JJBS

Volume 14, Number 5,December 2021
ISSN 1995-6673
Pages 933 – 937

Jordan Journal of Biological Sciences

Callus-mediated Somatic Embryogenesis and Plant Regeneration in *Vanda tricolor* Lindl. var. Pallida

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Received: May 20, 2021; Revised: June 18, 2021; Accepted: June22, 2021

Abstract

In this study, a protocol to induce indirect somatic embryogenesis from the basal leaf segments of *Vanda tricolor* Lindl. var. Pallida has been developed. The experiments consisted of two stages، i.e. induction of SEs from calli and regeneration of plantlet from SEs. Embryogenic calli obtained from previous experiment (0.05 mg L^{-1} NAA + 0.01 mg L^{-1} BAP) were used to induce somatic embryos (SEs) on half-strength Murashige and Skoog (MS) medium supplemented with (0.05 mg L^{-1} to 0.20 mg L^{-1}) 6-benzylaminopurine (BAP) alone or in combination with 0.01 mg L^{-1} α-naphtalene acetic acid (NAA). Embryogenic calli, those cultured on 0.05 mg L^{-1} BAP and 0.01 mg L^{-1} NAA resulted in 90 % induction of SE structures at 30 d of culture period. Histological observation exhibited development of pro-embryo to form completed embryo. The pattern of SEs development started from embryogenic callus to form pro-embryo, followed by globular phase at 10 d of culture. Globular embryo elongated to form suspensor at 20 d of incubation period, and completed embryo. Regeneration of SEs into complete plantlets was attained on the half-strength MS medium without addition of any plant growth regulator (PGR). Based on the results of the present study, it can be concluded that half-strength MS medium supplemented with NAA 0.01 mg L^{-1} and BAP 0.05 mg L^{-1} is the best medium for induction of SEs from embryogenic calli.

Keywords: Embryogenic calli, Histology, Half-strength Murashige-Skoog medium, Micropropagation, Orchid, Pro-embryo

1. Introduction

Vanda is a genus of orchids that consists of approximately 50 species. *Vanda tricolor,* just like its name, has fragrant flowers composed of three colors, i.e. white sepal, white with brown spot petal, and violet labellum. *Vanda tricolor* Lindl. in the slope of Mount Merapi located in Central Java, and *Vanda tricolor* Lindl. var. pallida in Amerta Jati Forest, Bali are local endemic species labelled endangered in Indonesia (Kurniawan *et al.*, 2020; Semiarti, 2018). These species are generally used as the parental for crossing to produce hybrids that have economic value.

The propagation of orchids, in most cases, is done by seed germination by *in vitro* culture. Thus, the result is not homogeneous and the flowers are varied. To overcome this problem, the clonal multiplication by using *in vitro* technique can be performed. Besides, *V. tricolor* as well as *V. testacea* are monopodial orchids that are not easy to be propagated vegetatively in conventional method (Sebastinraj *et al.*, 2014). It is necessary to develop a rapid and efficient micropropagation protocol in large quantities and in a short period of time. Micropropagation through callus has the potential for somaclonal variation, so it should be carried out in a shorter culture period as well as reducing costs (Melviana *et al.* 2021a).

Cardoso *et al.* (2020) stated that induction, proliferation and regeneration of protocorm-like bodies (PLBs) is one of the most advantageous methods for mass propagation of orchids. *In vitro* multiplication by direct or indirect embryogenesis through callus will result in somatic embryo(s). According to Naing *et al*. (2011) and Shen *et al*. (2018), embryogenesis in orchids, both direct and indirect, occurred through the development of protocorm-like bodies (PLBs). Thidiazuron cytokinin (< 1 µM) significantly stimulated formation and regeneration of PLBs compared to other plant growth regulators (Kundu and Gantait, 2018). Lee *et al*. (2013) stated that in early stage of PLBs development, the cells had characteristics that cytologically similar to that of the zygotic embryos. Therefore, it was concluded that PLBs were actually somatic embryos (SEs) in orchids. As reported by Jainol and Gansau (2017), embryogenic callus developed into nodular structures and progressed further formed into aggregates of PLBs. Somatic embryogenesis was a process where cells developed resemble the zygotic embryos with bipolar structure (Shen *et al*., 2018). The use of somatic embryo derived from callus tissue was chosen in this research because the produced propagules were unlimited where each somatic embryo originated from a somatic cell, i.e. callus cell.

Orchids propagation using protocorm-like bodies had been studied by Soe *et al.* (2014). In addition to that,

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somatic embryogenesis protocols from several orchids have been reported, for examples *Phalaenopsis bellina* (Rchb.f.) Christenson (Khoddamzadeh *et al.*, 2011), *Phalaenopsis amabilis* (L.) - Blume Orchid (Mose *et al*., 2017, Mose *et al*., 2020), *Tolumnia* Louise Elmore 'Elsa' (Shen *et al*., 2018). Concentration and combination of plant growth regulators play an important role throughout *in vitro* proliferation of different orchids. Auxins, especially α-naphtalene acetic acid (NAA) affect the process of regeneration in monopodial orchids, and act synergistically on the formation of PLBs (Jainol and Gansau, 2017). Hardjo and Savitri (2017) succeeded in callus induction from the basal part of *V. tricolor* var. pallida's leaf using half-strength MS medium (Melviana, *et al,* 2021b; Murashige and Skoog, 1962) with the addition of 0.05 mg L^{-1} NAA in collaboration with 0.01 mg L^{-1} BAP. Callus induction method which has been done by Hardjo and Savitri (2017) will be used and developed to induce somatic embryos and then to regenerate them into plantlets. The effect of NAA and BAP was studied for induction and maturation of somatic embryos. This study intended to develop a protocol to indirectly produce somatic embryo (SE) via callus, initiated from leaf basal segment of *V. tricolor* var. Pallida.

2. Materials and Method

2.1. Plant material

In vitro grown plantlets of *V. tricolor* var. Pallida were provided by Handoyo Budi Orchid, in Malang City, East Java, Indonesia. Three-month old *in vitro* plantlets, measuring 3 cm had four leaves and long roots. Leaf basal segments were used as explant source for callus induction.

2.2. 2.2 Induction and regeneration of somatic embryo

Basal segment of leaf was cultured on half-strength MS medium + 0.05 mg L^{-1} NAA + 0.01 mg L^{-1} BAP (following Hardjo and Savitri, 2017). Subculturing was carried out after 4 wk (week)s intervals. The experiments consisted of two stages, i.e. induction of SEs from calli and regeneration of plantlet from SEs. Embryogenic calli (approx. 5 mm in diameter), formed after subculturing, were transferred to half-strength MS medium added with various levels of BAP (0.05 mg L^{-1} to 0.20 mg L^{-1} and 0.01 mg L^{-1} NAA) to induce SEs.

2.3. Culture conditions

The half-strength MS basal medium contained half-strength macro- and micro-element of MS enriched with: myo-inositol (100 mg L^{-1}), niacin (0.5 mg L^{-1}), pyridoxine.HCl (0.5 mg L^{-1}), thiamine HCl (0.1 mg L^{-1}), glycine (2.0 mg L^{-1}), sucrose (10 000 mg), and phytagel (2 500 mg). Plant growth regulators as well as compulsory additives (according to the experimental objectives) were added to the media prior to autoclaving. The pH of the media was adjusted to 5.8 with 1 N KOH or HCl prior to autoclaving for 20 min at 121 °C. Explants were incubated under 16:8-h photoperiod at 24 °C to 26 °C. Subculturing was also executed every 4 wk.

2.4. Histological observation of SE

The tissue was fixed in formaldehyde-acetic acid - ethanol (FAA) solution (5 % formaldehyde: 5 % glacial acetic acid: 90 % ethanol 70 %) for 24 h, then continued with further processes (washing and gradual dehydration) until finally the tissue was covered in paraffin. The paraffin embedded tissue was cut at a thickness of 5 µm, and stained with hematoxylin-eosin. At the end, the specimen was then observed under the light microscope.

2.5. Complete plantlet regeneration and acclimatization

Regeneration of SE was performed on hormone-free MS medium and half strength MS + BAP (0 mg L^{-1} to 0.05 mg L^{-1}). The observations were recorded weekly to trace different stages of protocorm development. For stereo microscopy and histological observations, about five PLBs at every developmental stage were randomly collected from culture tubes. Plantlets with four leaves and three roots were transplanted into a 2-inch pot containing sphagnum moss and were covered with a clear plastic lid in greenhouse. Plastic lids were removed after 15 d. The moisture content of the pots was maintained by regular water spraying. Survival plantlets were recorded 2 mo after transplanting.

2.6. Experimental design and statistical analysis

The experiment was based on completely randomized design. Each experiment was composed of six treatments and 40 replications. Embryogenic callus induction from basal leaf explant was carried out as long as 4 wk. Embryogenic calli were transferred to various treatment media to promote somatic embryo formation. Observation parameters for SE induction were the initial time of SE formation, percentage of callus forming SE, and histological analysis of SE. For the observation of SE regeneration, the parameters were the initial time of emerging shoot, percentage of SE forming shoots, and percentage of SE forming plantlet. Data were analyzed with analysis of variance (ANOVA) and followed by Duncan's Multiple Range Test (DMRT) at $\alpha = 0.05$ (Adinurani, 2016)

3. Results and Discussions

3.1. Embryogenic callus induction and stages of SE

Basal leaf of *V. tricolor* var pallida's explant formed callus on half-strength MS medium + NAA 0.05 mg L^{-1} + BAP 0.01 mg L^{-1} (Figure 1A). Naing *et al*. (2011) reported the same thing that high auxin and cytokinin ratio has synergistic effect to induce callus on *Coelogyne cristata* Lindl. leaf explant. Figure 1B shows that after first subculture, callus structure shaped nodule and compact, with isodiametric size and shiny green in color. According to Jainol and Gansau (2017), these characteristics are typical in embryogenic callus and will be subsequently developed into embryo. Figure 1C represents callus that develop into SE, while the nodule size is bigger than others and color is dark green.

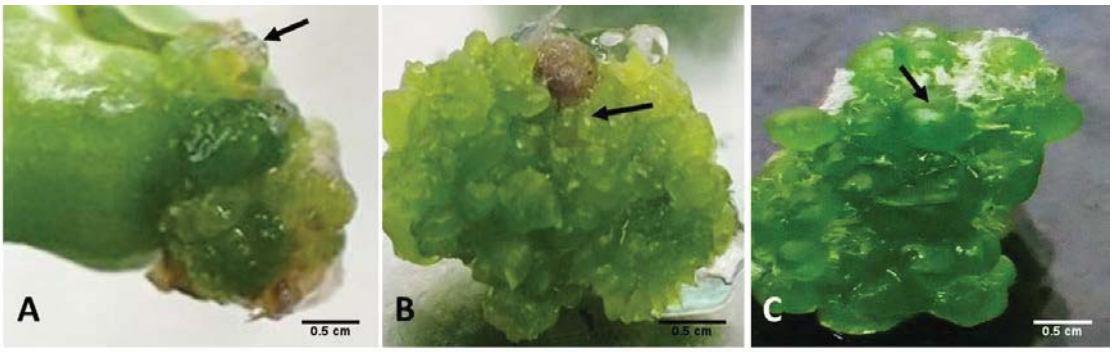

Figure 1. Callus formation on *V. tricolor* var. pallida basal leaf segment A. 8 wk of culture (½ MS medium + NAA 0.05 mg L^{-1} + BAP 0.01 mg L^{-1}); B. Embryogenic callus; C. SEs at globular phase

Treatment of 0.05 mg L^{-1} BAP and 0.01 mg L^{-1} NAA, all at once, was able to induce SE formation (90 %) in faster time rather than single BAP treatment (0.05 mg L^{-1} to 0.2 mg L^{-1}) as shown in the Table 1. Combination of NAA with cytokinin like BAP on various orchid species can increase the formation of PLBs such as Tolumnia cv. Snow Fairy (0.5 mg L^{-1} NAA with 4 mg L^{-1} BA) (Chookoh *et al*., 2019) while on leaf explant of *Phalaenopsis* 'Join Angle × Sogo Musadian' 2 mg L^{-1} 2,4-D was applied in combination with 1 mg L^{-1} TDZ but number of PLBs was less than the combination treatment of 0.5 mg L^{-1} NAA, 5 mg L^{-1} BAP, and 0.5 mg L^{-1} IAA that used root explant (Meilasari and Iriawati 2016). Furthermore, Mose *et al.* (2020) also reported combination of 3 mg L^{-1} TDZ with 1 mg L^{-1} NAA effectively induced *Phalaenopsis amabilis* L. (Blume) SEs formation from stem explants. Basal segment explants of *Dendrobium* (Sw) Sonia 'Earsakul' cultured on ½ MS medium supplemented with 1 mg L^{-1} TDZ alone could be stimulated to form PLBs (Juntada *et al*., 2015). In numerous orchids, SE induction has been completed with cytokinin alone or as a group with auxin at low concentration. The effect of various concentration is variable, depending on the type of explant and species. The decrease of auxin concentration results in inhibition of cell division and cells are encouraged to develop into embryos.

Table 1. Percentage of callus forming SE and time duration of SE maturation in *V. tricolor* var. pallida at various concentration and combination of BAP and NAA.

PGR treatment (mg L^{-1})	Time duration formed SEs (d)	Percentage of callus formed SEs after 30 d cultured (%)
BAP 0.2 + NAA 0.01	65.7 ± 0.65^{c}	60.2 ± 8.92^{bc}
BAP 0.1 + NAA 0.01	45.3 ± 0.52^{d}	70.2 ± 5.45^{b}
BAP 0.05 + NAA 0.01	30.8 ± 0.84^{e}	90.1 ± 4.72^{a}
BAP 0.2	112.3 ± 0.75^{b}	60.1 ± 7.65^{bc}
BAP 0.1	120.5 ± 0.64^{b}	55.4 ± 9.83^{c}
BAP 0.05	130.2 ± 0.54^{a}	50.3 ± 8.15^{c}

Note.: numbers followed by different letter in a column means that they are significantly different, tested with DMRT at $\alpha = 0.05$. There were 40 explants used for every treatment.

According to histological observation of longitudinal section of *V. tricolor* var. pallida's SE, it was noticeable that there were embryogenic cells in the form of proembryo which consisted of three cells (Figure 2A), and then developed into globular shape (Figure 2B), and differentiation occurred where cells in apical area were small while they were huge in basal area (Figure 2C). Furthermore, globular structure started to elongate at basal area, forming a suspensor-like structure and at apical area it formed a curvature, consequently there were two bulges (Figure 2D). At next development, the two bulges transformed into two leaves primordial, while the curvature developed into shoot apical meristem (Figure 2E). Figure 2F, exhibits that there was cell differentiation at the center of the specimen to form vascular tissue which will connect shoot apical meristem and root apical meristem. Embryogenic calli were able to form embryos because medium contained BAP cytokinin combined with low content of NAA (0.01 mg L^{-1}).

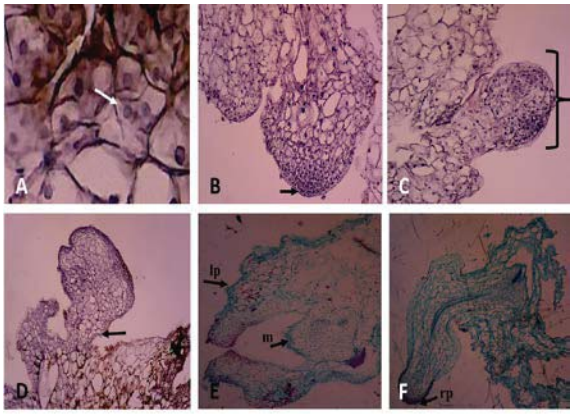

Figure 2. Histology of SE from callus of *V. tricolor* var. pallida.

A. Proembryo (three cells); B. SE on ½ MS medium supplemented with 0.05 mg L^{-1} BAP + 0.01 mg L^{-1} NAA; C. Globular stage of SE; D. Basal of SE with suspensor-like structure; E. Shoot apical meristem after 30 d on hormone-free ½ MS medium; F. Differentiation of the procambium at SE. (m = meristem; lp = leaf primordia; rp = root primordia).

3.2. Regeneration of SE to form plantlets

First and foremost, SE (Figure 3A) regenerated to form shoot (Figure 3B) occurred in a faster time on hormone-free half strength MS. Yet, it was not significantly different with half-strength MS supplemented with BAP at low concentration to 0.02 mg L^{-1} (Table 2). In line with that, there was a same report on protocorms in *Phalaenopsis* (Winarto *et al.*, 2016), regeneration of *Phalaenopsis bellina* (Rchb.f.) PLBs (Chew *et al*., 2018) and SE in *Tolumnia* Louise Elmore 'Elsa' (Shen *et al.*, 2018) which regenerated into plantlets after being transferred to hormone-free half-strength MS medium. The addition of BAP in higher concentration from 0.02 mg L^{-1} evidently had an effect to slow down germination of SE to form plantlets and caused germination time to be delayed.

High concentration of BAP actually inhibit SE germination. Morphological observation (Figure 3C) showed that the roots emerge after the first and second leaf develop. In a period of 30 d of culture (Table 2) all of SEs (100 %) form whole plantlets, while on MS medium and half-strength MS + BAP ≥ 0.03 mg L^{-1} only 80 % SEs form plantlets. Low concentration cytokinin has beneficial effect on SE germination of *V. tricolor* var. pallida. Naing *et al*. (2011) stated that BAP effectively promote regeneration on *Coelogyne cristata* (Lindl.)'s PLBs to form shoots, and it was similar to the result reported by Zhao *et al*. (2013) using *Dendrobium wangliangii* (G.W.Hu, C.L.Long & X.H.Jin). Generally, cytokinins have the effect of encouraging cell division and regeneration to form shoots, and the optimum concentration is specific for each species. Cytokinin BA are also commonly used for *in vitro* shoot multiplication of various species such as for example *Ammi visnaga* (L.) Lam. (Al-Saleh *et al*., 2019).

Table 2. Regeneration of SEs of *V. tricolor* var. Pallida forming plantlets on half-strength MS and MS supplemented with PGRs after 30 d of culture period

Medium and PGR (mg L^{-1})	SE forming shoot (d)	Percentage of SE forming shoot (%)	Percentage of SE forming plantlet (%)
MS	25.5 ± 2.27[a]	80	85
½ MS	17.8 ± 3.45[c]	100	100
½ MS + BAP 0.01	15.2 ± 2.68[c]	100	100
½ MS + BAP 0.02	16.4 ± 2.85[c]	100	100
½ MS + BAP 0.03	20.8 ± 2.85[b]	90	80
½ MS + BAP 0.05	22.4 ± 2.85[b]	80	80

Note.: numbers followed by different letter in a column means that they are significantly different, tested with DMRT at α = 0.05. There were 40 explants used for every treatment.

The percentage of plantlets (Figure. 3D) that were well acclimatized in the glasshouse with survival rate 95 %. These plantlets were grown in sphagnum moss medium and exhibited normal developmental (Figure 3E). Sphagnum moss was used to maintain the moisture.

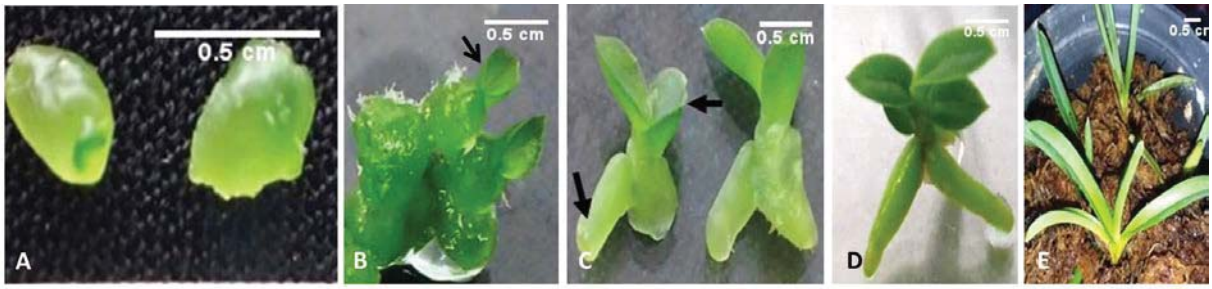

Figure 3. Mature SE of *Vanda tricolor* Lindl. var. pallida transformed into plantlet on hormone-free half-strength MS medium. A. Somatic embryo (SE); B. SE with shoot; C. SE with shoot and root; D. Plantlet; E. Acclimatized plantlets (2 mo old after planting)

4. Conclusion

Embryogenic calli formed SEs on ½ MS medium + NAA 0.01 mg L^{-1} + BAP 0.05 mg L^{-1} at 30 d of culture period. The development of embryo started with the formation of proembryo structure from embryogenic callus and then developed into globular structure with suspensor. Somatic embryo could germinate to form shoots firstly and subsequently form root on hormone-free half-strength MS medium after 30 d of culture period. Plantlets regenerated through somatic embryogenesis would be a new procedure of clonal propagation in *V. tricolor*.

The success in somatic embryo formation from callus followed by the establishment of *Vanda tricolor* Lindl. var. Pallida plantlets opened the chance to develop orchid synthetic seed and advanced plant breeding.

Acknowledgement

This research was funded by Campus Intellectual Product Business Development Program Grant [*Hibah Program Pengembangan Usaha Produk Intelektual Kampus* (PPUPIK)] Kemendikbud-Ristek 2021 with contract no. 004/SPP-PPM/LPPM-02/DRPM/FTB/IV/ 2021 (on behalf of Dr.rer.nat. Sulistyo Emantoko Dwi Putra). All experiments were conducted in Faculty of Biotechnology University of Surabaya. The authors would like to thank to our laboratory assistants and students for their intensive helped to support this research.

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Best Regards, SCImago Team

M **MERIEM** 1 year ago

please I sent my manuscript 4 days ago and I didn't receive the submission number and now I would like to withdraw my manuscript, I sent an email to the Editor but I didn't receive anything from the journal.

reply

SCImago Team

Melanie Ortiz 1 year ago

Dear Meriem,
thank you for contacting us.
Unfortunately, we cannot help you with your request.
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S **Shatha** 1 year ago

تحية طيبة
ارغب بنشر بحث في مجلتكم-والي كم هي اجوار النشر لطفا
خالص تحياتي

reply

SCImago Team

Melanie Ortiz 1 year ago

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A **Adebayo Osesusi** 2 years ago

Please it seems Jordan Journal of Biological Science is not covered in 2019-2020, if yes why?

reply

SCImago Team

Melanie Ortiz 2 years ago

Dear Adebayo, thank you very much for your comment, unfortunately we cannot help you with your request. We suggest you to consult the Scopus database directly. Keep in mind that the SJR is a static image (the update is made one time per year) of a database (Scopus) which is changing every day.
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N **Neti Yuliana** 2 years ago

Dear Elena

Do all journals listed on Scimago journal rank also have to be indexed by Scopus?
Pls your information. Thank you

reply

G **girin** 2 years ago

what is the thomson reuters impact factor of this journal

reply

SCImago Team

Melanie Ortiz 2 years ago

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A **ahmed aziz** 2 years ago

please, I need your mail?

reply

SCImago Team

Melanie Ortiz 2 years ago

Dear Ahmed, Could you please expand your comment? Best Regards, SCImago Team

E **Ella** 2 years ago

I am PhD student and I would like to ask if publishing is free or not

reply

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K **Khaled H. Abu-Elteen** 3 years ago

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reply

M **Mostefa FODIL** 3 years ago

Dear Editor,
i would like to know how many time it takes to get an acceptation or an answer after submission.
Thanks.

reply

A **asmaa omar** 2 years ago

could you please, tell me the cost of publication

K **Khaled H. Abu-Elteen** 3 years ago

The publication process depends on Referee's to send their Evaluation Reports, and it takes between 4-5 weeks.
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I **Ibrahim** 3 years ago

Dear authors I have a research paper and I want to publish please I need your advises
I am from Iraq please help.

reply

K **Khaled H. Abu-Elteen** 3 years ago

You can send by the e mail to jjbs@hu.edu.jo

K **Khaled H. Abu-Elteen- Editor in Chief** 3 years ago

You can send your MS by e-mail to the Jordan Journal of Biological Sciences.
jjbs@hu.edu.jo

S **sumayah** 3 years ago

I am a PhD student. I want to publish my paper in your journal.

reply

K **Khaled H. Abu-Elteen- Editor in Chief** 3 years ago

Dear Sumayah
You can send your manuscript by e-mail to the Jordan Journal of Biological Sciences.
jjbs@hu.edu.jo
Regards

SCImago Team

Elena Corera 3 years ago

Thank you very much for the comment!

SCImago Team

Elena Corera 3 years ago

Please, check comments below.

Best regards,
SCImago Team

H **Haitham** 3 years ago

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SCImago Team

Elena Corera 3 years ago

Please, check comments abobe.

Best regards,
SCImago Team

A **adnan jasim** 3 years ago

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please, can you send paper pdf to publishing in your journal?

regard

reply

K **Khaled H. Abu-Elteen- Editor in Chief** 3 years ago

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K **Khaled H. Abu-Elteen** 3 years ago

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Thanks
Prof. Khaled H. Abu-Elteen
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Elena Corera 3 years ago

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The abstract should be concise and informative. It should not exceed **350 words** in length for full manuscript and Review article and **150 words** in case of Case Report and/ or Short Communication. It should briefly describe the purpose of the work, techniques and methods used, major findings with important data and conclusions. No references should be cited in this part. Generally non-standard abbreviations should not be used, if necessary they should be clearly defined in the abstract, at first use.

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Hilly MO, Adams MN and Nelson SC. 2009. Potential fly-ash utilization in agriculture. *Progress in Natural Sci.*, **19**: 1173-1186.

Reference to a book:

Brown WY and White SR.1985. **The Elements of Style**, third ed. MacMillan, New York.

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Mettam GR and Adams LB. 2010. How to prepare an electronic version of your article. In: Jones BS and Smith RZ (Eds.), **Introduction to the Electronic Age**. Kluwer Academic Publishers, Netherlands, pp. 281–304.

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EDITORIAL PREFACE

Jordan Journal of Biological Sciences (JJBS) is a refereed, quarterly international journal financed by the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research in cooperation with the Hashemite University, Jordan. JJBS celebrated its 12th commencement this past January, 2020. JJBS was founded in 2008 to create a peer-reviewed journal that publishes high-quality research articles, reviews and short communications on novel and innovative aspects of a wide variety of biological sciences such as cell biology, developmental biology, structural biology, microbiology, entomology, molecular biology, biochemistry, medical biotechnology, biodiversity, ecology, marine biology, plant and animal biology, plant and animal physiology, genomics and bioinformatics.

We have watched the growth and success of JJBS over the years. JJBS has published 11 volumes, 45 issues and 479 articles. JJBS has been indexed by SCOPUS, CABI's Full-Text Repository, EBSCO, Clarivate Analytics- Zoological Record and recently has been included in the UGC India approved journals. JJBS Cite Score has improved from 0.18 in 2015 to 0.7 in 2019 (Last updated on 1 March, 2021) and with Scimago Institution Ranking (SJR) 0.18 (Q3) in 2019.

A group of highly valuable scholars have agreed to serve on the editorial board and this places JJBS in a position of most authoritative on biological sciences. I am honored to have six eminent associate editors from various countries. I am also delighted with our group of international advisory board members coming from 15 countries worldwide for their continuous support of JJBS. With our editorial board's cumulative experience in various fields of biological sciences, this journal brings a substantial representation of biological sciences in different disciplines. Without the service and dedication of our editorial; associate editorial and international advisory board members, JJBS would have never existed.

In the coming year, we hope that JJBS will be indexed in Clarivate Analytics and MEDLINE (the U.S. National Library of Medicine database) and others. As you read throughout this volume of JJBS, I would like to remind you that the success of our journal depends on the number of quality articles submitted for review. Accordingly, I would like to request your participation and colleagues by submitting quality manuscripts for review. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscripts or not, is the feedback of our review process. JJBS provides authors with high quality, helpful reviews to improve their manuscripts.

Finally, JJBS would not have succeeded without the collaboration of authors and referees. Their work is greatly appreciated. Furthermore, my thanks are also extended to The Hashemite University and the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research for their continuous financial and administrative support to JJBS.

Professor Atoum, Manar F.
March, 2021

JJBS

Volume 14, Number 5,December 2021
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Jordan Journal of Biological Sciences

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