product. In this context, the submerged fermentation of the mycelial form of the mushroom has received attention as an alternative for the efficient production of biomass and, therefore, of polysaccharides. Technical problems and forms of cultivation are studied so that the standardization of cultivation is more effective. This review describes some aspects about the submerged fermentation technique in bottles and bioreactors, exploring the two main ones, which are the stirred tank fermentator and the air lift reactors and the species that are in these cultivated systems.

Key words: Mushrooms, polysaccharides, submersed fermentation, biorreactor.

INTRODUCTION

In recent decades, a large number of publications have presented the possibility of using mushrooms in the production of several metabolites of interest of low molar mass (Shu and Lung, 2004; Zou, 2006; Tang et al., 2007) and high molar mass, such as polysaccharides, which are shown as potent pharmacological agents with diverse activities. In addition, mushrooms have been widely consumed for centuries in the East and Europe, given their high nutritional value, with around 2000 edible species recognized and approximately 25 grown on a commercial scale (Jong and Donovick, 1989; Furnali and Godoy, 2007). In its composition, there are essential and non-essential amino acids, carbohydrates, organic acids, minerals, vitamins low lipids and high protein and fiber contents (Ghorai et al., 2009; Kawagoe et al., 2004; Sales-Campos et al., 2011a, 2013).

Special attention is given to fungal polysaccharides because they have biological activity as antioxidant, antitumor, hypoglycemic, aintinflammatory and immunomodulatory (Bai et al., 2020; Fan et al., 2007;

*Corresponding author. E-mail: lcampestrini@gmail.com.

Zhang et al., 2011; Silveira et al., 2015; Wang et al., 2019). Polysaccharides are metabolites of great interest in the cultivation of basidiomycetes. The molecular arrangement of these polysaccharides is well distributed, but most belong to the groups of β-glucans, whose main chain connections are of the type β-(13) and varying number of β-(16) branches (Seo et al., 2019). However, at least one example of a β-(13) glucan, containing β-(12) and β-(16) branches has already been described (Ruiz-Herrera and Ortiz-Castellanos, 2019).

Traditionally, mushrooms are grown on a solid substrate under controlled temperature and humidity conditions. However, this method requires a long cultivation time and low yield, in addition to the possibility that the culture is contaminated by other microorganisms (El-Enshasy et al., 2010).

In addition to solid-state crops, fungi are grown in liquid media in bottles or bioreactors. This type of cultivation presents a fast, efficient and less expensive alternative form (Wu et al., 2003, 2004; Tang and Zhong, 2002), in which organisms can be obtained in limited physical spaces under conditions controlled and optimized for the production of biomass, exopolysaccharides (EPS) and endopolysaccharides (IPS), simultaneously (Cui et al., 2006; Confortin et al., 2008; Liu and Zhang, 2019; Guillén-Navarro et al., 1998), also showing the ease of separating the mycelium from the culture medium, obtaining products of uniform quality, in addition to the possibility of being cultivated all year round (Leonowicz et al., 1991; Rosado et al., 2002).

Many species are grown in flasks of liquid medium or even in bioreactors, including *Lentinus edodes, Ganoderma, lucidum, Schizophyllum commune, Trametes versicolor, Inonotus obliquus* and *Flammulina velutipes*. For the production of polysaccharides, an important step in submerged culture is the type of bioreactor used, since it directly influences the development of the mycelium. When it comes to flasks or stirred tank reactors, even optimized conditions can behave differently (Burns et al., 1994; Maziero et al., 1999; Rosado et al., 2003; Zhang et al., 2003).

Many studies are carried out with the aim of optimizing culture media for the production of fungal metabolites using statistical techniques (Fan et al., 2007; Joo et al., 2004; Assis et al., 2013). Studies, such as the one carried out using the fungus *Grifola frondosa*, were conducted in order to optimize the production of biomass and exopolysaccharide by response surface techniques (RSM) (Cui et al., 2006).

The bioprocesses used in the cultivation of fungi are carried out under optimal conditions such as temperature, pH, aeration, types of nutrients, pressure, type of reactor used, shear stress, the use of surfactants or defoamers and produced biomass. These processes involve complex and multiple biochemical reactions as well as a particular kinetics for each type of organism grown (Garcia-Ochoa and Gomez, 2009).

Thus, the objective of this work is to review some fungi of interest grown in a submerged manner for the production of polysaccharides, in flasks and bioreactors in agitated and pneumatic tanks.

CULTIVATION IN SUBMERGED FERMENTATION: A NEW PERSPECTIVE OF CULTIVATION

The submerged fermentation process is the process of cultivating microorganisms in liquid culture medium. This type of cultivation has several advantages over solid state fermentation. One of the main advantages is the possibility and ease of homogenization during the process. Homogenization is possible, as the medium is easily mixed, eliminating the differences that are generated as the microorganism grows. The liquid medium allows important factors, such as temperature and pH, to be easily measured and controlled. In addition, the consumption of nutrients is more complete, allowing maximum use of nutrients and maximum growth. These variables can be measured and controlled due to the greater precision of the samples taken during cultivation (Rossi et al., 2002; Elisashvili, 2012). Another advantage associated with submerged fermentation is the speed with which crops are grown, reducing production time and, consequently, the chances of contamination occurring. This is due to the ease with which nutrients are dispersed in the environment, maximizing the contact area between microorganisms and nutrients (Wu et al., 2003).

Cultivation can be carried out in static and agitated flasks, with forced aeration and also in bioreactors. The studies in small flasks aim to evaluate the best cultivation conditions so that the process can be scaled, reducing the risk of failure in the process at the industrial level. The cultivation of filamentous fungi by submerged fermentation is already a widespread practice, with a primary focus on obtaining products from primary or secondary metabolism secreted in the medium such as proteins, enzymes, acids, antibiotics, exopolysaccharides (EPS) intracellular polysaccharides (IPS) and other bioactive, medicinal and/or industrial compounds and also biomass for use as a food supplement (Saeki et al., 2011; Elisashvili, 2012; Vamanu, 2012; Singh et al., 2013).

Cultivation in flasks

The medium used for the cultivation of microorganisms must contain in a balanced way all the elements and nutrients for the synthesis of cellular substances and for the production of metabolites. Chemically defined culture media are used in laboratory research, but on an industrial scale, for economic reasons, complex substrates of variable composition are used, by-products of other industries. These substrates must be adapted for

cultivation by means of extraction, supplementation and balancing the composition of the medium in order to increase the production yield of the selected microorganism, using screening experiments prior to cultivation in bioreactors or even aiming at the production of polysaccharides in the plants themselves (Crueger and Crueger, 1990).

Submerged fermentation techniques are widely developed to be applied to most medicinal mushrooms for the purpose of mycelium propagation (Smith et al., 2002). Factors such as nutrients (macronutrients and micronutrients), temperature, pH and agitation are factors that can influence the growth rate of macromycetes, the biomass yield and the production of metabolites (Sales-Campos et al., 2011b). Thus, it is essential to optimize the parameters in advance and to maintain them during the cultivation period (Singh et al., 2013).

Fungi of the genus *Pleurotus*, are widely studied and reports for cultivation methodologies are described for several strains (Table 1).

Pleurotus sajor-caju, grown in flasks, were studied for the optimization of the medium, with the different variables being a source of nitrogen $(NH_4)_2SO_4$, yeast extract and soy peptone. For this fungus, two concentrations of $(NH_4)_2SO_4$ and yeast extract were tested in addition to the high concentration of soy peptone, which caused the EPS concentration to reach 0.60 g/L (Assis et al., 2013). The cultivation of this species served as a model for an optimization process based on the response surface methodology, taking into account three parameters, with a number of combinations of 2^3. In 500 mL flasks, Erlenmeyer flasks containing 100 mL of medium were established that the highest concentrations of soy protein and yeast extract, combined with the lowest concentration of $(NH_4)_2SO_4$, were more efficient in biomass production (Confortin et al., 2008).

Also for *Pleurotus djamour*, whose optimization of the carbon source (glucose) and pH were tested as a function of biomass and EPS production, showed that the initial combination of 40 g/L of glucose and pH 3.0 decreased the consumption time of carbohydrate when compared with the other conditions (Borges et al., 2013).

Maftoun et al. (2013) cultivated *Pleurotus ostreatus* of 250 and 50 mL of medium. 7 different means were tested in order to select the most suitable one. For the growth of biomass and generation of EPS, the medium containing glucose and corn steep liquor as a source of carbon and peptone and yeast extract as a source of nitrogen, favored the production of EPS over the other media. In this study, the kinetics of obtaining EPS showed a dependence on the carbon source, especially glucose. Another similar experiment conducted by Gern et al. (2008) studied the effect of different culture media on the production of biomass and polysaccharides in the same species. The media placed in 500 mL Erlenmeyer flasks containing 100 mL of the media showed different growth rates, affected by the interaction of the factors. Differently from what was previously reported, the growth rate is affected by the interaction between the factors, with organic nitrogen increasing the studied variables and the inorganic nitrogen having a negative effect, except in the global polysaccharide productivity.

In two other strains, *P. ostreatus* “Florida” and *Pleurotus ostreatoroseus*, Rosado et al. (2003) used a medium containing a high concentration of glucose as a carbon and peptone source, yeast extract, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$ in pH 6.0, in 500 mL flasks with 100 mL of medium. The results showed that for this medium, low concentrations of $(NH_4)_2SO_4$ and high concentration of glucose favored both fungi with higher EPS production, 22.8 g/L for *P. ostreatus* “Florida” and 16.8 g/L for *P. ostreatoroseus*.

The cultivation of *Agaricus brasiliensis* was performed, aiming at the production of EPS, in which sucrose, yeast extract, pH at 6.1 and temperature of 30°C were the most effective in increasing EPS production (Fan et al., 2007). This type of behavior shows that different species have different cultivation behavior and, therefore, there is a need to adapt the culture medium.

Ganoderma lucidum, widely cultivated for its nutritional characteristics, is also among the most cultivated by the submerged environment. In flask cultivation, to develop effective and economical solid seeds suitable for use in submerged culture for the production of EPS, four supplements were examined as sources of nitrogen for the formulation of substrate with sawdust as the basal ingredient. The higher biomass and EPS yield obtained for the four solid test seeds on the liquid seed were attributed to the formation of a large number of growth points of hyphae in cultures, resulting from the dissociation of inoculated solid seeds into numerous tiny particles (Liu and Zhang, 2019).

Cultivation in biorreactors

Larger-scale crops use bioreactors, which are closed growing equipment, in which fermentation processes take place. The size of the equipment varies according to its use and the object of use, since they are adapted to the research and industrial scale. The term fermenters is used because primarily, bacteria and yeasts were cultivated and as a rule, almost all are anaerobic (Oosterhuis et al., 2013, Camelini et al., 2014).

In the same way that flask culture media are optimized for the production of the metabolites of interest and for switching to production in a bioreactor, in the latter, there will also be a need for scaling in relation to thermodynamic aspects, fluid dynamics, transfers of gases and mass, need for aeration and agitation must be carried out (Schmidell, 2001).

Within the universe of cultivation in bioreactors, there is a series of equipment that can be used for the cultivation of fungi. The materials from which they are manufactured

Table 1. Current state of cultivation of mushrooms and polysaccharides production in submerged culture.

Mushroom	Cultive type	Result	Reference
Agaricus brasiliensis	Flasks 250 - 50 mL medium	382 mg/L	Fan et al. ,(2007)
Agaricus brasiliensis	Stirred tank bioreactor - 5 L working volume	1.67 g/L	Zou (2006)
Agaricus brasiliensis	Stirred tank bioreactor (Inceltech) - 1 L	321.2 mg/L	Fan et al. ,(2007)
Agaricus blaezei	Flasks 500 - 100 mL medium	0.84 and 1.269 g/L	Hamedi et al., (2007)
Agrocybe cylindraceaa	Stirred tank reactor (KoBiotech - South Korea) - 5 L	3.0 g/L	Kim et al. ,(2005a)
Antrodia camphorata	Stirred tank reactor (Biostat B, B. Braum, Germany) - 5 L	5.05 mg/g	Shu and Lung (2004)
Auricularia polytricha	Stirred tank reactor (KoBiotech - South Korea) - 5 L	3.1 g/L	Xu and Yun (2003)
Auricularia polytricha	Flasks 250 - 50 mL médium	2.12 g/L	Xu and Yun (2003)
Ganoderma lucidum	Flasks 250 - 50 mL medium	1.33 g/L	Liu and Zhang (2019)
Ganoderma lucidum	Biorreactor in lab scale	5.23 mg/mL	Petre et al., (2010)
Ganoderma lucidum	Flasks 250 - 40 mL medium	IPS: 0.130 g/L; EPS:1.20 g/L	Fang and Zhong (2002)
Grifola frondosa	Stirred tank reactor (KoBiotech - South Korea) - 5 L	EPS: 7.2 g/L	Kim et al., (2007)
Grifola frondosa	Reactor with three six blade discs (Biostat C10-3, Germany) - 15 L	EPS: 1.326 g/L	Cui et al., (2006)
Inonutus obliquus	Stirred tank reactor (KoBiotech - South Korea) - 5 L	0.495 g/L	Kim et al., (2005b)
Lentinula edodes	Stirred tank reactor (Inaltec) - 1 L	1.44 g/L	Garcia-Cruz et al., (2020)
Lentinula edodes	Biorreactor in lab scale	4.75 mg/mL	Petre et al., (2010)
Lentinus edodes	Biorreactor – 10L	Not informed	Wang et al., 2019
Phellinus gilvus	Stirred tank reactor (KoBiotech – South Korea) - 5 L	5.3 g/L	Hwang et al. , (2004)
Phellinus baumii	Stirred tank reactor (KoBiotech – South Korea) - 5 L	3.59 g/L	Hwang et al., (2004)
Phellinus linteus	Stirred tank reactor (KoBiotech – South Korea) - 5 L	2.43 g/L	Hwang et al., (2004)
Pleurotus Sajor-caju	Flasks 2 L	3.84 g/L per h	Assis et al., (2013)
Pleurotus Sajor-caju	Stirred tank reactor (Biostat B, B. Braum, Germany) - 5 L	0.94 g/L	Silveira et al., (2015)
Pleutorus djamour	Stirred tank reactor (Biostat B, B. Braum, Germany) - 5 L	32.2 mg/L h	Borges et al.,(2013)
Pleurotus ostreatus	BioFlo 310 biorreactor (New Brunswick) - 5L	IPS: 1.15 g/L; EPS: 2.0 g/L	Vamanu (2012)
Pleurotus ostreatus	Biorreactor in lab scale	5.10 mg/mL	Petre et al., (2010)
Pleurotus ostreatus	Flasks 250 - 50 mL medium	1.48 a 1.52 g/L	Maftoun et al., (2013)
Pleurotus ostreatus	Stirred tank reactor 16L (Bioengeneering, Switzerland) - 8 L	EPS: 0.55g g/g	Maftoun et al., (2013)
Pleurotus ostreatus	Flasks 500 - 100 mL medium	20.05 mg/L	Gern et al., (2008)
Pleurotus ostreatus	Stirred tank reactor (Biostat C15 L, Germany) - 15 L	0.69 g/L	El-Enshasy et al.,(2010)
Pleurotus ostreatus	Flasks 250 - 50 mL medium	2.1 g/L	El-Enshasy et al., (2010)
Sarcodon aspratus	Stirred tank reactor (KoBiotech - South Korea) - 5 L	2.68 g/L	Joo et al., (2004)
Tuber melanosporum	Flasks 250 - 180 mL medium	EPS: 7.09 g/L; IPS: 4.43 g/L	Liu et al., (2009)
Tuber sinense	Flasks 250 - 180 mL medium	EPS: 5.45 g/L; IPS: 2.40 g/L	Tang et al., (2008)

also vary from stainless steel to glass, which can withstand variations in pressure and temperature, especially at the time of sterilization. Another advantage of bioreactors over flask culture lies in the control factor. The bioreactors are equipped with temperature, pH and oxygen availability control systems by coupling sensors, which are also sterilizable. Unlike flask cultivation, bioreactors allow sampling and thus the process

can be followed over the culture period (Oosterhuis et al., 2013; Xu et al., 2013).

As the culture is established, the rheological parameters in the medium are modified by the increase in mycelial mass and by the production of metabolites secreted into the medium. This modification also alters the transference and in the case of filamentous fungi, this viscosity determines its growth mode in the free form or forming pellets. The form of pellets is widely observed in the cultivation of fungi and this morphology helps to avoid variations in the density of the medium. At the same time, this formation interferes with the rheological properties that can cause difficulty in transferring oxygen to the interior of the pellets, generating anaerobic conditions (Prosser and Tough, 1991; Rossi et al., 2002).

In the industry, two types of bioreactors are widely used, according to the characteristics of the cultivation and the microorganism used: those with agitated tanks and tires (Chisti, 1989; Kavanagh 2005). Currently, what limits the full use of the potential of bioreactors and the scheduling of processes for some types of microorganisms is the limited knowledge of bioprocess engineering (Elisashvili, 2012).

The cultivation of fungi in bioreactors

The most mentioned type of bioreactor used in fungus cultivation is the stirred tank reactor (STR), which is equipped with a system of blades or turbines to agitate and aerate the culture medium (Table 1). This model has a sophisticated construction that includes axles, supports and blades, which influences the cost. The aseptic mode of this model also makes it a choice, since the set of the vessel, sensors and culture medium can be sterilized in a single step and remains aseptic for a long time. A problem in relation to sterilization lies in the many moving parts that increase the points of contamination and the complexity of the mechanical seals of the equipment (Nienow, 2014).

Even though they are the most common, agitated tank bioreactors are not always the most suitable. The agitation generated by the equipment's blades necessary for aeration and mass transfer can cause damage to cells due to high shear (Duobin et al., 2013).

Mushrooms like *P. sajor-caju* showed good growth in an optimized medium with glucose, soy protein, yeast extract and ammonium sulfate, producing a good amount of biomass quickly even without pH control in agitated tank bioreactors (Confortin et al., 2008). In other strains of fungi of the genus *Pleurotus* such as *P. ostreatus* grown in an 18 L volume bioreactor with an 8 L working volume, two experiments with pH 5.5 and uncontrolled were studied in terms of biomass production and EPS.

This study showed the importance of pH control in cultures, where at 5.5 there was an increase in EPS in the order of 0.445 g/g (Maftoun et al., 2013), as well as influencing glucose consumption rates (Kurosumi et al., 2006). Changes in the pH of the culture are usually caused by the presence of organic acids and other metabolites excreted in the medium, produced by the fungi themselves (Xiao et al., 2006; Fang et al., 2002; Kim et al., 2005a).

Other variables are also important in cultivation in bioreactors. This is demonstrated for *P. ostreatus* grown in a stirring bioreactor, where the cultivation conditions varied in terms of agitation (120-180 rev min^{-1}); the initial pH (pH, 4.5-5.5) and keeping the temperature fixed at 25°C. This type of test, allowed the variation of the constitution of the medium, when it is desired to study the optimization, separating them from the physical factors (Petre et al., 2010).

Bioreactors from agitated tanks of different volumes are reported in experiments in the cultivation of fungi of the genus *Agaricus* in which the optimization of exopolysaccharide production conditions was determined by response surface, in which starch was established as the best source of carbon and yeast extract as a source of nitrogen (Hamedi et al., 2007). Likewise, *Agaricus brasiliensis* showed a similar behavior when grown in the presence of glucose (Fan et al., 2007).

For other species of fungi, such as the *Ganoderma* genus, the optimization processes established in STR also show the importance of controlling the inoculum density in the growth process, biomass production and intra and extra-cellular polysaccharides. In the study conducted by Fan and Zhong (2002), the higher inoculum density promoted a stimulus in production. This species when cultivated at an initial pH 6.5 showed lower biomass and intra and extra-cellular polysaccharides compared to pH 3.5 (Fang and Zhong, 2002).

A less used type is represented by pneumatic bioreactors, which do not have mechanical agitation, as is the case of bubble column and airlifts bioreactors (AR). Pneumatic bioreactors are bioreactors with high height, usually above 10 times the diameter, in contrast to the STR that have a height of two to three times their diameter. This is necessary because the bubbles need time to contact the liquid to transfer oxygen and absorb carbon dioxide. In STR, forced agitation keeps the bubbles in contact longer with the liquid, in addition to being continuously broken, increasing the transfer area (Merchuk, 2003).

The first is made in a single part, with an internal column that receives the injection of air, whereas the airlift bioreactor is composed of a column, which is divided into the region that contains the upward flow of liquid gas (the riser) and the region containing thedownward flow of liquid gas (the downcomer) and in this model, the difference in liquid density between these regions causes the fluid to circulate in the bioreactor by an air-lift action (Znad et al., 2004; Merchuk and Yunger, 1990; Chisti, 1989). The circulation of liquid within the bioreactor is essential for its proper performance, as it is

related to the most important variables of the process, such as the turbulence that affects the size of the bubbles and the mixing time.

The cultivation of fungi in this type of bioreactor has an advantage when studying the shear force imposed on the pellets or in the cell. These biological processes, another advantage of airlift over the bubble column and agitated tank reactors is related to the shear force imposed by the turbulent field in cells or “pellets”. Numerous studies have been conducted investigating the effects of shearing on microorganisms and cells in an effort to quantify the level of shear variation that microorganisms can tolerate (Merchuk and Yunger, 1990). Thus, in biological processes the shear force generated by the turbulence in the pellets generated during the growth of the fungi is less than in the agitation tanks, causing less damage.

Many fungi of medicinal and food interest were grown in bioreactors (Table 1). According to work by Xu et al., (2013), the cultivation of fungi of the genus *Trametes* demonstrated a similar behavior, regardless of the design of the bioreactor or the agitation rate, since the yield of products was similar. Higher EPS yield was achieved in STR than AR, and higher EPS production was related to advantageous morphological characteristics of the fungus defined by lower shear forces in AR. Recently, the cultivation of *T. ochracea* was optimized according to the different carbon sources, in a bioreactor of the stirred tank type, where sucrose proved to be the best sugar for the development of the fungus and the production of IPS and EPS (Bai et al., 2020). Other species, such as *Phellinus vaninii* grown in a stirred tank bioreactor, using lactose as a carbon source, proved to be efficient, producing EPS at a rate of 3.75 g/L (Li et al., 2019).

Pneumatic systems based on airfilt-type bioreactors showed important effects on *Agaricus subrufascens* crops when compared with agitated tank bioreactors, even when pH parameters were not controlled (Camelini et al., 2013). Likewise, different formulations of culture media for *Pleurotus flabellatus*, grown in an airlift bioreactor, showed the highest biomass productivity (0.180 g/L/day), with potato extract and a β-glucan yield of 7.70±1.11 g/100 g (Mohamad et al., 2015).

In some cases, as in *Trametes trogii* cultivation, the pneumatic airlift system did not promote biomass and EPS production in 6 days of cultivation, when compared with STR cultivated for 5 days. In this case, agitation can be beneficial to the growth of fungi and performance, improving the characteristics of mass transfer in relation to substrates, products by-products and oxygen (Xu et al., 2013).

One application of pneumatic bioreactors lies in the production of mycelium for use as an inoculant for growing mushrooms. Another great advantage is that due to the simplicity of these bioreactors, they can be manufactured at a lower cost and applying only basic techniques of turning and welding in stainless steel. The reduced diameters of these equipment also imply the need for less thick walls than the STR with consequent material savings. For these simple reasons, you can easily build a cultivation system that can be used by mushroom producers or companies that work with this type of inoculant.

CONCLUSION AND PERSPECTIVES

The experimental results described for submerged fermentation methods are important to direct and plan equipment configurations, improve the culture media according to the species studied or that have not yet been cultivated using this methodology. When aiming at greater productivity in terms of biomass and polysaccharide production, the definition of the type and quantity of the culture media and the inoculum are particularly important. The submerged culture systems described in this review include fungi that have a medicinal or edible use and are cultivated in that system. In the works described, the system proved to be quite efficient when compared with what is described in solid state fermentation, regarding the control of parameters. Certainly, the results described demonstrate that much can still be improved even if other elements are inserted in the system, such as extracts, and broths.

The biotechnological processes that involve the cultivation of fungi have a potential yet to be explored. Tropical forests are a repository of fungi that can be explored for the metabolites of interest, especially polysaccharides. For these species, the described methods can be a guide to direct the cultivation of these species in order to take advantage of their maximum potential. The polysaccharides obtained from these species represent a promising and attractive field and can be used in future research projects, where they will be used against a wide range of diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This research work was supported by CAPES and Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) [grant number 062.00143/2020] from Brazil.

REFERENCES

Assis IS, Chaves MB, Silveira MLL, Gern RMM, Wisbeck E, Furigo Junior A, Furlan SA (2013). Production of bioactive compounds with antitumor activity against Sarcoma 180 by *Pleurotus sajor-caju*. Journal of Medicinal Food 16(11):1004-1012.

Bai J, Jia X, Chen Y, Ning Z, Liu, S, Xu C (2020).. Effect of carbon source on properties and bioactivities of exopolysaccharide produced by *Trametes ochracea* (Agaricomycetes). International Journal of

Mushrooms 23(3):289-297.
Borges GM, de Barba FFM, Schiebelbein AP, Pereira BP, Chaves MB, Silveira MLL, Pinho, MLS, Furlan SA, Wisbeck E (2013). Extracellular polysaccharide production by a strain of *Pleurotus djamor* isolated in the south of Brazil and antitumor activity on sarcoma 180. Brazilian Journal of Microbiology 44(4):1059-1065.
Burns PJ, Yeo P, Keshavarz T, Roller S, Evans CS (1994). Physiological studies of exopolysaccharide production from basidiomycetes *Pleurotus* sp. Florida. Enzyme Microbiology Technology 16(7):566-572.
Camelini CM, Rezzadori K, Benedetti S, Proner MC, Fogaça L, Azambuja AA, Giachini AJ, Rossi MJ, Petrus JCC (2013). Nanofiltration of polysaccharides from *Agaricus subrufescens*. Applied Microbiology and Biotechnology 97:9993-10002.
Camelini CM, Rossi MJ. Cardozo, FTGS, Gomes A, Sales-Campos C, Giachini AJ (2014). Fungal cultivation and production of polysaccharides. In: Ramawat K, Mérillon JM (eds) Polysaccharides pp. 1-34.
Chisti MY (1989). Airlift Bioreactors. London: Elsevier Applied Sciences 345 p.
Confortin FG, Marchetto R, Bettin F, Camassola M, Salvador M, Dillon, AJP (2008). Production of *Pleurotus sajor-caju* strain PS-2001 biomass in submerged culture. Journal of Industrial Microbiology and Biotechnology 35(10):1149-1155.
Crueger W, Crueger A (1990). Substrates for industrial fermentation. In: Biotechnology: A textbook of industrial microbiology. Wisconsin: Science Tech Publishers 59-63.
Cui, FJ, Xu ZH, Xu HY, Sun K, Tao WY (2006). Optimization of the medium composition for production of mycelial biomass and exo-polymer by *Grifola frondosa* GF9801 using response surface methodology. Bioresource Technology 97(10):1209-1216.
Duobin M, Yupping MA, Lujing G, Aijing Z, Jianqiang Z, Chunping X (2013). Fermentation characteristics in stirred-tank reactor of exopolysaccharides with hypolipidemic activity produced by *Pleurotus geesteranus* 5#. Anais da Academia Brasileira de Ciências 85(4):1473-1481.
El-Enshasy H, Daba A, El Demellawy M, Ibrahim A, El Sayed S, Elabary I (2010). Bioprocess development for large scale production of anticancer exopolysaccharide by *Pleurotus ostreatus* in submerged culture. Journal of Applied Sciences 10(21):2523-2529.
Elisashvili V (2012). Submerged cultivation of medicinal mushrooms: bioprocesses and products. International Journal of Medicinal Mushrooms 14(3):211-239.
Fan L, Soccol AT, Pandey A, Soccol CR (2007). Effect of nutritional and environmental conditions on the production of exo-polysaccharide of *Agaricus brasiliensis* by submerged fermentation and its antitumor activity. LWT 40(1):30-35.
Fang, QH, Zhong JJ (2002). Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum.* Process Biochemistry 37(7):769-774.
Furnali, RPZ, Godoy HT (2007). Valor nutricional de cogumelos comestíveis. Ciencia e Tecnologia de Alimentos 27(1):154-157.
García-Cruz F, Durán-Páramo E, Garín-Aguilar MA, del Toro GV, Chairez I (2020). Parametric characterization of the initial pH effect on the polysaccharides production by Lentinula edodes in submerged culture. Food and Bioproducts Processing 119:170-178.
Garcia-Ochoa F, Gomez E (2009). Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. Biotechnology Advance 27(2):153-176.
Gern RMM, Wisbeck E, Rampinelli J, Ninow JL, Furlan SA (2008). Alternative medium for production of *Pleurotus ostreatus* biomass and potential antitumor polysaccharides. Bioresource Technology 99(1):76-82.
Ghorai S, Banik SP, Verma D, Chowdhury S, Mukherjee S, Khowala S (2009). Fungal biotechnology in food and feed processing. Food Research International 42(5-6):577-587.
Guillén-Navarro GK, Márquez-Rocha FJ, Sanchez-Vázquez JE (1998). Producción de biomasa y enzimas ligninolíticas por *Pleurotus ostreatus* en cultivo sumergido. Revista Iberoamericana de Mycologia 15:302-306.
Hamedi A, Vahid H, Ghanati F (2007) Optimization of the medium composition for production of mycelia biomass and exo-polysaccharide by *Agaricus blazei* Murill DPPh 131 using response surface methodology. Biotechnology 6(4):456-464.
Hwang HJ, Kim SW, XU CP, Choi JW, Yum JW (2004). Morphological and rheological properties of the three different species of basidiomycetes *Phellinus* in submerged cultures. Journal of Applied Microbiology 96(6):1296-1305.
Jong SC, Donovick R (1989). Antitumor and antiviral substances from fungi. Advances in Applied Microbiology 34:183-261.
Joo JH, Lim JM, Kim HO, Kim SW, Hwang HJ, Choi, JW, Yun JW (2004). Optimization of submerged culture conditions for exopolysaccharide production in *Sarcodon aspratus* (Berk) S. Ito TG-3. World Journal of Microbiology and Biotechnology 20:767-773.
Kavanagh K (2005). Fungal fermentation systems and products. In: Kavanagh K. Fungi: Biology and Applications. West Sussex: Wiley pp. 89-111.
Kawagoe M, Nagaoka Y, Araki M, Yamagaki K, Naoe K, Noda H (2004) Submerged culture of Agaricus blazei mycelium in a bubble column fermentor. Journal of Chemical Engineering of Japan 37(8):1056-1061.
Kim HO, Lim JM, Joo JH, Kim SW, Hwang HJ, Choi JW, Youn JW (2005a). Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*. Bioresource Technology 96:1175-1182.
Kim YO, Han SB, Lee HW, Ahn HJ, Yoon YD, Jung JK, Kim HM, Shin CS (2005b). Immuno-stimulating effect of the endo-polysaccharide produced by submerged culture of *Inonotus obliquus*. Life Science 77(19):2438-2456.
Kim SW, Hwang HJ, Lee BC, Yun JW (2007). Grifola frondosa polysaccharides as cosmeceuticals, Food Technology and Biotechnology 45(3):295-305.
Kurosumi A, Kobayasi F, Mtui G, Nakamura Y (2006). Development of optimal culture method of *Sparassis crispa* mycelia and a new extraction method of antineoplastic constituent. Biochemical. Engineering Journal 30(1):109-113.
Leonowicz A, Wojias-Wasilewska M, Rogalski J, Luterek J (1991). Higher fungi as a potential feed and food source from lignocellulosic wastes. Studies in Environmetal Science 42:229-255.
Li J, Jia Z, Yao Y, Bai Y (2019). Characterization of Exopolysaccharide Produced by *Phellinus vaninii* (Agaricomycetes) and Antioxidant Potential for Meat Batter. International Journal of Mushrooms 21(5):459-467.
Liu QN, Liu RS, Wang YH, Mi ZY, Li DS, Zhong JJ (2009). Fed-batch fermentation of Tuber melanosporum for the hyperproduction of mycelia and bioactive *Tuber* polysaccharides. Bioresource Technology 100:3644-3649.
Liu SR, Zhang WR (2019). Optimization of submerged culture conditions involving a developed fine powder solid seed for exopolysaccharide production by the medicinal mushroom *Ganoderma lucidum*. Food Science and Biotechnology 28:1135-1145.
Maftoun P, Malek R, Mahmoud AS, Abdel-Sadek M, Aziz R, El Enshasy H (2013). Bioprocess for semi-industrial production of immunomodulator polysaccharide Pleuran by *Pleurotus ostreatus* in submerged culture. Journal of Scientific and Industrial Research 72(11):655-662.
Maziero R, Cavazzoni V, Bononi VLR (1999). Screening of basidiomycetes for the production of exopolysaccharide and biomass in submerged culture. Revista de Microbiologia 30:77-84.
Merchuk JC, Yunger R (1990). The role of the gas-liquid separator of airlift reactors in the mixing process. Chemical Engineering Science 45(9):2973-2976.
Merchuk JC (2003). Airlift Bioreactors: Review of Recent Advances. The Canadian Journal of Chemical Engineering 81(3):324-337.
Mohamad SA, Awang MR, Ibrahim R, Keong CY, Hamzah MY, Rashid A, Hussein S, Rahim KA, Daud F, Hamid AB, Yusoff MW (2015). Production of endopolysaccharides from Malaysia's local mushrooms in air-lift bioreactor. Advances in Bioscience and Biotechnology 6:456-462.
Nienow AW (2014). Stirring and Stirred-Tank Reactors. Chemie Ingenieur Technik 86(12):1-13.
Oosterhuis NMG, Neubauer P, Junne S (2013). Single-use bioreactors for microbial cultivation. Pharmeutical Bioprocess 1(2):167-177.
Petre M, Teodorescu A, Tuluca E, Bejan C, Andronescu A (2010).

Biotechnology of mushroom pellets producing by controlled submerged fermentation. Romanian Biotechnological Letters 15(2):50-55.
Prosser JI, Tough (1991). Growth mechanisms and growth kinetics of filamentous microorganisms. Critical Reviews in Biotechnology 10(4):253-274.
Rosado FR, Carbonero ER, Kemmelmeier C, Tischer CA, Gorin PAJ, Iacomini M (2002). A partially 3-O-methylated (14)-linked-D-galactan and alpha-D-mannan from *Pleurotus ostreatoroseus* Sing. FEMS Microbiology Letters 212:261-265.
Rosado FR, Germano S, Carbonero, ER, Da Costa SM, Iacomini M, Kemmelmeier C (2003). Biomass and exopysaccharide production in submerged cultures of *Pleurotus ostreatoroseus* Sing. and *Pleurotus ostreatus* florida (Jack.:Fr.) Kummer. Journal of Basic Microbiology 43(3):230-237.
Rossi MJ, Souza JAR, Oliveira VL (2002). Inoculum production of the ectomycorrhizal fungus *Pisolithus microcarpus* in an airlift bioreactor. Applied Microbiology and Biotechnology 59:175-181.
Ruiz-Herrera J, Ortiz-Castellanos L (2019). Cell wall glucans of fungi. A review. The Cell Surface 5:100022.
Saeki, N, Takeda, H, Tanesaka E, Yoshida M, Takeda H (2011). Induction of manganese peroxidase and laccase by *Lentinula edodes* under liquid culture conditions and their isozyme detection by enzymatic staining on native-PAGE. Mycoscience 52(2):132-136.
Sales-Campos C, Vianez BF, de Abreu RLS (2011a). Productivity and nutritional composition of *Lentinus strigosus* (Svhwinitz) fries mushroom from the Amazon region cultivated in sawdust supplemented with soy bran. Recent Trends for Enhancing the Diversity and Quality of Soybean Products pp. 227-250.
Sales-Campos C, Araújo LM, Minhoni MTDA, Andrade MCND (2011b). Physiochemical analysis and centesimal composition of *Pleurotus ostreatus* mushroom grown in residues from the Amazon. Food Science and Technology 31(2):456-461.
Sales-Campos C, Araujo LM, Minhoni MTA, Andrade MCN (2013). Centesimal composition and physical-chemistry analysis of the edible mushroom *Lentinus strigosus* occurring in the Brazilian Amaz. Anais da Academia Brasileira de Ciências 85(4):1537-1544.
Schmidell W (2001). Agitação e aeração em biorreatores. In: Schmidell W. Biotecnologia Industrial Vol II: Engenharia Bioquímica. São Paulo: Edgard Blücher Ltda. pp. 277-331.
Seo YR, Patel DK, Shin WC, Sim WS, Lee OH, Lim KT (2019). Structural Elucidation and Immune-Enhancing Effects of Novel Polysaccharide from *Grifola frondosa*. BioMed Research International 2019:1-7.
Shu CH, Lung MY (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures. Process Biochemistry 39(8):931-937.
Silveira MLL, Smiderle FR, Agostini F, Pereira EM, Bonatti-Chavesa M, Wisbeck E, Ruthes AC, Sassaki GL, Cipriani TR, Furlan, AS, Iacomini M (2015). Exopolysaccharide produced by *Pleurotus sajor-caju*: Its chemical structure and anti-inflammatory activity. International Journal of Biological Macromolecules 75:90-96.
Singh MP, Vishwakarma SK, Srivastava (2013). Bioremediation of direct blue 14 and extracellular ligninolytic enzyme production by white rot fungi: *Pleurotus* spp. BioMed Research International 2013:1-4.
Smith JE, Rowan NJ, Sullivan R (2002). Medicinal mushrooms: a rapidly developing area of biotechnology for cancer therapy and other bioactivities. Biotechnology Letters 24:1839-1845.
Tang YJ, Zhong JJ (2002). Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharides and ganoderic acid. Enzyme and Microbial Technology 31(1-2):20-28.
Tang YJ, Zhu LW, Li HM, Li DS (2007). Submerged culture of mushrooms in bioreactors – Challenges, current state-of-the-art, and future prospects. Food Technology and Biotechnology 45(3):221-229.
Tang YJ, Zhu LL, Liu RS, Li HM, Li DS, Mi ZY (2008). Quantitative response of cell growth and Tuber polysaccharides biosynthesis by medicinal mushroom chinese truffle *Tuber sinense* to metal ion in culture medium Bioresource Technology 99(16):7606-7615.
Vamanu E (2012). Biological activities of the polysaccharides produced in submerged culture of two edible *Pleurotus ostreatus* mushrooms. Journal of Biomedicine and Biotechnology 2012:56597.
Wang T, He H, Liu X, Liu C, Liag Y, Mei Y (2019). Mycelial polysaccharides of *Lentinus edodes* (shiitake mushroom) in submerged culture exert immunoenhancing effect on macrophage cells via MAPK pathway. International Journal of Biological Macromolecules 130:745-754.
Wu JZ, Cheung PCK, Wong K-H, Huang N-L (2003) Studies on submerged fermentation of *Pleurotus tuber-regium* (Fr.) Singer. Part 1: physical and chemical factors affecting the rate of mycelial growth and bioconversion efficiency. Food Chemistry 81(3):389-393.
Wu JZ, Cheung PCK, Wong KH, Huang N-L (2004) Studies on submerged fermentation of *Pleurotus tuber-regium* (Fr.) Singer. Part 2: effect of carbon-to-nitrogen ratio of the culture medium on the content and composition of the mycelial dietary fibre. Food Chemistry 85(1):101-105.
Xiao JH, Chen DX, Wan WH, Hu XJ, Qi Y, Liang ZQ (2006). Enhanced simultaneous production of mycelia and intracellular polysaccharide in submerged cultivation of *Cordyceps jiangxiensis* using desirability functions. Process Biochemistry 41(8):1887-1893.
Xu CP, Yun JW (2003). Optimization of submerged-culture conditions for mycelial growth and exo-biopolymer production by *Auricularia polytricha* (wood ears fungus) using the methods of uniform design and regression analysis. Biotechnology and Applied Biochemistry. 38(2):193-199.
Xu C, Geng L, Zhang W (2013). Production of Extracellular Polysaccharides by the Medicinal Mushroom *Trametes trogii* (Higher Basidiomycetes) in Stirred-Tank and Airlift Reactors. International Journal of Medicinal Mushrooms 15(2):183-189.
Znad HV, Báleš VJ, Markoš J, Kawaseb Y (2004). Modeling and simulation of airlift bioreactors. Biochemical Engineering Journal 21(1):73-81.
Zhang M, Zhang L, Cheung PC (2003). Molecular mass and chain conformation of carboxymethylate derivatives of β-glucan from sclerotia of *Pleurotus tuber-regium.* Biopolymers 68(2):150-159.
Zhang Z, Guoying LV, Pan H, dos Santos LF, Fan M, Soccol CR, Soccol, VT, Lacerda LG, Carvalho Filho MAS, Fan L (2011). Antidiabetic activities of ethanol extract of dry matters of culture broth of *Coriolus versiolor* in submerged culture. Brazilian Archives of Biology and Technology 54(4):701-708.
Zou X (2006). Fed-batch fermentation for hyperproduction of polysaccharide and ergosterol by medicinal mushroom *Agaricus brasiliensis*, Process Biochemistry 41(4):970-974.

Related Journals: