Gmail fauziyah harahap <fauziyahharahap@gmail.com>

Be the Identified Member

1 message

Science Alert <support@scialert.com> Mon, Jul 1, 2019 at 5:02 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear FAUZIYAH HARAHAP

Thanks for being registered with Science Alert Online Manuscript Submission and Peer Reviewed System.

Science Alert integrated with the liveDNA to accurately identify and disambiguated all authors of all articles in Science alert Database.

LiveDNA is dedicated to provide a transparent and improvised tracking of the research and researcher by issuing each researcher a Numerological ID called his/her LiveDNA. It's one of the basic objective is to establish and develop strong relationship between research and researchers, contributors and the work. Having a LiveDNA will help the researcher in his/her manuscript submission, grant applications, professional society membership, to have association with other identifiers or profiles and to display it on CV, webpage and more. LiveDNA is devoted to the openness, transparency and the protection of scholars' privacy.

Therefore, please send us your CV to be the identified member of the Science Alert.

Regard
Science Alert

Gmail **fauziyah harahap <fauziyahharahap@gmail.com>**

Associate your profile with your LiveDNA

2 messages

Science Alert <support@scialert.com> Tue, Jul 2, 2019 at 11:25 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear Dr. FAUZIYAH HARAHAP,

I hope you will receive this email with good health.

I would like to inform you that now it is mandatory to associate your Science Alert author profile with your digital identifier. Your name as an author is a key to establishing a unique public profile for enhancing your research and for attribution purposes. Author identifiers are unique identifiers assigned to researchers to prevent the author ambiguity problem within the scholarly community because of researchers having the same first and last names.

An identifier will simplify updating your CV or preparing an annual report because it streamlines the process of compiling a bibliography of your work. Searchable author identifier registries contain researcher profiles and lists of citations.

Therefore, it is requested to please submit your CV to generate your digital identifier so that we may associate your author profile with your digital identifier.

Regard
Sudha Pandey
Member Profile Manager
Science Alert

Diky Setya Diningrat <dikysetyadiningrat@gmail.com> Wed, Jul 3, 2019 at 12:43 PM
To: fauziyah harahap <fauziyahharahap@gmail.com>

Formatnya mengikuti LIve DNA nantulang

On Wed, 3 Jul 2019, 12:32 fauziyah harahap, <fauziyahharahap@gmail.com> wrote:

> assalamu'alaikum
>
> ky publishernya minta cv
> ada formatnya ky ?
> [Quoted text hidden]

Gmail **fauziyah harahap <fauziyahharahap@gmail.com>**

my LiveDNA , article submission

2 messages

fauziyah harahap <fauziyahharahap@gmail.com> Sat, Jul 6, 2019 at 11:10 PM
To: support@scialert.com

Dear Manager
Science Alert

Thank you for replying to my request.
Here I want to convey that, I have connected my profile with LiveDNA

Here I also send my curriculum vitae.
I hope, I get an account soon and can send articles to Pakistan journal of biological sciences

Thank you for your kindness

Best Regard
Fauziyah Harahap
Biology Depart
Universitas Negeri Medan, Indonesia

Curriculum Vitae, Fauziyah Harahap, English.docx
48K

fauziyah harahap <fauziyahharahap@gmail.com> Sat, Jul 6, 2019 at 11:16 PM
Draft

[Quoted text hidden]

Curriculum Vitae, Fauziyah Harahap, English.docx
48K

Gmail

fauziyah harahap <fauziyahharahap@gmail.com>

Forgot password email

1 message

Science Alert <no-reply@scialert.com> Thu, Jul 11, 2019 at 10:51 AM
To: fauziyahharahap@gmail.com

Your username and password information is as under:

Username: **fauziyahharahap@gmail.com**
Password: **473024**

The manuscript processing site is http://www.scialert.com. You need to login there for new submission or monitoring of your submitted manuscripts.

Please enable cookies and javascript in your web browser.

For further clarification or inquiry you can contact us at http://www.scialert.com/contact.php

Thanking you
Executive - Editorial Office

Gmail **fauziyah harahap <fauziyahharahap@gmail.com>**

Suggest Reviewers - Article No. 97358-PJBS-ANSI

1 message

Science Alert <support@scialert.com> Tue, Jul 16, 2019 at 2:13 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Received on: July 12, 2019
Manuscript No.: 97358-PJBS-ANSI
Submitted to: Pakistan Journal of Biological Sciences
Title: Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique

Dear FAUZIYAH HARAHAP,

Thanks for considering Pakistan Journal of Biological Sciences to publish your valuable research findings.

We have sent a request letter to few of the reviewers available in our database for the evaluation of your submitted manuscript. As early as we receive the positive response from any reviewer(s) we will send your article for evaluation purpose.

You can also help us to accelerate the evaluation process by suggesting at least 3 reviewers for the evaluation of your article. So, you please suggest three reviewers who can review your article in a timely manner, please send us their name with complete postal and email address by replying this email.

Your quick response would be highly appreciated.

Regard
Zunaira Mahmood
Academic Editor
Pakistan Journal of Biological Sciences

Gmail fauziyah harahap <fauziyahharahap@gmail.com>

Acknowledgement of a New Manuscript

4 messages

Science Alert <support@scialert.com> Tue, Jul 16, 2019 at 2:13 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Received on: July 12, 2019
Manuscript No.: 97358-PJBS-ANSI
Submitted to: Pakistan Journal of Biological Sciences
Title: Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique

Dear FAUZIYAH HARAHAP,

Thank you very much for submitting your above mentioned manuscript. Your paper has been assigned with an ID of 97358-PJBS-ANSI. Please refer to this ID whenever you communicate with our Editorial Office in the future.

Your paper will undergo the NORMAL REVIEW PROCESS of the Journal. The process normally takes 3 to 4 weeks to complete depending on the number of rounds the reviews need to take place.

Please do expect slight delay if the review period overlaps with a long holiday or Summer/Winter break.

Once again, thank you very much for your submission to the Pakistan Journal of Biological Sciences.

Regard
Zunaira Mahmood
Academic Editor
Pakistan Journal of Biological Sciences

Science Alert <support@scialert.com> Tue, Jul 16, 2019 at 2:13 PM
To: Fauziyah Harahap <fauziyahharahap@gmail.com>

Dear Fauziyah Harahap,

We have received the following article for publication in Pakistan Journal of Biological Sciences on July 12, 2019 and your good name is listed as co-author in this article.

Article Number:
97358-PJBS-ANSI

Title:
Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique

Author(s) Name:
Fauziyah Harahap, Diky Setya Diningrat, Roedhy Poerwanto, Nanda Eska Anugrah Nasution

Submitted to:
Pakistan Journal of Biological Sciences

Corresponding Author:
FAUZIYAH HARAHAP

Your paper will undergo the NORMAL REVIEW PROCESS. The process normally takes 3 to 4 weeks to complete depending on the number of rounds the reviews need to take place.

Please do expect slight delay if the review period overlaps with a long holiday or Summer/Winter

break.

Once again, thank you very much for your submission to the Pakistan Journal of Biological Sciences.

Regard
Zunaira Mahmood
Academic Editor
Pakistan Journal of Biological Sciences

---

fauziyah harahap <fauziyahharahap@gmail.com> Tue, Jul 16, 2019 at 6:46 PM
To: Science Alert <support@scialert.com>

Dear Editor of Pakistan Journal of Biological Sciences
U

Thank you very much for accepting my article.
I will wait for the review process from the editorial board in the Pakistan Journal of Biological Sciences.

Best regards

Fauziyah Harahap
Universitas Negeri Medan,
Indonesia
[Quoted text hidden]

---

fauziyah harahap <fauziyahharahap@gmail.com> Fri, Jul 26, 2019 at 4:53 PM
To: iyulharahap@gmail.com, Nabila Afifaturraihana Hasibuan <nabelhasibuan@gmail.com>

[Quoted text hidden]

Gmail **fauziyah harahap <fauziyahharahap@gmail.com>**

Status has been changed for your article No. 97358-PJBS-ANSI

3 messages

Science Alert <no-reply@scialert.com> Wed, Jul 24, 2019 at 11:56 AM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear FAUZIYAH HARAHAP,

Status of your above mentioned manuscript has been changed. Current status of your manuscript is as under:

Manuscript assigned to Reviewer for Scientific Review

For further information, please logon the system at http://www.scialert.com/login.php with your user id and password.

Best Regards
Science Alert Support Team

---

fauziyah harahap <fauziyahharahap@gmail.com> Fri, Jul 26, 2019 at 5:12 PM
To: Science Alert <no-reply@scialert.com>

Dear Science Alert Support Team

I am very thankful for this notification.
ABOUT MY JOURNAL STATUS

Thank very much for your kindness

BEST REGARDS

Fauziyah Harahap
Biology Departement
Universitas Negeri Medan, Indonesia

[Quoted text hidden]

Mail Delivery Subsystem <mailer-daemon@googlemail.com> Fri, Jul 26, 2019 at 5:14 PM
To: fauziyahharahap@gmail.com

Address not found

Your message wasn't delivered to **no-reply@scialert.com** because the address couldn't be found, or is unable to receive mail.

The response from the remote server was:

```
550 No Such User Here"
```

Final-Recipient: rfc822; no-reply@scialert.com
Action: failed
Status: 5.0.0
Remote-MTA: dns; mail.scialert.com. (198.1.111.209, the server for the domain scialert.com.)
Diagnostic-Code: smtp; 550 No Such User Here"
Last-Attempt-Date: Fri, 26 Jul 2019 03:14:50 -0700 (PDT)

---------- Forwarded message ----------
From: fauziyah harahap <fauziyahharahap@gmail.com>
To: Science Alert <no-reply@scialert.com>
Cc:
Bcc:
Date: Fri, 26 Jul 2019 17:12:24 +0700
Subject: Re: Status has been changed for your article No. 97358-PJBS-ANSI

Dear Science Alert Support Team

I am very thankful for this notification.
ABOUT MY JOURNAL STATUS

Thank very much for your kindness

BEST REGARDS

Fauziyah Harahap
Biology Departement
Universitas Negeri Medan, Indonesia

On Wed, Jul 24, 2019 at 11:56 AM Science Alert <no-reply@scialert.com> wrote:

> Dear FAUZIYAH HARAHAP,
>
> Status of your above mentioned manuscript has been changed. Current status of your manuscript is as under:
>
> **Manuscript assigned to Reviewer for Scientific Review**
>
> For further information, please logon the system at http://www.scialert.com/login.php with your user id and password.
>
> Best Regards
> Science Alert Support Team

Gmail

fauziyah harahap <fauziyahharahap@gmail.com>

Status has been changed for your article No. 97358-PJBS-ANSI

1 message

Science Alert <no-reply@scialert.com> Mon, Jul 29, 2019 at 3:02 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear FAUZIYAH HARAHAP,

Status of your above mentioned manuscript has been changed. Current status of your manuscript is as under:

Revision required for manuscript acceptance

For further information, please logon the system at http://www.scialert.com/login.php with your user id and password.

Best Regards
Science Alert Support Team

Gmail **fauziyah harahap <fauziyahharahap@gmail.com>**

Ecologia - Free Publication

1 message

Science Alert <support@scialert.com> Sun, Aug 4, 2019 at 8:54 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear Dr. FAUZIYAH HARAHAP

Ecologia is a peer-reviewed, Open Access journal that publishes original research as well as review articles in all areas of ecological sciences. Articles focusing on behavioral, environmental, evolutionary, and population ecology will be considered, as well new findings relating to biodiversity, conservation, and paleoecology.

Currently Econologia is indexed in Asian Digital Library, IndexONE, ASCI Database, and Google Scholar.

Submit your best paper to Ecologia for publication in the coming issue of the journal. Currently Econologia accepted the manuscript without Article Processing Charges.

For further information, please visit at https://scialert.net/jhome.php?issn=1996-4021

Regard
M. Imran Pasha
Publication Manager

Gmail **fauziyah harahap <fauziyahharahap@gmail.com>**

97358-PJBS-ANSI - Request for Revised Article

5 messages

Science Alert <support@scialert.com> Wed, Aug 14, 2019 at 8:49 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear Dr. FAUZIYAH HARAHAP

This is with regard to your submitted manuscript, 97358-PJBS-ANSI, titled Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique, submitted to Pakistan Journal of Biological Sciences on 12 July, 2019 for consideration as a Research Article.

The article has been accepted for publication after revision. A Peer Review report is available online and you can access this report after log in to your account with User ID: fauziyahharahap@gmail.com.

If you have forgot your password, you may retrieve your password from the following link by providing your User ID fauziyahharahap@gmail.com.

http://scialert.com/forgot_password.php

It is therefore, requested to please submit revised version of your article urgently for further processing.

Please let us know when we can expect the revised version of your manuscript.

We look forward to hearing from you.

Regard
Academic Editor
Pakistan Journal of Biological Sciences

fauziyah harahap <fauziyahharahap@gmail.com> Wed, Aug 14, 2019 at 10:50 PM
To: Science Alert <support@scialert.com>

Dear Academic Editor

Pakistan Journal of Biological Sciences

I just sent 2 files, namely 1. article revision no 97358-PJBS-ANSI Revision, 2. comments from reviewers

I beg to check whether it has been sent or not

Best regards

Fauziyah Harahap
Biology Department
Medan State University
Indonesia

[Quoted text hidden]

Science Alert <support@scialert.com> Fri, Aug 16, 2019 at 11:30 AM
To: fauziyah harahap <fauziyahharahap@gmail.com>

Dear Dr. Fauziyah Harahap,

Greetings of the day!

It is to inform you that we did not receive your revised article. Therefore, it is requested to send us your revised manuscript as urgent as possible.

Awaiting for your quick response.

Regard
Academic Editor
Pakistan Journal of Biological Sciences

---

fauziyah harahap <fauziyahharahap@gmail.com> Fri, Aug 16, 2019 at 8:47 PM
To: Science Alert <support@scialert.com>

Dear Academic Editor
Pakistan Journal of Biological sciences

Thanks for your information
I want to explain that I have resubmitted my article, file namely

1. article revision no 97358-PJBS-ANSI Revision,

2. comments from reviewers

on the same date, August 14 2019, at 11.10 pm.

Here I attach my file:

1. Evidence has been uploaded journal revision

2) article revision no 97358-PJBS-ANSI Revision,

3) comments from reviewers

and I shall re-submitted my
1. article revision no 97358-PJBS-ANSI Revision
2. and my answer to reviewer comment

Best Regard

Fauziyah Harahap
Biology Departement
Universitas Negeri Medan
Indonesia

[Quoted text hidden]

3 attachments

evidence has been uploaded journal revision.docx
129K

97358-PJBS-ANSI_1, review.docx
26K

97358-PJBS-ANSI_1 REVISION.docx
4137K

---

fauziyah harahap <fauziyahharahap@gmail.com> Fri, Aug 16, 2019 at 9:09 PM
To: Science Alert <support@scialert.com>

Dear Academic Editor
Pakistan Journal of Biological sciences

I just now, to try login, want to re-sent

1. article revision no 97358-PJBS-ANSI Revision
2. and my answer to reviewer comment

but
it's closed. Please re check again

Best Regard
Fauziyah Harahap
Biology departement
Universitas Negeri Medan
Indonesia
[Quoted text hidden]

Gmail

fauziyah harahap <fauziyahharahap@gmail.com>

Account Information

1 message

Science Alert <support@scialert.com> Sun, Aug 18, 2019 at 8:12 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear FAUZIYAH HARAHAP

Your username and password information is as under:

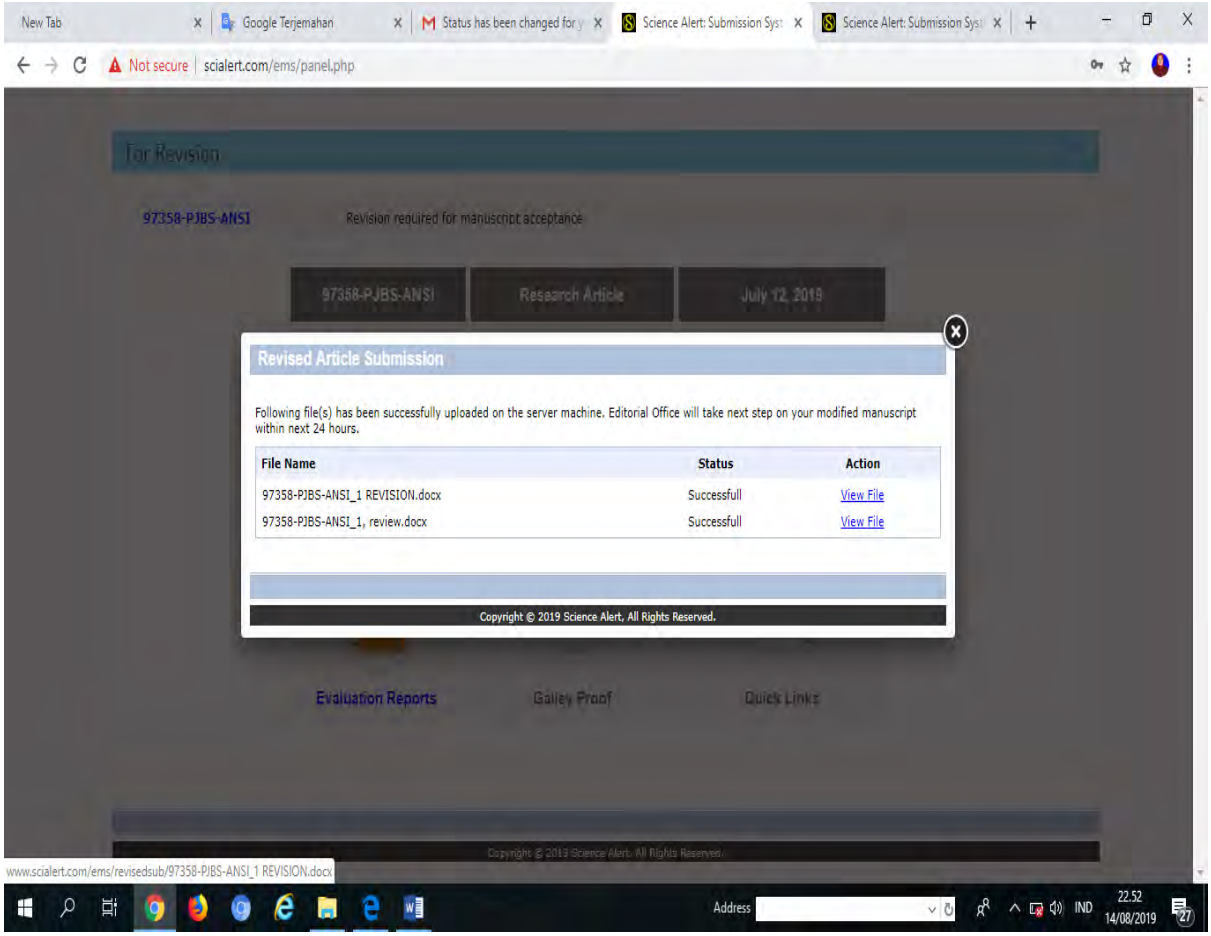
Username : fauziyahharahap@gmail.com
Password : 473024

The manuscript processing site is http://www.scialert.com. You need to login there for new submission or monitoring of your submitted manuscripts.

Please enable cookies and javascript in your web browser.

For further clarification or inquiry you can contact us at http://www.scialert.com/contact.php

Thanking you
Executive - Editorial Office


New Tab
Google Terjemahan
Status has been changed for
Science Alert: Submission Syst
Science Alert: Submission Syst
Not secure
scialert.com/ems/panel.php
For Revision
97358-PJBS-ANSI
Revision required for manuscript acceptance
97358-PJBS-ANSI
Research Article
July 12, 2019
Revised Article Submission
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www.scialert.com/ems/revisedsub/97358-PJBS-ANSI_1 REVISION.docx
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22.52
14/08/2019

Gmail

fauziyah harahap <fauziyahharahap@gmail.com>

Asian Journal of Biotechnology

1 message

Science Alert <support@scialert.com> Wed, Aug 21, 2019 at 10:35 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear Dr. FAUZIYAH HARAHAP

Asian Journal of Biotechnology is an international peer-reviewed scientific journal dedicated to publishing cutting edge research work in all areas of biotechnology. The scope of the journal includes: Molecular biology, genetic engineering, microbial biotechnology, plant biotechnology, animal biotechnology, marine biotechnology, environmental biotechnology, biological processes, industrial applications, bioinformatics, stress physiology, proteomics, biochemistry and biochemical engineering.

Currently Asian Journal of Biotechnology is indexed in Asian Digital Library, IndexONE, ASCI Database, and Google Scholar.

Submit your best paper to Asian Journal of Biotechnology for publication in the coming issue of the journal.

Currently Asian Journal of Biotechnology accepted the manuscript without Article Processing Charges.

For further information, please visit at https://scialert.net/jhome.php?issn=1996-4021

Regard
Fariha Sattar
Academic Editor

Gmail

fauziyah harahap <fauziyahharahap@gmail.com>

Asian Journal of Agricultural Research

1 message

Science Alert <support@scialert.com>
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Tue, Aug 27, 2019 at 11:04 AM

Dear Dr. FAUZIYAH HARAHAP

Asian Journal of Agricultural Research dedicated to publish outstanding research on all aspect of agricultural science. Scope of the journal includes: Agricultural entomology, crop and animal physiology, modelling of crop and animal systems, the scientific underpinning of agronomy and husbandry, engineering solutions, decision support systems, land use, environmental impacts of agriculture and forestry, impacts of climate change, rural biodiversity, experimental design and statistical analysis, the application of new analytical and study methods (including molecular studies). Asian Journal of Agricultural Research now accepting new submissions.

Currently Asian Journal of Agricultural Research is indexed in Asian Digital Library, IndexONE, ASCI Database, and Google Scholar.

Submit your best paper to Asian Journal of Agricultural Research for publication in the coming issue of the journal.

For further information, please visit at https://scialert.net/jhome.php?issn=1819-1894

Regard
Sabeen Saher
Academic Editor

Gmail

fauziyah harahap <fauziyahharahap@gmail.com>

Open for Submission - Journal of Biological Sciences

1 message

Science Alert <support@scialert.com> Fri, Aug 30, 2019 at 11:49 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear Dr. FAUZIYAH HARAHAP

Journal of Biological Sciences is a peer-reviewed and well indexed scientific journal dedicated to publish and disseminate the high quality scientific research work in the broad field of biological sciences. Scope of the journal includes: Cell biology, developmental biology, structural biology, microbiology, molecular biology & genetics, biochemistry, biotechnology, biodiversity, entomology, toxicology, ecology, freshwater biology, marine biology, environmental Biology, plant biology, Ethno-medicines and bioinformatics.

Currently Journal of Biological Sciences is indexed in AGRIS, Asian Digital Library, IndexONE, ASCI Database, EMBASE and Google Scholar.

Submit your best paper to Journal of Biological Sciences for publication in the coming issue of the journal via online submission system at http://scialert.com

For further information, please visit at https://scialert.net/jhome.php?issn=1727-3048

Regard
Sabeen Saher
Academic Editor

Gmail **fauziyah harahap <fauziyahharahap@gmail.com>**

Status has been changed for your article No. 97358-PJBS-ANSI

1 message

Science Alert <no-reply@scialert.com> Wed, Sep 11, 2019 at 12:27 AM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear FAUZIYAH HARAHAP,

Status of your above mentioned manuscript has been changed. Current status of your manuscript is as under:

Manuscript has been accepted for publication (Pending for Payment)

For further information, please logon the system at http://www.scialert.com/login.php with your user id and password.

Best Regards
Science Alert Support Team

Gmail science alert

99

Transaction confirmation

Inbox ×

Science Alert <no-reply@telr.com>
to me

Transaction confirmation

Science Alert

Transaction reference:	030023515930
Transaction type:	Sale
Amount:	$325.00
Description:	Publication / Processing Charges
Time:	12:38 PM on Friday the 13th of September, 2019
Authorisation Code:	008375
Card:	Visa Credit ending 2656

Please retain this receipt for your records.

For more information, please visit http://sciencealert.ae/ or contact sarwarm@sciencealert.ae

Sig

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Science Alert
scialert.net

LiveDNA: 62.29499
Member Status: Author
Primary Email: fauziyahharahap@gmail.com
VAT / TRN: 100372069300003

Billing Address

nill

TAX INVOICE

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27-Jul-2019	IMMEDIATE	97358INV19

Order Number	Description	Amount (USD)
97358-PJBS-ANSI	Article Processing Charges for manuscript No. 97358-PJBS-ANSI **Title:** Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique **Author(s):** Fauziyah Harahap, Diky Setya Diningrat, Roedhy Poerwanto and Nanda Eska Anugrah Nasution	325 USD
	Net Amount	325 USD
	VAT Amount	0
	Amount Payable	325 USD

Kesiya Johnson
Account Manager

Science Alert
1 Yonge Street, Suite#1801,
Toronto, ON M5E 1W7, Canada

METHOD OF PAYMENT

☐ Pay Via Bank

Telegraphic Transfer / Wire Transfer

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Bank Name:	Royal Bank of Canada
Beneficiary Account Number:	Transit # 02157 Institution # 003 Account # 1000371
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Link	https://www.paypal.me/scialert/325

Gmail **fauziyah harahap <fauziyahharahap@gmail.com>**

97358-PJBS-ANSI - Request for Payment

2 messages

Science Alert <support@scialert.com> Sat, Sep 14, 2019 at 12:00 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear Dr. FAUZIYAH HARAHAP

This is with regard to your submitted manuscript, 97358-PJBS-ANSI, titled Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique, submitted to Pakistan Journal of Biological Sciences on 12 July, 2019 for consideration as a Research Article.

The above mentioned manuscript has been finally accepted by the Reviewer for publication in Pakistan Journal of Biological Sciences as Research Article. You may download the final acceptance letter after log in to your account with User ID fauziyahharahap@gmail.com.

Before we can continue with final production, it is mandatory to pay Article Processing Charges. All articles published in our journals are open access and freely available online, immediately upon publication. This is made possible by an article-processing charge (APC) that covers the range of publishing services we provide. This includes provision of online tools for editors and authors, article production and hosting, liaison with abstracting and indexing services, and customer services. The APC, payable when your manuscript is editorially accepted and before publication, is charged to either you, or your funder, institution or employer.

You may download the invoice after log in to your account with User ID: fauziyahharahap@gmail.com.

If you have forgot your password, you may retrieve your password from the following link by providing your User ID fauziyahharahap@gmail.com.

http://scialert.com/forgot_password.php

Therefore, It is requested to please pay the Article Processing Charges urgently so that we may send your article to the production department for final publication.

We look forward to hearing from you.

Regard
Academic Editor
Pakistan Journal of Biological Sciences

Science Alert <support@scialert.com> Sat, Sep 14, 2019 at 12:04 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

[Quoted text hidden]

SCIENCE ALERT
www.scialert.com
P.O.Box 126208 Deira Dubai, UAE

Sep 14, 2019

Dr. FAUZIYAH HARAHAP,
Jl. Willem Iskandar Pasar V Medan Estate, Medan, Indonesia

Subject: Acceptance Letter for Article No. 97358-PJBS-ANSI

It's a great pleasure for us to inform you that below mentioned manuscript has been accepted for publication in Pakistan Journal of Biological Sciences as Research Article on the recommendation of the reviewers.

Title: Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique

Author's Name: Fauziyah Harahap, Diky Setya Diningrat, Roedhy Poerwanto and Nanda Eska Anugrah Nasution

Receiving Date: July 12, 2019

Regards

M. Imran Pasha
Publication Manager

Gmail

fauziyah harahap <fauziyahharahap@gmail.com>

Re: Processing cost (scialert.com)

5 messages

Science Alert <support@scialert.com> Fri, Sep 20, 2019 at 1:51 PM
To: FAUZIYAH HARAHAP <fauziyahharahap@gmail.com>

Dear Author

Please send us payment Transaction reference number

Regard
Abd al Hakim
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Research Article
In vitro Callus Induction of Sipahutar Pineapple (*Ananas comosus* L.) from North Sumatra Indonesia

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Abstract

Background and Objective: Sipahutar pineapple (*Ananas comosus* L.) is a indigenous of pineapple grown in Sipahutar district, north Sumatra, Indonesia. Propagation of Sipahutar pineapple that being done traditionally is less effective, because the number of seeds that produced is very limited and requires a long time. Propagation through *in vitro* culture is an alternative solution to solve this problem. It is necessary to add plant growth regulator (PGR) to stimulate callus formation in Sipahutar pineapple explants (*Ananas comosus* L.). Callus induction of pineapple from Sipahutar was carried out by PGR treatment on MS medium. The purpose of this study was to determine the effect MS medium treatment with added dichlorophenoxyacetic acid (2,4-D) and benzyl amino purin (BAP) PGR on Sipahutar pineapple callus formation (*Ananas comosus* L.) with light and dark treatment. **Materials and Methods:** This callus induction research used a completely randomized design (CRD) with 2 factors, the first factor was treatment 2,4-D (0, 1, 2) ppm. The second factor is BAP (0, 0.5, 1) ppm. **Results:** Nine combinations of treatments are obtained. Each combination of treatments is treated in both light and dark conditions. The parameters of this study were the percentage (%) of explants that formed callus, the time of callus formed, callus texture, callus biomass, callus surface height and callus surface area. Data were analyzed with two-way ANOVA, followed by Duncan Multiple Rate Test (DMRT). **Conclusion:** The study showed that the interaction between 2,4-D and BAP significantly affected the time of callus formed but 2,4-D and BAP did not significantly affect callus biomass, callus surface height and callus surface area. All explants can form callus, except explants without the addition of 2,4-D and BAP. The callus formed on 10 days after induction (DAI) and 12 DAI with the treatment of light and dark. The color of the produced callus were white, yellowish white, greenish white, brown, brownish yellow, brownish white, brownish green, yellowish green and greenish white. The callus formed is generally compact textures, except for explants which by giving 1 ppm 2,4-D produce friable callus.

Key words: 2,4-D, BAP, *in vitro*, pineapple, callus

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sipahutar pineapple is a indegeneus, local pineapple that is famous for its sweet taste, watery, large and yellow skin color[1]. This pineapple has long been cultivated, has prospects, has the potential to be developed. Sipahutar Pineapple provides good prospects, to help increase agricultural production, especially for food crop needs. Efforts to develop Sipahutar pineapple plant continue to be carried out, especially in the supply of seeds.

Usually farmers grow pineapple traditionally. At present, Sipahutar Pineapple has been planted like a pineapple plantation but the supply of seeds has always been a big problem[1]. In order to achieve large scale development, traditional propagation is not effective, because the number of seedlings produced is measly and takes long time. Propagation through tissue culture is an alternative technique for solving this problem[1-3], specifically using callus culture. Callus is a collection of amorphous cell masses which divide continuously, composed by parenchymal cells which bonds are very tenuous[2,3]. Callus culture aims to obtain callus from grown explants on a culture medium continuously, by *in vitro* technique, one of the methods to develop of reproducible of plantlets through callus because it was the most suitable material used for genetic transformation in plant[4], induction of somatic embryogenesis[5]. Callus culture is important to do with various purposes including to study cell metabolism and differentiation, cell morphogenesis, somaclonal variation, genetic transformation, secondary metabolite production[6]. In this study, callus culture was carried out to obtain the best combination of media and to produce the best callus that could be regenerated, becoming a source of explants that would eventually be produced in large numbers of Sipahutar pineapple plantlets.

In terms of inducing callus, growing media are needed, generally using Murashige and Skoog (MS) media, PGR is combined with basic media. The most commonly used compounds for callus induction is 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetate acid (NAA), indole acetate acid (IAA), indole butyric acid (IBA). Amin *et al.*[7] states that there is an effect of pineapple callus growth of 75% by adding 2,4-D of PGR 2.0 mg L^{-1}, the combination between 2.4-D 2.0 mg L^{-1} and BAP 2.0 mg L^{-1} showed an effect of 95% callus growth.

This study aims to determine the effect of (1) PGR 2,4-D, (2) PGR BAP, (3) Combination of PGR 2,4-D and BAP and (4) Dark and light treatment, on induction of Sipahutar pineapple callus (*Ananas comosus* L.).

MATERIALS AND METHODS

This research was conducted at YAHDI Tissue Culture Laboratory, Perum Pelabuhan Jl. Lambung No. 16 Tanah 600 Medan Marelan, Medan and Universitas Negeri Medan Biology Laboratory, for 8 months from March-October 2018. Tools that being used in this study were standard tissue culture tools. The material used in this study are: *in vitro* Sipahutar pineapple, Murashige and Skoog (MS) media, PGR 2,4-D, PGR BAP, alcohol, 0.1 N HCl, 0.1 N NaOH, sterile aquadest, detergent, chlorox.

Sterilization and making the media: All tools sterilized using an autoclave, at 121°C for 1 h at a pressure of 17.5 psi. Everything is according to the amount listed in the composition of making 1 L MS media, all ingredients are mixed. 2,4-D and BAP were added according to the treatment.

Callus induction: The plant material was used as of this study was 1 cm *in vitro* Sipahutar pineapple bulb. This study was carried out in completely randomized design (CRD) with 9 treatment combinations. This study used MS basic media with added PGR, namely (2,4-dichlorophenoxyacetic acid (0, 1, 2 ppm) and benzyl amino purine (0, 0.5, 1 ppm), with 4 replications, therefore there are 36 experimental units. All combinations of treatment are placed in both dark and light treatment, hence experimental units are 72 bottles.

Callus induction was carried out in a laminar air flow cabinet (LAFC) using *in vitro* Sipahutar pineapple bulb. *In vitro* shoots are taken, placed on petridish, *in vitro* leaves are removed. Buds cut into 1 cm size for each treatment media according to the concentration that has been made.

Maintenance was carried out by placing bottles filled with explants on culture racks at a temperature range of 22°C for 36 bottles of light treatment by application of fluorescent light of 3000-3200 lux in a 16 h photoperiod and 36 bottles closed using black cloth as a dark treatment. These samples were incubated, maintained at 24°C by regulating the room air conditioner in the culture room.

Observation parameters

Percentage of explants that formed callus: Explants forming callus were observed from the 1st day after induction to 35th day of observation. The percentage of explants that formed callus calculated by the equation:

$$\text{Explants (\%) form callus} = \frac{\text{No. of explants that make up the callus}}{\text{Total No. of explants}} \times 100\%$$

Time of the callus formation: The time of the callus formation, characterized by the emergence of irregular amorphous cells, were observed from the 1st day after induction to 35th day of observation.

Callus biomass: Callus biomass measurements in Sipahutar pineapple explants on light and dark treatments were carried out after 35 days after induction (DAI). The callus was removed from the culture bottle and weighed using a digital scale.

Callus color: The color of callus was observed after the formation of callus, 20th day and 35th day. Determination of callus color was based on Andaryani[8] with the researchers modifications, namely: Brown (1), brownish yellow (2), brownish white (3), greenish white (4), brownish green (5), yellowish green (6), whitish green (7) and green (8).

Callus texture: Callus texture was observed 35 days after induction. Characterized by a compact and friable callus texture. Friable callus is marked by the form of callus that is easily separated. While the compact callus is marked by the callus that is not easily separated.

Callus height stack: Callus stack height was measured after 35 days after induction. Callus was removed from the culture bottle and measured using millimeter paper and a ruler.

Callus surface area: The callus surface area was measured after 35 days after induction. Callus was removed from the culture bottle and measured using millimeter paper.

Statistical analysis: This research uses factorial completely randomized design model and analysis with factorial ANOVA, the equation:

$$Y_{ijk} = \mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ijk}$$

Where:

Y_{ijk} = Observations on the k test, which received 2,4-D treatment the-i and BAP treatment the -j

μ = Middle value

α_i = Effect of 2,4-D concentration on the i level

β_j = Effect of BAP concentration at the j level

$(\alpha\beta)_{ij}$ = Effect of the interaction of 2,4-D treatment at the i-level and the BAP-j treatment

ε_{ijk} = Effect of the error with 2,4-D treatment at the i level and BPA treatment at the j level at the k-replication

If the hypothesis testing obtained significantly different, then proceed with the Duncan Multiple Range Test (DMRT).

RESULT

Percentage of explants forming callus: Both light and dark treatment, all explants (100%) formed callus. Only callus treated with MS media without the addition of PGR formed callus of 75%, the rest of the explants were able to form callus (Table 1). The highest percentage of explants forming callus came from the treatment of MS media with an additional 1 ppm 2,4-D and 0.5 ppm BAP. The treatment of 1 ppm 2,4-D and 1 ppm BAP was also able to induce rapid and good callus formation.

Time of callus formed: From Table 1, it can be obtained that the combination treatment of MS medium with the addition of 1 ppm PGR 2,4-D and 1 ppm BAP was able to induce the fastest callus at 10 days after induction (DAI) in the light treatment and 12 DAI in the dark treatment.

With the same treatment of PGR 2,4-D, which is 1 ppm and addition of the lower concentration of BAP (0.5 ppm) causing delayment to form callus, that is on day 12 in the

Table 1: Percentage of explants forming callus and time of callus formation for light and dark treatment

PGR treatments		Explant forming callus (%)		Callus texture	
2,4-D (ppm)	BAP (ppm)	Light treatment	Dark treatment	Light treatment	Dark treatment
0	0.0	75	75	Compact	Compact
0	0.5	100	100	Compact	Compact
0	1.0	100	100	Compact	Friable
1	0.0	100	100	Compact	Friable
1	0.5	100	100	Friable	Friable
1	1.0	100	100	Friable	Friable
2	0.0	100	100	Compact	Compact
2	0.5	100	100	Friable	Compact
2	1.0	100	100	Compact	Compact

light treatment and day 14 in dark treatment. Increasing 2,4-D concentration to 2 ppm with combination of 0, 0.5 and 1 ppm BAP was not able to accelerate in forming callus, callus emergence was delayed to days 13-16 for light treatment and days 17-20 for dark treatment. While the longest form of callus is without added of PGR, to be exact at 25 DAI in light treatment and 27 DAI in dark treatment (Fig. 1).

Callus color: The treatment of 2,4-D and BAP PGR for light treatment resulted in a variety of callus colors (brownish white, greenish white and others) (Table 2). Observations at 20 days after induction, explants without additional and additional of low-dose 2,4-D single or combined with low-dose BAP (0, 0.5 ppm), both with light and dark treatment did not show callus formation.

Explants without the addition of 2,4-D with the addition of BAP 1 ppm produced white callus in light treatment meanwhile dark treatment did not produce callus. Addition of 1-2 ppm 2,4-D produced variety of callus colors which varied from white, yellowish white, greenish white (Fig. 2).

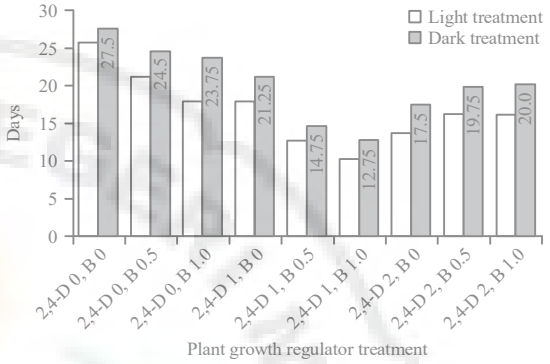

Fig. 1: Average time of callus formed in dark and light treatment
2,4-D: 2,4 dichlorophenoxyacetic acid, B: Benzyl amino purin

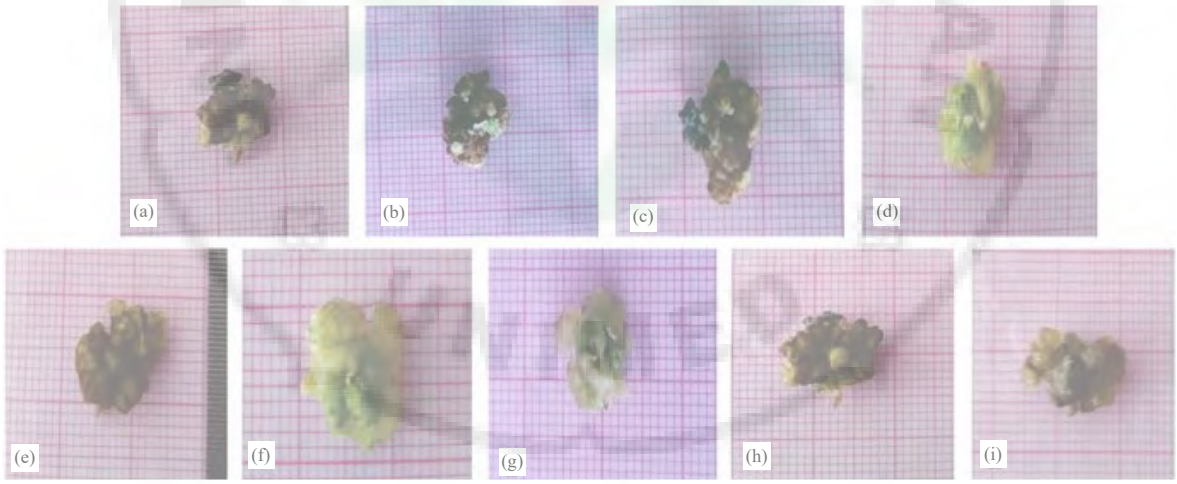

Fig. 2(a-i): Performance of callus at 35 DAI in a row starting from treatment, (a) 2,4-D 0 ppm and BAP 0 ppm, (b) 2,4-D 0 ppm and BAP 0.5 ppm, (c) 2,4-D 0 ppm and BAP 1 ppm, (d) 2,4-D 1 ppm and BAP 0 ppm, (e) 2,4-D-1 ppm and BAP 0.5 ppm, (f) 2,4-D 1 ppm and BAP 1 ppm, (g) 2,4-D 2 ppm and BAP 0 ppm, (h) 2,4-D 2 ppm and BAP 0.5 ppm and (i) 2,4-D 2 ppm and BAP 1 ppm

Table 2: Color of callus age 20 and 35 days after induction for light and dark treatment

PGR treatments		Color of callus (20 DAI)		Color of callus (35 DAI)	
2,4-D (ppm)	BAP (ppm)	Light treatment	Dark treatment	Light treatment	Dark treatment
0	0.0	Callus hasn't appeared yet	Callus hasn't appeared yet	Brown (1)	Brown (1)
0	0.5	Callus hasn't appeared yet	Callus hasn't appeared yet	Brownish yellow (2)	Brownish white (3)
0	1.0	Brownish white (3)	Callus hasn't appeared yet	Brown (1)	Brownish white (3)
1	0.0	Greenish white (7)	Callus hasn't appeared yet	Brownish green (5)	Brownish green (5)
1	0.5	Greenish white (7)	Greenish white (7)	Brownish green (5)	Brownish green (5)
1	1.0	Greenish white (7)	Greenish white (7)	Yellowish green (6)	Yellowish green (6)
2	0.0	Yellowish white (3)	Yellowish white (3)	Brown (1)	Brown (1)
2	0.5	Yellowish white	Yellowish white	Brown	Brownish yellow
2	1.0	Brownish white	Yellowish white	Brown	Greenish white

Callus biomass: Based on the results of the analysis of variance, 2,4-D PGR addition had huge effect on callus biomass for light treatment and dark but BAP in the light and dark treatment and the interaction of 2,4-D and BAP for light and dark treatment have no gave effect. The highest callus biomass was produced from the treatment of 2,4-D 1 ppm and BAP 1 ppm in the light and dark treatment that is 3.32 and 2.94 g. While the lowest callus biomass was 1.67 g (light treatment) and 1.46 g (dark treatment) from the treatment of 2,4-D 0 ppm and BAP 0 ppm (Fig. 3).

The Duncan's Multiple Range Test (DMRT) results showed that the average callus biomass was not different. It is seen that in both treatments (light and dark), the heaviest biomass is 3.32 g (bright) and 2.94 g (dark), the results of 2,4-D 1 ppm treatment and BAP 1 ppm. The lightest callus biomass is the result of 2,4-D 0 ppm and BAP 0 ppm, which is 1.67 g from light and 1.46 from dark treatment (Fig. 3).

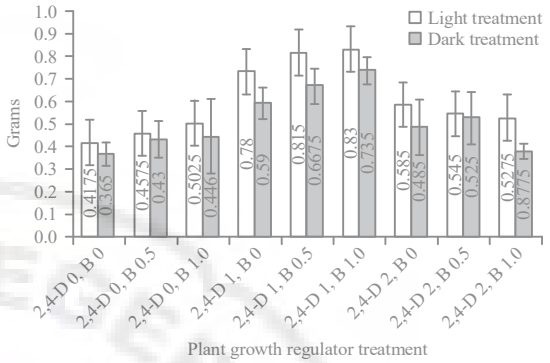

Fig. 3: Average callus biomass (grams) for light and dark treatment
2,4-D: 2,4 dichlorophenoxyacetic acid, B: Benzyl amino purin

Callus texture: The formed callus texture is differentiated into callus with friable texture and compact texture callus (Fig. 4). Friable callus is characterized by an easily separated callus texture, compact callus is in the form of a solid lump which is difficult to separate. Based on the observation of PGR 2,4-D and BAP treatment, it produced 2 types of callus texture, namely compact and friable (Fig. 2). Light and dark treatment shows that the most dominant texture is compact callus texture. Friable texture callus is generally found in 2,4-D PGR treatment with a concentration of 1 ppm both in light and dark treatments. The results of observations carried out in this study indicate that 2,4-D added to the media has an effect on the appearance of callus texture (Table 1).

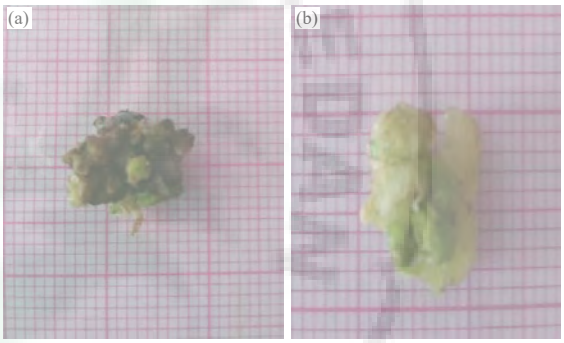

Fig. 4(a-b): Color and texture of callus, (a) Brown (2,4-D 0 ppm+BAP 1 ppm and (b) Yellowish green (2,4-D 1 ppm+BAP 1 ppm) and compact callus texture

Height stack of callus: The treatment of 2,4-D 2 ppm and BAP 0 ppm produced the highest callus stack which was 1.7 cm. The lowest callus stack height is the result of 2,4-D 0 ppm treatment and 0 ppm BAP with a stack height of 1.28 cm. Dark treatment, 2,4-D 0 ppm and 0 ppm BAP produced the highest callus stack height of 1.7 cm. The lowest callus height was treatment of 2,4-D 0 ppm and BAP 0 ppm in the light treatment, with a stack height of 1.28 cm callus (Fig. 5).

Results of analysis of variance, treatment of PGR 2,4-D; BAP; the interaction of 2,4-D and BAP on the height of the callus stack with light and dark treatment did not have effect.

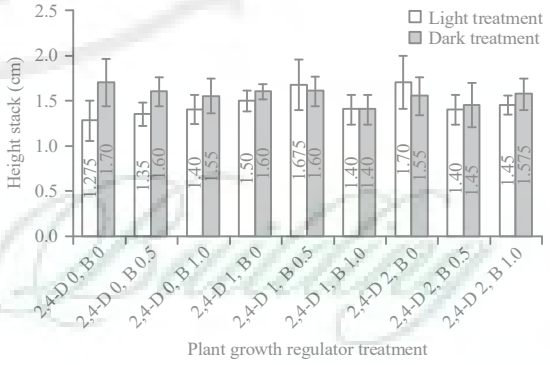

Fig. 5: Average height stack (cm) of callus for light and dark treatment
2,4-D: 2,4 dichlorophenoxyacetic acid, B: Benzyl amino purin

Callus surface area: According to the results of analysis of variance analysis, the addition of 2,4-D affected the surface area of callus both in light and dark treatment (Table 3, 4). Meanwhile, the BAP treatment did not affect the surface area of callus in both light and dark treatment. The interaction 2,4-D and BAP did not affect callus biomass for light or dark treatments (Table 4). The 2,4-D 1 ppm and BAP 0.5 ppm treatment produced the highest callus surface area of 0.95 cm

Table 3: Analysis of variance (ANOVA) effect of interaction between 2,4-D and BAP on the callus surface area of 35 DAI at light treatment

Main effect	Degree of freedom	Sum squared	Middle squared	F-count	F-table (0.05)	F-table (0.01)
Main effect						
2,4-D treatment	2	0.22	0.11	4.23*	3.35	5.49
BAP treatment	2	0.06	0.03	1.15^{tn}	3.35	5.49
Interaction of 2 factors						
2,4-D, BAP	4	0.13	0.032	1.23^{tn}	2.73	4.11
Error	27	0.70	0.026			
Total	35					

2,4-D treatment is significant, while for BAP treatment and interaction between 2,4-D and BAP is not significant, ^{tn}Not significantly different, *Significantly different

Table 4: Analysis of variance (ANOVA) effect of interaction between 2,4-D and BAP towards the surface area of callus of 35 DAI at dark treatment

Variants effect	Degree of freedom	Sum squared	Middle squared	F-count	F-table (0.05)	F-table (0.01)
Main effect						
2,4-D treatment	2	0.11	0.06	3.55*	3.35	5.49
BAP treatment	2	0.03	0.015	0.97^{tn}	3.35	5.49
Interaction of 2 factors						
2,4-D, BAP	4	0.16	0.04	1.29^{tn}	2.73	4.11
Error	27	0.83	0.031			
Total	35					

2,4 D treatment is significant, while for BAP Treatment and interaction between 2,4 D and BAP is not significant, ^{tn}Not significantly different, *Significantly different

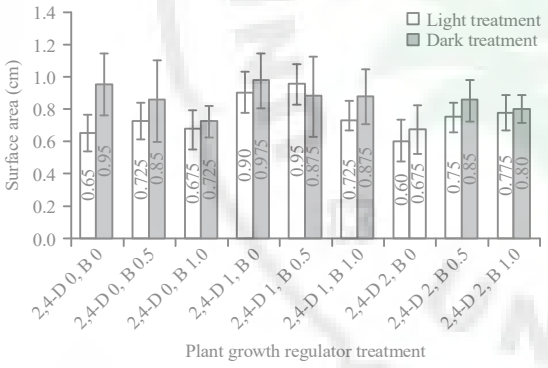

Fig. 6: Average callus surface area (cm^2) in dark and light treatment

2,4-D: 2,4 dichlorophenoxyacetic acid, B: Benzyl amino purin

(light) and PGR 2,4-D ppm and BAP 0 ppm, resulting 0.98 cm (dark) callus surface area. While the lowest surface area of callus is 0.6 cm (light) and 0.68 cm (dark) the results of 2,4-D 2 ppm and BAP 0 ppm (Fig. 6).

DISCUSSION

The data shows that, explants are generally able to form callus, except explants that were not given 2,4-D and BAP. The PGR is absolutely necessary for good callus formation, in this research was 2,4-D and BAP which will effect on increasing the percentage of explants that are able to form callus, callus appearance and acceleration of callus time formed. Callus formed in explants is formed due to the presence of openings on the tissue and response to hormones or growth regulators. The appearance of callus in the injured part is thought to be due to the stimulation of the tissue in the explants to cover the wound. One of the main characteristic of plant cells is having high plasticity for cell differentiation. Ikeuchi *et al.*[9] said, plants produce unorganized cell masses, such as callus or tumors, in response to pressure, such as wounds or pathogenic infections.

Dalila *et al.*[10] said the addition of 2,4-D and kinetin on basic MS medium gave better results of callus compared to using MS medium added with sucrose or phytagel. This is characterized by high frequency of callus induction, the callus produced is friable, beige color and grows intensively. Anita and Kumari[11] states auxiliary as IAA and IBA were not effective for callus induction in all explants tested but 2,4-D was very effective for inducing callus with sources of petiole, leaf, cotyledon and hypocotyl of *Rauvolfia tetraphylla* L., while cytokines singularly it cannot induce this plant's callus. This statement is in line with the statement of Chakraborty *et al.*[12] which resulted the maximum callus obtained on MS medium with addition of a combination of 2,4-D (0.5 mg L^{-1}) and kinetin 0.2 mg L^{-1}.

Based on the this research, the concentration of 2,4-D, BAP gave an effect on the time of callus formed. 2,4-D is a PGR that is most often used in callus culture because of its stable activity to stimulate cell multiplication, suppress organogenesis and maintain callus growth. This strong and optimal 2,4-D activity is caused by carboxyl groups separated by carbon and oxygen[11]. Each growth regulator has an influence on the induction of pineapple callus. Yifter *et al.*[13]

stated that MS media that supplemented with BAP 2 ppm and NAA 1 ppm in *Sesamum indicum* L. Hirhir variety was the best composition for accelerating time to grow this plant.

In this study, the addition of 2,4-D and BAP which is getting higher, causing the delayment of callus being formed. It appears that the optimum concentration for Sipahutar pineapple callus formation is 1 ppm 2,4-D with 1 ppm BAP. It can be understand that too high the concentration of auxin and cytokine PGR in cells, causing cells to keep on racing to make elongation and stretching. This activity takes place repeatedly without giving the cell a chance to do normal, so in the end it will cause no expression of the normal callus formation process. This study also in line with Tahir *et al.*[14] which explains that the addition of 2,4-D 3.5 mg L^{-1} gives the best effect in the formation of the callus sugarcane then the growth of callus decreases with the addition of 2,4-D above 3.5 mg L^{-1} and Mostafiz and Wagiran[15], the formation of rice callus shows better as the addition of concentration 2, 4-D but declining growth if exceeding 3 mg L^{-1}.

The dark treatment did not show a positive effect for accelerating formation of callus. As the latest study known that auxin works maximally on dark situations. Most likely there is another factor that affected the delayment of forming the callus in the dark treatment. Auxin works optimally in dark conditions and will be disturbed if there is light. From the results of this study there may be other factors that affect the formation of callus. Harahap[2] stated the ratio of auxin and cytokinin in the cell will determine the direction of induction in the tissue. If inside the cell, the auxin:cytokinin ratio is 1:1 so the tendency that occurs is callus formation. From this statement, the possibility is not only light factor which inhibits the formation of callus but there are other factors, in this case for example the balance of 2,4-D and BAP in the cell has not reached the desired ratio to form callus in the treated explants. Light in general is not giving strong effect for callus growth[2]. However, light affects the cell metabolism and effectiveness of PGR in the media. Light can damage auxin and can also cause the transfer of auxin in a direction away from light[16]. Tissue culture method in dark conditions is one of the way to make auxin effective in order to accelerate callus formation.

In vitro plant culture growth is not always hampered by the presence of light, whereas light is actually needed for optimal results. George and Sherrington[3] stated that in most cultures, cells will be able to do division in light conditions with the presence of external auxin in the media. In this study 2,4-D PGR was very effective for inducing callus of Sipahutar pineapple. As the literatures stated that IAA and IBA are not effective for inducing callus but 2,4-D is more effective for inducing callus with sources of petiole, leaf, cotyledonary leaf, hypocotyl explants[11].

The speed of growth that occurs in explants is due to the proper interaction between endogenous hormones explants and exogenous hormones given. This is reinforced by Urfiana[17] and Maciel *et al.*[5], stating that the interaction and balance of PGR given to the media and endogenously produced by plants determines the direction of development of a culture, Wahyuni *et al.*[18] say the interaction and the balance between each plant growth regulator which provided to the medium and produced by the plant cells indigenously determinated the direction of the culture development, this also in line with research from Chakraborty *et al.*[13] that stated BAP treatment alone was not all suitable for induction of callus.

The emergence of callus obstructed and also the emergence of the brown callus in treatment without 2,4-D and low dose either in single or combined with BAP, indicating that there is no addition of auxin PGR in the treatment of both light and dark treatments will inhibit callus growth and affect the color of the callus to brown, as well as the addition of sugar. Harahap and Solim[19] stated that the high content of sugar and carbohydrates in the medium can spur the occurrence of browning. The addition and increasement in the dose of 2,4-D and BAP both with light and dark treatment, will delay the change in callus color to brown.

Growth is characterized by one of which is increasing weight, so that measurements of callus biomass can represent variable callus growth originating from explants. According to Wahyuni *et al.*[18] said the fresh weight is an increase in the callus fresh weights is due to an increasing number of cells (cell division) and the increase in the cell size (cell enlargement). In conclusion the result of fresh weight is depend on the speed at which the cells divide, multiply themselves and continue with the enlargement of the callus. Through this study, it showed in order to induce callus maximally, besides 2,4-D, BAP was also needed so that the resulting callus biomass was maximal. This is in line with the statement from Harahap *et al.*[20] and Qosim[21] that BAP is needed to regulate cell division, which is characterized by increased production of number of leaves, number of segments and nodules of mangosteen callus.

2,4-D is a growth regulating agent in the auxin group which functions to boost callus induction and has ability to affect plant genetic stability. This is in accordance with the research results of the Dalila *et al.*[10], indicating that PGR 2,4-D

and kinetin with various combinations in MS Medium produced better callus compared to other basic media. Harahap[2], stated that 2,4-D is effective for forming callus because its strong activity spurred cell dedifferentiation processes, suppressing organogenesis. Tang *et al.*[22] obtained that the highest frequency of callus formation was acquired on MS medium with 0.5 mg L^{-1} BA and 3.0 mg L^{-1} 2, 4-D. The ratio between endogenous hormones explants and exogenous hormones given will determine the direction of the culture development and organ type formation[20].

Auxin affects the division, enlargement and elongation of cells. Auxin is usually applied to stimulate callus growth, cell suspension and organs and root initiation. While cytokines play a role in regulating cell division, tissue and organogenesis[23]. According to Dalila *et al.*[10], Harahap and Solim[19], that the addition of basic media without auxin as growth regulator substances or only given kinetin cannot induce callus growth. Overall showed that 2,4-D was essential for inducing callus, this is in line with the Romeida and Ganefianti[24] study, MS medium that supplemented with 1 mg L^{-1} 2,4-D produced the highest callus diameter, friable callus structure and transparent green callus and the addition of kinetin are very useful for increasing callus growth. Many researchers report that the size of the callus that being transferred to the regeneration medium also determines the success of regeneration. Callus measuring 1-2 mm is the best callus to be transferred to the regeneration medium, while callus measuring less than 1 mm will be difficult to regenerate or die[19,20]. Another study obtained, the addition of 2,-D with kinetin on MS media produced better callus than only by giving MS basic media[10].

Callus texture is one of the indicators used to assess the growth of a callus. Lizawati[25] obtained a yellowish-white, friable callus, which is characteristic of embryogenic callus, obtained from 2.5 ppm of 2,4-D treatment with the addition Tridiazuron (TDZ), this is in accordance with the results of this study. In addition, compact texture callus is a good producer of secondary metabolites. Compact callus texture is considered good because it can accumulate more secondary metabolites[26], the adding of 2,4-D around 0.25-1.00 mg L^{-1} was able to maintain the green color of explants, friable callus quality and transparent green color[23].

According to Harahap[2] friable callus is a callus that composed of long tubular cells where the structure of cells is tenuous, irregular and fragile. Dwi *et al.*[27] stated that the callus induced with cytokinin has a compact texture than the callus compared to callus that is not induced by cytokines. The compact callus texture is the effect of cytokinin and auxin which affect the water potential in cells. This causes the absorption of water from the medium into the cell to increase, so the cell becomes more rigid. 2,4-D concentrations of 1-2 ppm can produce friablely textured callus. This is in accordance with what was revealed by Dalila *et al.*[10], MS medium was added 1.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kinetin, produced friable callus textured and Khatak *et al.*[28] get result 2,4-D generate green friable callus and inclusion of BAP as a cytokinin, the days to callus induction decrease and Dharmayanti *et al.*[29] also gets the same results, namely giving a combination 2 ppm BA and 1-4 ppm 2,4-D can induce good callus formation and inhibits shoots and roots growth.

CONCLUSION

This study found that 2,4 D and BAP plant growth regulator is needed to induce callus on Sipahutar pineapple bulb. All explants can form callus, except explants without the addition of 2,4-D and BAP. The concentration of 2,4-D and BAP PGR of 1 ppm gave the best results for callus growth. Increased dose of 2,4-D and BAP causes the delayment of callus being formed. The dark treatment did not accelerated the formation of Sipahutar pineapple. This study will help the researchers to uncover the critical areas of auxin use (2,4-D) in dark and light treatments for callus induction, that many researchers were not able to explore. Thus a new theory on auxin ratio: cytokines in cells for callus induction may be arrived at.

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Research Article
In vitro Callus Induction of Sipahutar Pineapple (*Ananas comosus* L.) from North Sumatra Indonesia

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Abstract

Background and Objective: Sipahutar pineapple (*Ananas comosus* L.) is a indigenous of pineapple grown in Sipahutar district, North Sumatra, Indonesia. Propagation of Sipahutar pineapple that being done traditionally is less effective, because the number of seeds that produced is very limited and requires a long time. Propagation through *in vitro* culture is an alternative solution to solve this problem. It is necessary to add plant growth regulator (PGR) to stimulate callus formation in Sipahutar pineapple explants (*Ananas comosus* L.). Callus induction of pineapple from Sipahutar was carried out by PGR treatment on MS medium. The purpose of this study was to determine the effect MS medium treatment with added dichlorophenoxyacetic acid (2,4-D) and benzyl amino purin (BAP) PGR on Sipahutar pineapple callus formation (*Ananas comosus* L.) with light and dark treatment. **Materials and Methods:** This callus induction research used a completely randomized design (CRD) with 2 factors, the first factor was treatment 2,4-D (0, 1, 2) ppm. The second factor is BAP (0, 0.5, 1) ppm. **Results:** Nine combinations of treatments are obtained. Each combination of treatments is treated in both light and dark conditions. The parameters of this study were the percentage (%) of explants that formed callus, the time of callus formed, callus texture, callus biomass, callus surface height and callus surface area. Data were analyzed with two-way ANOVA, followed by Duncan Multiple Rate Test (DMRT). **Conclusion:** The study showed that the interaction between 2,4-D and BAP significantly affected the time of callus formed but 2,4-D and BAP did not significantly affect callus biomass, callus surface height and callus surface area. All explants can form callus, except explants without the addition of 2,4-D and BAP. The callus formed on 10 days after induction (DAI) and 12 DAI with the treatment of light and dark. The color of the produced callus were white, yellowish white, greenish white, brown, brownish yellow, brownish white, brownish green, yellowish green and greenish white. The callus formed is generally compact textures, except for explants which by giving 1 ppm 2,4-D produce friable callus.

Key words: 2,4-D, BAP, *in vitro*, pineapple, callus

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sipahutar pineapple is a indegeneus, local pineapple that is famous for its sweet taste, watery, large and yellow skin color[1]. This pineapple has long been cultivated, has prospects, has the potential to be developed. Sipahutar Pineapple provides good prospects, to help increase agricultural production, especially for food crop needs. Efforts to develop Sipahutar pineapple plant continue to be carried out, especially in the supply of seeds.

Usually farmers grow pineapple traditionally. At present, Sipahutar Pineapple has been planted like a pineapple plantation but the supply of seeds has always been a big problem[1]. In order to achieve large scale development, traditional propagation is not effective, because the number of seedlings produced is measly and takes long time. Propagation through tissue culture is an alternative technique for solving this problem[1-3], specifically using callus culture. Callus is a collection of amorphous cell masses which divide continuously, composed by parenchymal cells which bonds are very tenuous[2,3]. Callus culture aims to obtain callus from grown explants on a culture medium continuously, by *in vitro* technique, one of the methods to develop of reproducible of plantlets through callus because it was the most suitable material used for genetic transformation in plant[4], induction of somatic embryogenesis[5]. Callus culture is important to do with various purposes including to study cell metabolism and differentiation, cell morphogenesis, somaclonal variation, genetic transformation, secondary metabolite production[6]. In this study, callus culture was carried out to obtain the best combination of media and to produce the best callus that could be regenerated, becoming a source of explants that would eventually be produced in large numbers of Sipahutar pineapple plantlets.

In terms of inducing callus, growing media are needed, generally using Murashige and Skoog (MS) media, PGR is combined with basic media. The most commonly used compounds for callus induction is 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetate acid (NAA), indole acetate acid (IAA), indole butyric acid (IBA). Amin *et al.*[7] states that there is an effect of pineapple callus growth of 75% by adding 2,4-D of PGR 2.0 mg L^{-1}, the combination between 2.4-D 2.0 mg L^{-1} and BAP 2.0 mg L^{-1} showed an effect of 95% callus growth.

This study aims to determine the effect of (1) PGR 2,4-D, (2) PGR BAP, (3) Combination of PGR 2,4-D and BAP and (4) Dark and light treatment, on induction of Sipahutar pineapple callus (*Ananas comosus* L.).

MATERIALS AND METHODS

This research was conducted at YAHDI Tissue Culture Laboratory, Perum Pelabuhan Jl. Lambung No. 16 Tanah 600 Medan Marelan, Medan and Universitas Negeri Medan Biology Laboratory, for 8 months from March-October 2018. Tools that being used in this study were standard tissue culture tools. The material used in this study are: *in vitro* Sipahutar pineapple, Murashige and Skoog (MS) media, PGR 2,4-D, PGR BAP, alcohol, 0.1 N HCl, 0.1 N NaOH, sterile aquadest, detergent, chlorox.

Sterilization and making the media: All tools sterilized using an autoclave, at 121°C for 1 h at a pressure of 17.5 psi. Everything is according to the amount listed in the composition of making 1 L MS media, all ingredients are mixed. 2,4-D and BAP were added according to the treatment.

Callus induction: The plant material was used as of this study was 1 cm *in vitro* Sipahutar pineapple bulb. This study was carried out in completely randomized design (CRD) with 9 treatment combinations. This study used MS basic media with added PGR, namely (2,4-dichlorophenoxyacetic acid (0, 1, 2 ppm) and benzyl amino purine (0, 0.5, 1 ppm), with 4 replications, therefore there are 36 experimental units. All combinations of treatment are placed in both dark and light treatment, hence experimental units are 72 bottles.

Callus induction was carried out in a laminar air flow cabinet (LAFC) using *in vitro* Sipahutar pineapple bulb. *In vitro* shoots are taken, placed on petridish, *in vitro* leaves are removed. Buds cut into 1 cm size for each treatment media according to the concentration that has been made.

Maintenance was carried out by placing bottles filled with explants on culture racks at a temperature range of 22°C for 36 bottles of light treatment by application of fluorescent light of 3000-3200 lux in a 16 h photoperiod and 36 bottles closed using black cloth as a dark treatment. These samples were incubated, maintained at 24°C by regulating the room air conditioner in the culture room.

Observation parameters

Percentage of explants that formed callus: Explants forming callus were observed from the 1st day after induction to 35th day of observation. The percentage of explants that formed callus calculated by the equation:

$$\text{Explants (\%) form callus} = \frac{\text{No. of explants that make up the callus}}{\text{Total No. of explants}} \times 100\%$$

Time of the callus formation: The time of the callus formation, characterized by the emergence of irregular amorphous cells, were observed from the 1st day after induction to 35th day of observation.

Callus biomass: Callus biomass measurements in Sipahutar pineapple explants on light and dark treatments were carried out after 35 days after induction (DAI). The callus was removed from the culture bottle and weighed using a digital scale.

Callus color: The color of callus was observed after the formation of callus, 20th day and 35th day. Determination of callus color was based on Andaryani[8] with the researchers modifications, namely: Brown (1), brownish yellow (2), brownish white (3), greenish white (4), brownish green (5), yellowish green (6), whitish green (7) and green (8).

Callus texture: Callus texture was observed 35 days after induction. Characterized by a compact and friable callus texture. Friable callus is marked by the form of callus that is easily separated. While the compact callus is marked by the callus that is not easily separated.

Callus height stack: Callus stack height was measured after 35 days after induction. Callus was removed from the culture bottle and measured using millimeter paper and a ruler.

Callus surface area: The callus surface area was measured after 35 days after induction. Callus was removed from the culture bottle and measured using millimeter paper.

Statistical analysis: This research uses factorial completely randomized design model and analysis with factorial ANOVA, the equation:

$$Y_{ijk} = \mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ijk}$$

Where:

Y_{ijk} = Observations on the k test, which received 2,4-D treatment the-i and BAP treatment the -j

μ = Middle value

α_i = Effect of 2,4-D concentration on the i level

β_j = Effect of BAP concentration at the j level

$(\alpha\beta)_{ij}$ = Effect of the interaction of 2,4-D treatment at the i-level and the BAP-j treatment

ε_{ijk} = Effect of the error with 2,4-D treatment at the i level and BPA treatment at the j level at the k-replication

If the hypothesis testing obtained significantly different, then proceed with the Duncan Multiple Range Test (DMRT).

RESULT

Percentage of explants forming callus: Both light and dark treatment, all explants (100%) formed callus. Only callus treated with MS media without the addition of PGR formed callus of 75%, the rest of the explants were able to form callus (Table 1). The highest percentage of explants forming callus came from the treatment of MS media with an additional 1 ppm 2,4-D and 0.5 ppm BAP. The treatment of 1 ppm 2,4-D and 1 ppm BAP was also able to induce rapid and good callus formation.

Time of callus formed: From Table 1, it can be obtained that the combination treatment of MS medium with the addition of 1 ppm PGR 2,4-D and 1 ppm BAP was able to induce the fastest callus at 10 days after induction (DAI) in the light treatment and 12 DAI in the dark treatment.

With the same treatment of PGR 2,4-D, which is 1 ppm and addition of the lower concentration of BAP (0.5 ppm) causing delayment to form callus, that is on day 12 in the

Table 1: Percentage of explants forming callus and time of callus formation for light and dark treatment

PGR treatments		Explant forming callus (%)		Callus texture	
2,4-D (ppm)	BAP (ppm)	Light treatment	Dark treatment	Light treatment	Dark treatment
0	0.0	75	75	Compact	Compact
0	0.5	100	100	Compact	Compact
0	1.0	100	100	Compact	Friable
1	0.0	100	100	Compact	Friable
1	0.5	100	100	Friable	Friable
1	1.0	100	100	Friable	Friable
2	0.0	100	100	Compact	Compact
2	0.5	100	100	Friable	Compact
2	1.0	100	100	Compact	Compact

light treatment and day 14 in dark treatment. Increasing 2,4-D concentration to 2 ppm with combination of 0, 0.5 and 1 ppm BAP was not able to accelerate in forming callus, callus emergence was delayed to days 13-16 for light treatment and days 17-20 for dark treatment. While the longest form of callus is without added of PGR, to be exact at 25 DAI in light treatment and 27 DAI in dark treatment (Fig. 1).

Callus color: The treatment of 2,4-D and BAP PGR for light treatment resulted in a variety of callus colors (brownish white, greenish white and others) (Table 2). Observations at 20 days after induction, explants without additional and additional of low-dose 2,4-D single or combined with low-dose BAP (0, 0.5 ppm), both with light and dark treatment did not show callus formation.

Explants without the addition of 2,4-D with the addition of BAP 1 ppm produced white callus in light treatment meanwhile dark treatment did not produce callus. Addition of 1-2 ppm 2,4-D produced variety of callus colors which varied from white, yellowish white, greenish white (Fig. 2).

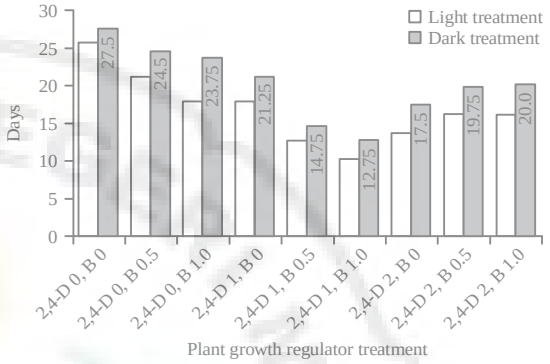

Fig. 1: Average time of callus formed in dark and light treatment
2,4-D: 2,4-dichlorophenoxyacetic acid, B: Benzyl amino purin

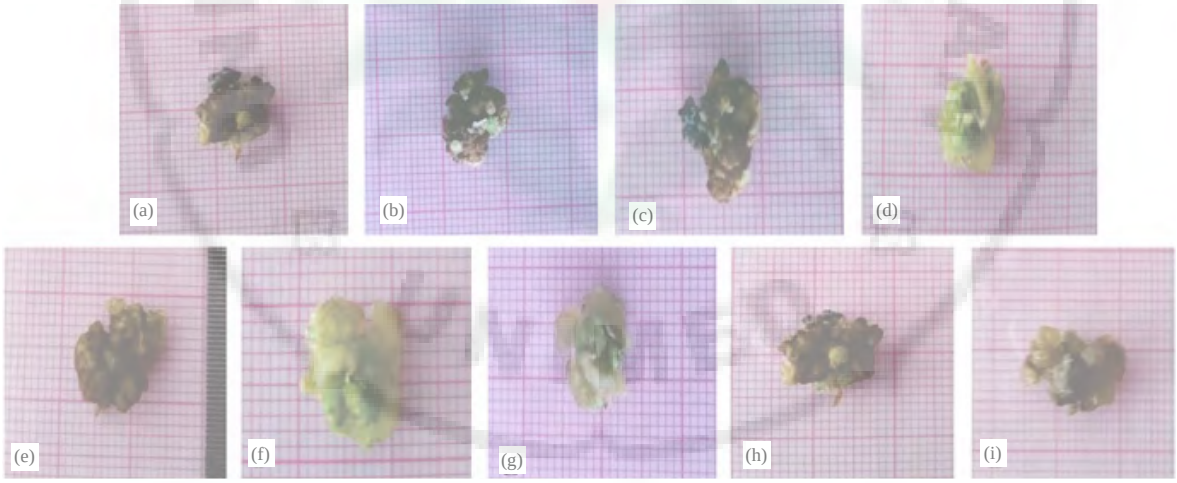

Fig. 2(a-i): Performance of callus at 35 DAI in a row starting from treatment, (a) 2,4-D 0 ppm and BAP 0 ppm, (b) 2,4-D 0 ppm and BAP 0.5 ppm, (c) 2,4-D 0 ppm and BAP 1 ppm, (d) 2,4-D 1 ppm and BAP 0 ppm, (e) 2,4-D-1 ppm and BAP 0.5 ppm, (f) 2,4-D 1 ppm and BAP 1 ppm, (g) 2,4-D 2 ppm and BAP 0 ppm, (h) 2,4-D 2 ppm and BAP 0.5 ppm and (i) 2,4-D 2 ppm and BAP 1 ppm

Table 2: Color of callus age 20 and 35 days after induction for light and dark treatment

PGR treatments		Color of callus (20 DAI)		Color of callus (35 DAI)	
2,4-D (ppm)	BAP (ppm)	Light treatment	Dark treatment	Light treatment	Dark treatment
0	0.0	Callus hasn't appeared yet	Callus hasn't appeared yet	Brown (1)	Brown (1)
0	0.5	Callus hasn't appeared yet	Callus hasn't appeared yet	Brownish yellow (2)	Brownish white (3)
0	1.0	Brownish white (3)	Callus hasn't appeared yet	Brown (1)	Brownish white (3)
1	0.0	Greenish white (7)	Callus hasn't appeared yet	Brownish green (5)	Brownish green (5)
1	0.5	Greenish white (7)	Greenish white (7)	Brownish green (5)	Brownish green (5)
1	1.0	Greenish white (7)	Greenish white (7)	Yellowish green (6)	Yellowish green (6)
2	0.0	Yellowish white (3)	Yellowish white (3)	Brown (1)	Brown (1)
2	0.5	Yellowish white	Yellowish white	Brown	Brownish yellow
2	1.0	Brownish white	Yellowish white	Brown	Greenish white

Callus biomass: Based on the results of the analysis of variance, 2,4-D PGR addition had huge effect on callus biomass for light treatment and dark but BAP in the light and dark treatment and the interaction of 2,4-D and BAP for light and dark treatment have no gave effect. The highest callus biomass was produced from the treatment of 2,4-D 1 ppm and BAP 1 ppm in the light and dark treatment that is 3.32 and 2.94 g. While the lowest callus biomass was 1.67 g (light treatment) and 1.46 g (dark treatment) from the treatment of 2,4-D 0 ppm and BAP 0 ppm (Fig. 3).

The Duncan's Multiple Range Test (DMRT) results showed that the average callus biomass was not different. It is seen that in both treatments (light and dark), the heaviest biomass is 3.32 g (bright) and 2.94 g (dark), the results of 2,4-D 1 ppm treatment and BAP 1 ppm. The lightest callus biomass is the result of 2,4-D 0 ppm and BAP 0 ppm, which is 1.67 g from light and 1.46 from dark treatment (Fig. 3).

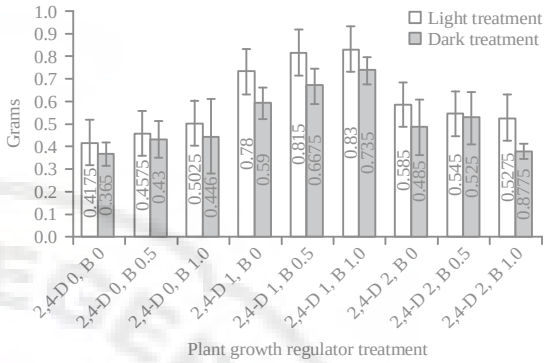

Fig. 3: Average callus biomass (grams) for light and dark treatment
2,4-D: 2,4-dichlorophenoxyacetic acid, B: Benzyl amino purin

Callus texture: The formed callus texture is differentiated into callus with friable texture and compact texture callus (Fig. 4). Friable callus is characterized by an easily separated callus texture, compact callus is in the form of a solid lump which is difficult to separate. Based on the observation of PGR 2,4-D and BAP treatment, it produced 2 types of callus texture, namely compact and friable (Fig. 2). Light and dark treatment shows that the most dominant texture is compact callus texture. Friable texture callus is generally found in 2,4-D PGR treatment with a concentration of 1 ppm both in light and dark treatments. The results of observations carried out in this study indicate that 2,4-D added to the media has an effect on the appearance of callus texture (Table 1).

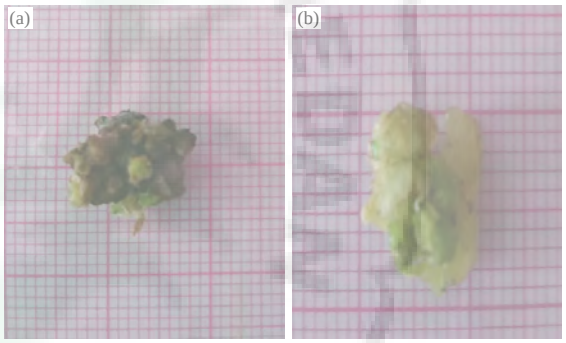

Fig. 4(a-b): Color and texture of callus, (a) Brown (2,4-D 0 ppm+BAP 1 ppm and (b) Yellowish green (2,4-D 1 ppm+BAP 1 ppm) and compact callus texture

Height stack of callus: The treatment of 2,4-D 2 ppm and BAP 0 ppm produced the highest callus stack which was 1.7 cm. The lowest callus stack height is the result of 2,4-D 0 ppm treatment and 0 ppm BAP with a stack height of 1.28 cm. Dark treatment, 2,4-D 0 ppm and 0 ppm BAP produced the highest callus stack height of 1.7 cm. The lowest callus height was treatment of 2,4-D 0 ppm and BAP 0 ppm in the light treatment, with a stack height of 1.28 cm callus (Fig. 5).

Results of analysis of variance, treatment of PGR 2,4-D; BAP; the interaction of 2,4-D and BAP on the height of the callus stack with light and dark treatment did not have effect.

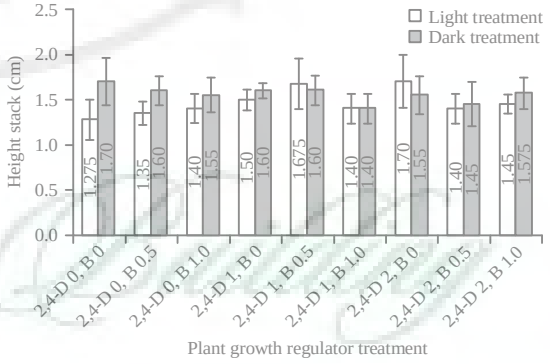

Fig. 5: Average height stack (cm) of callus for light and dark treatment
2,4-D: 2,4-dichlorophenoxyacetic acid, B: Benzyl amino purin

Callus surface area: According to the results of analysis of variance analysis, the addition of 2,4-D affected the surface area of callus both in light and dark treatment (Table 3, 4). Meanwhile, the BAP treatment did not affect the surface area of callus in both light and dark treatment. The interaction 2,4-D and BAP did not affect callus biomass for light or dark treatments (Table 4). The 2,4-D 1 ppm and BAP 0.5 ppm treatment produced the highest callus surface area of 0.95 cm

Table 3: Analysis of variance (ANOVA) effect of interaction between 2,4-D and BAP on the callus surface area of 35 DAI at light treatment

Main effect	Degree of freedom	Sum squared	Middle squared	F-count	F-table (0.05)	F-table (0.01)
Main effect						
2,4-D treatment	2	0.22	0.11	4.23*	3.35	5.49
BAP treatment	2	0.06	0.03	1.15^{ns}	3.35	5.49
Interaction of 2 factors						
2,4-D, BAP	4	0.13	0.032	1.23^{ns}	2.73	4.11
Error	27	0.70	0.026			
Total	35					

2,4-D treatment is significant, while for BAP treatment and interaction between 2,4-D and BAP is not significant, ns: Not significantly different, *Significantly different

Table 4: Analysis of variance (ANOVA) effect of interaction between 2,4-D and BAP towards the surface area of callus of 35 DAI at dark treatment

Variants effect	Degree of freedom	Sum squared	Middle squared	F-count	F-table (0.05)	F-table (0.01)
Main effect						
2,4-D treatment	2	0.11	0.06	3.55*	3.35	5.49
BAP treatment	2	0.03	0.015	0.97^{ns}	3.35	5.49
Interaction of 2 factors						
2,4-D, BAP	4	0.16	0.04	1.29^{ns}	2.73	4.11
Error	27	0.83	0.031			
Total	35					

2,4-D treatment is significant, while for BAP treatment and interaction between 2,4-D and BAP is not significant, ns: Not significantly different, *Significantly different

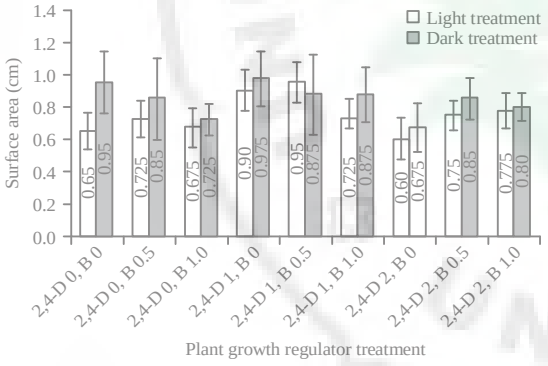

Fig. 6: Average callus surface area (cm^2) in dark and light treatment

2,4-D: 2,4-dichlorophenoxyacetic acid, B: Benzyl amino purin

(light) and PGR 2,4-D ppm and BAP 0 ppm, resulting 0.98 cm (dark) callus surface area. While the lowest surface area of callus is 0.6 cm (light) and 0.68 cm (dark) the results of 2,4-D 2 ppm and BAP 0 ppm (Fig. 6).

DISCUSSION

The data shows that, explants are generally able to form callus, except explants that were not given 2,4-D and BAP. The PGR is absolutely necessary for good callus formation, in this research was 2,4-D and BAP which will effect on increasing the percentage of explants that are able to form callus, callus appearance and acceleration of callus time formed. Callus formed in explants is formed due to the presence of openings on the tissue and response to hormones or growth regulators. The appearance of callus in the injured part is thought to be due to the stimulation of the tissue in the explants to cover the wound. One of the main characteristic of plant cells is having high plasticity for cell differentiation. Ikeuchi *et al.*[9] said, plants produce unorganized cell masses, such as callus or tumors, in response to pressure, such as wounds or pathogenic infections.

Dalila *et al.*[10] said the addition of 2,4-D and kinetin on basic MS medium gave better results of callus compared to using MS medium added with sucrose or phytagel. This is characterized by high frequency of callus induction, the callus produced is friable, beige color and grows intensively. Anita and Kumari[11] states auxiliary as IAA and IBA were not effective for callus induction in all explants tested but 2,4-D was very effective for inducing callus with sources of petiole, leaf, cotyledon and hypocotyl of *Rauvolfia tetraphylla* L., while cytokines singularly it cannot induce this plant's callus. This statement is in line with the statement of Chakraborty *et al.*[12] which resulted the maximum callus obtained on MS medium with addition of a combination of 2,4-D (0.5 mg L^{-1}) and kinetin 0.2 mg L^{-1}.

Based on the this research, the concentration of 2,4-D, BAP gave an effect on the time of callus formed. 2,4-D is a PGR that is most often used in callus culture because of its stable activity to stimulate cell multiplication, suppress organogenesis and maintain callus growth. This strong and optimal 2,4-D activity is caused by carboxyl groups separated by carbon and oxygen[11]. Each growth regulator has an influence on the induction of pineapple callus. Yifter *et al.*[13]

stated that MS media that supplemented with BAP 2 ppm and NAA 1 ppm in *Sesamum indicum* L. Hirhir variety was the best composition for accelerating time to grow this plant.

In this study, the addition of 2,4-D and BAP which is getting higher, causing the delayment of callus being formed. It appears that the optimum concentration for Sipahutar pineapple callus formation is 1 ppm 2,4-D with 1 ppm BAP. It can be understand that too high the concentration of auxin and cytokine PGR in cells, causing cells to keep on racing to make elongation and stretching. This activity takes place repeatedly without giving the cell a chance to do normal, so in the end it will cause no expression of the normal callus formation process. This study also in line with Tahir *et al.*[14] which explains that the addition of 2,4-D 3.5 mg L^{-1} gives the best effect in the formation of the callus sugarcane then the growth of callus decreases with the addition of 2,4-D above 3.5 mg L^{-1} and Mostafiz and Wagiran[15], the formation of rice callus shows better as the addition of concentration 2, 4-D but declining growth if exceeding 3 mg L^{-1}.

The dark treatment did not show a positive effect for accelerating formation of callus. As the latest study known that auxin works maximally on dark situations. Most likely there is another factor that affected the delayment of forming the callus in the dark treatment. Auxin works optimally in dark conditions and will be disturbed if there is light. From the results of this study there may be other factors that affect the formation of callus. Harahap[2] stated the ratio of auxin and cytokinin in the cell will determine the direction of induction in the tissue. If inside the cell, the auxin:cytokinin ratio is 1:1 so the tendency that occurs is callus formation. From this statement, the possibility is not only light factor which inhibits the formation of callus but there are other factors, in this case for example the balance of 2,4-D and BAP in the cell has not reached the desired ratio to form callus in the treated explants. Light in general is not giving strong effect for callus growth[2]. However, light affects the cell metabolism and effectiveness of PGR in the media. Light can damage auxin and can also cause the transfer of auxin in a direction away from light[16]. Tissue culture method in dark conditions is one of the way to make auxin effective in order to accelerate callus formation.

In vitro plant culture growth is not always hampered by the presence of light, whereas light is actually needed for optimal results. George and Sherrington[3] stated that in most cultures, cells will be able to do division in light conditions with the presence of external auxin in the media. In this study 2,4-D PGR was very effective for inducing callus of Sipahutar pineapple. As the literatures stated that IAA and IBA are not effective for inducing callus but 2,4-D is more effective for inducing callus with sources of petiole, leaf, cotyledonary leaf, hypocotyl explants[11].

The speed of growth that occurs in explants is due to the proper interaction between endogenous hormones explants and exogenous hormones given. This is reinforced by Urfiana[17] and Maciel *et al.*[5], stating that the interaction and balance of PGR given to the media and endogenously produced by plants determines the direction of development of a culture, Wahyuni *et al.*[18] say the interaction and the balance between each plant growth regulator which provided to the medium and produced by the plant cells indigenously determinated the direction of the culture development, this also in line with research from Chakraborty *et al.*[13] that stated BAP treatment alone was not all suitable for induction of callus.

The emergence of callus obstructed and also the emergence of the brown callus in treatment without 2,4-D and low dose either in single or combined with BAP, indicating that there is no addition of auxin PGR in the treatment of both light and dark treatments will inhibit callus growth and affect the color of the callus to brown, as well as the addition of sugar. Harahap and Solim[19] stated that the high content of sugar and carbohydrates in the medium can spur the occurrence of browning. The addition and increasement in the dose of 2,4-D and BAP both with light and dark treatment, will delay the change in callus color to brown.

Growth is characterized by one of which is increasing weight, so that measurements of callus biomass can represent variable callus growth originating from explants. According to Wahyuni *et al.*[18] said the fresh weight is an increase in the callus fresh weights is due to an increasing number of cells (cell division) and the increase in the cell size (cell enlargement). In conclusion the result of fresh weight is depend on the speed at which the cells divide, multiply themselves and continue with the enlargement of the callus. Through this study, it showed in order to induce callus maximally, besides 2,4-D, BAP was also needed so that the resulting callus biomass was maximal. This is in line with the statement from Harahap *et al.*[20] and Qosim[21] that BAP is needed to regulate cell division, which is characterized by increased production of number of leaves, number of segments and nodules of mangosteen callus.

2,4-D is a growth regulating agent in the auxin group which functions to boost callus induction and has ability to affect plant genetic stability. This is in accordance with the research results of the Dalila *et al.*[10], indicating that PGR 2,4-D

and kinetin with various combinations in MS Medium produced better callus compared to other basic media. Harahap[2], stated that 2,4-D is effective for forming callus because its strong activity spurred cell dedifferentiation processes, suppressing organogenesis. Tang *et al.*[22] obtained that the highest frequency of callus formation was acquired on MS medium with 0.5 mg L^{-1} BA and 3.0 mg L^{-1} 2, 4-D. The ratio between endogenous hormones explants and exogenous hormones given will determine the direction of the culture development and organ type formation[20].

Auxin affects the division, enlargement and elongation of cells. Auxin is usually applied to stimulate callus growth, cell suspension and organs and root initiation. While cytokines play a role in regulating cell division, tissue and organogenesis[23]. According to Dalila *et al.*[10], Harahap and Solim[19], that the addition of basic media without auxin as growth regulator substances or only given kinetin cannot induce callus growth. Overall showed that 2,4-D was essential for inducing callus, this is in line with the Romeida and Ganefianti[24] study, MS medium that supplemented with 1 mg L^{-1} 2,4-D produced the highest callus diameter, friable callus structure and transparent green callus and the addition of kinetin are very useful for increasing callus growth. Many researchers report that the size of the callus that being transferred to the regeneration medium also determines the success of regeneration. Callus measuring 1-2 mm is the best callus to be transferred to the regeneration medium, while callus measuring less than 1 mm will be difficult to regenerate or die[19,20]. Another study obtained, the addition of 2,-D with kinetin on MS media produced better callus than only by giving MS basic media[10].

Callus texture is one of the indicators used to assess the growth of a callus. Lizawati[25] obtained a yellowish-white, friable callus, which is characteristic of embryogenic callus, obtained from 2.5 ppm of 2,4-D treatment with the addition Tridiazuron (TDZ), this is in accordance with the results of this study. In addition, compact texture callus is a good producer of secondary metabolites. Compact callus texture is considered good because it can accumulate more secondary metabolites[26], the adding of 2,4-D around 0.25-1.00 mg L^{-1} was able to maintain the green color of explants, friable callus quality and transparent green color[23].

According to Harahap[2] friable callus is a callus that composed of long tubular cells where the structure of cells is tenuous, irregular and fragile. Dwi *et al.*[27] stated that the callus induced with cytokinin has a compact texture than the callus compared to callus that is not induced by cytokines. The compact callus texture is the effect of cytokinin and auxin which affect the water potential in cells. This causes the absorption of water from the medium into the cell to increase, so the cell becomes more rigid. 2,4-D concentrations of 1-2 ppm can produce friablely textured callus. This is in accordance with what was revealed by Dalila *et al.*[10], MS medium was added 1.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kinetin, produced friable callus textured and Khatak *et al.*[28] get result 2,4-D generate green friable callus and inclusion of BAP as a cytokinin, the days to callus induction decrease and Dharmayanti *et al.*[29] also gets the same results, namely giving a combination 2 ppm BA and 1-4 ppm 2,4-D can induce good callus formation and inhibits shoots and roots growth.

CONCLUSION

This study found that 2,4 D and BAP plant growth regulator is needed to induce callus on Sipahutar pineapple bulb. All explants can form callus, except explants without the addition of 2,4-D and BAP. The concentration of 2,4-D and BAP PGR of 1 ppm gave the best results for callus growth. Increased dose of 2,4-D and BAP causes the delayment of callus being formed. The dark treatment did not accelerated the formation of Sipahutar pineapple. This study will help the researchers to uncover the critical areas of auxin use (2,4-D) in dark and light treatments for callus induction, that many researchers were not able to explore. Thus a new theory on auxin ratio: cytokines in cells for callus induction may be arrived at.

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Research Article
In vitro Callus Induction of Sipahutar Pineapple (*Ananas comosus* L.) from North Sumatra Indonesia

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Abstract

Background and Objective: Sipahutar pineapple (*Ananas comosus* L.) is a indigenous of pineapple grown in Sipahutar district, north Sumatra, Indonesia. Propagation of Sipahutar pineapple that being done traditionally is less effective, because the number of seeds that produced is very limited and requires a long time. Propagation through *in vitro* culture is an alternative solution to solve this problem. It is necessary to add plant growth regulator (PGR) to stimulate callus formation in Sipahutar pineapple explants (*Ananas comosus* L.). Callus induction of pineapple from Sipahutar was carried out by PGR treatment on MS medium. The purpose of this study was to determine the effect MS medium treatment with added dichlorophenoxyacetic acid (2,4-D) and benzyl amino purin (BAP) PGR on Sipahutar pineapple callus formation (*Ananas comosus* L.) with light and dark treatment. **Materials and Methods:** This callus induction research used a completely randomized design (CRD) with 2 factors, the first factor was treatment 2,4-D (0, 1, 2) ppm. The second factor is BAP (0, 0.5, 1) ppm. **Results:** Nine combinations of treatments are obtained. Each combination of treatments is treated in both light and dark conditions. The parameters of this study were the percentage (%) of explants that formed callus, the time of callus formed, callus texture, callus biomass, callus surface height and callus surface area. Data were analyzed with two-way ANOVA, followed by Duncan Multiple Rate Test (DMRT). **Conclusion:** The study showed that the interaction between 2,4-D and BAP significantly affected the time of callus formed but 2,4-D and BAP did not significantly affect callus biomass, callus surface height and callus surface area. All explants can form callus, except explants without the addition of 2,4-D and BAP. The callus formed on 10 days after induction (DAI) and 12 DAI with the treatment of light and dark. The color of the produced callus were white, yellowish white, greenish white, brown, brownish yellow, brownish white, brownish green, yellowish green and greenish white. The callus formed is generally compact textures, except for explants which by giving 1 ppm 2,4-D produce friable callus.

Key words: 2,4-D, BAP, *in vitro*, pineapple, callus

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sipahutar pineapple is a indegeneus, local pineapple that is famous for its sweet taste, watery, large and yellow skin color[1]. This pineapple has long been cultivated, has prospects, has the potential to be developed. Sipahutar Pineapple provides good prospects, to help increase agricultural production, especially for food crop needs. Efforts to develop Sipahutar pineapple plant continue to be carried out, especially in the supply of seeds.

Usually farmers grow pineapple traditionally. At present, Sipahutar Pineapple has been planted like a pineapple plantation but the supply of seeds has always been a big problem[1]. In order to achieve large scale development, traditional propagation is not effective, because the number of seedlings produced is measly and takes long time. Propagation through tissue culture is an alternative technique for solving this problem[1-3], specifically using callus culture. Callus is a collection of amorphous cell masses which divide continuously, composed by parenchymal cells which bonds are very tenuous[2,3]. Callus culture aims to obtain callus from grown explants on a culture medium continuously, by *in vitro* technique, one of the methods to develop of reproducible of plantlets through callus because it was the most suitable material used for genetic transformation in plant[4], induction of somatic embryogenesis[5]. Callus culture is important to do with various purposes including to study cell metabolism and differentiation, cell morphogenesis, somaclonal variation, genetic transformation, secondary metabolite production[6]. In this study, callus culture was carried out to obtain the best combination of media and to produce the best callus that could be regenerated, becoming a source of explants that would eventually be produced in large numbers of Sipahutar pineapple plantlets.

In terms of inducing callus, growing media are needed, generally using Murashige and Skoog (MS) media, PGR is combined with basic media. The most commonly used compounds for callus induction is 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetate acid (NAA), indole acetate acid (IAA), indole butyric acid (IBA). Amin *et al.*[7] states that there is an effect of pineapple callus growth of 75% by adding 2,4-D of PGR 2.0 mg L^{-1}, the combination between 2.4-D 2.0 mg L^{-1} and BAP 2.0 mg L^{-1} showed an effect of 95% callus growth.

This study aims to determine the effect of (1) PGR 2,4-D, (2) PGR BAP, (3) Combination of PGR 2,4-D and BAP and (4) Dark and light treatment, on induction of Sipahutar pineapple callus (*Ananas comosus* L.).

MATERIALS AND METHODS

This research was conducted at YAHDI Tissue Culture Laboratory, Perum Pelabuhan Jl. Lambung No. 16 Tanah 600 Medan Marelan, Medan and Universitas Negeri Medan Biology Laboratory, for 8 months from March-October 2018. Tools that being used in this study were standard tissue culture tools. The material used in this study are: *in vitro* Sipahutar pineapple, Murashige and Skoog (MS) media, PGR 2,4-D, PGR BAP, alcohol, 0.1 N HCl, 0.1 N NaOH, sterile aquadest, detergent, chlorox.

Sterilization and making the media: All tools sterilized using an autoclave, at 121°C for 1 h at a pressure of 17.5 psi. Everything is according to the amount listed in the composition of making 1 L MS media, all ingredients are mixed. 2,4-D and BAP were added according to the treatment.

Callus induction: The plant material was used as of this study was 1 cm *in vitro* Sipahutar pineapple bulb. This study was carried out in completely randomized design (CRD) with 9 treatment combinations. This study used MS basic media with added PGR, namely (2,4-dichlorophenoxyacetic acid (0, 1, 2 ppm) and benzyl amino purine (0, 0.5, 1 ppm), with 4 replications, therefore there are 36 experimental units. All combinations of treatment are placed in both dark and light treatment, hence experimental units are 72 bottles.

Callus induction was carried out in a laminar air flow cabinet (LAFC) using *in vitro* Sipahutar pineapple bulb. *In vitro* shoots are taken, placed on petridish, *in vitro* leaves are removed. Buds cut into 1 cm size for each treatment media according to the concentration that has been made.

Maintenance was carried out by placing bottles filled with explants on culture racks at a temperature range of 22°C for 36 bottles of light treatment by application of fluorescent light of 3000-3200 lux in a 16 h photoperiod and 36 bottles closed using black cloth as a dark treatment. These samples were incubated, maintained at 24°C by regulating the room air conditioner in the culture room.

Observation parameters

Percentage of explants that formed callus: Explants forming callus were observed from the 1st day after induction to 35th day of observation. The percentage of explants that formed callus calculated by the equation:

$$\text{Explants (\%) form callus} = \frac{\text{No. of explants that make up the callus}}{\text{Total No. of explants}} \times 100\%$$

Time of the callus formation: The time of the callus formation, characterized by the emergence of irregular amorphous cells, were observed from the 1st day after induction to 35th day of observation.

Callus biomass: Callus biomass measurements in Sipahutar pineapple explants on light and dark treatments were carried out after 35 days after induction (DAI). The callus was removed from the culture bottle and weighed using a digital scale.

Callus color: The color of callus was observed after the formation of callus, 20th day and 35th day. Determination of callus color was based on Andaryani[8] with the researchers modifications, namely: Brown (1), brownish yellow (2), brownish white (3), greenish white (4), brownish green (5), yellowish green (6), whitish green (7) and green (8).

Callus texture: Callus texture was observed 35 days after induction. Characterized by a compact and friable callus texture. Friable callus is marked by the form of callus that is easily separated. While the compact callus is marked by the callus that is not easily separated.

Callus height stack: Callus stack height was measured after 35 days after induction. Callus was removed from the culture bottle and measured using millimeter paper and a ruler.

Callus surface area: The callus surface area was measured after 35 days after induction. Callus was removed from the culture bottle and measured using millimeter paper.

Statistical analysis: This research uses factorial completely randomized design model and analysis with factorial ANOVA, the equation:

$$Y_{ijk} = \mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ijk}$$

Where:

Y_{ijk} = Observations on the k test, which received 2,4-D treatment the-i and BAP treatment the -j

μ = Middle value

α_i = Effect of 2,4-D concentration on the i level

β_j = Effect of BAP concentration at the j level

$(\alpha\beta)_{ij}$ = Effect of the interaction of 2,4-D treatment at the i-level and the BAP-j treatment

ε_{ijk} = Effect of the error with 2,4-D treatment at the i level and BPA treatment at the j level at the k-replication

If the hypothesis testing obtained significantly different, then proceed with the Duncan Multiple Range Test (DMRT).

RESULT

Percentage of explants forming callus: Both light and dark treatment, all explants (100%) formed callus. Only callus treated with MS media without the addition of PGR formed callus of 75%, the rest of the explants were able to form callus (Table 1). The highest percentage of explants forming callus came from the treatment of MS media with an additional 1 ppm 2,4-D and 0.5 ppm BAP. The treatment of 1 ppm 2,4-D and 1 ppm BAP was also able to induce rapid and good callus formation.

Time of callus formed: From Table 1, it can be obtained that the combination treatment of MS medium with the addition of 1 ppm PGR 2,4-D and 1 ppm BAP was able to induce the fastest callus at 10 days after induction (DAI) in the light treatment and 12 DAI in the dark treatment.

With the same treatment of PGR 2,4-D, which is 1 ppm and addition of the lower concentration of BAP (0.5 ppm) causing delayment to form callus, that is on day 12 in the

Table 1: Percentage of explants forming callus and time of callus formation for light and dark treatment

PGR treatments		Explant forming callus (%)		Callus texture	
2,4-D (ppm)	BAP (ppm)	Light treatment	Dark treatment	Light treatment	Dark treatment
0	0.0	75	75	Compact	Compact
0	0.5	100	100	Compact	Compact
0	1.0	100	100	Compact	Friable
1	0.0	100	100	Compact	Friable
1	0.5	100	100	Friable	Friable
1	1.0	100	100	Friable	Friable
2	0.0	100	100	Compact	Compact
2	0.5	100	100	Friable	Compact
2	1.0	100	100	Compact	Compact

light treatment and day 14 in dark treatment. Increasing 2,4-D concentration to 2 ppm with combination of 0, 0.5 and 1 ppm BAP was not able to accelerate in forming callus, callus emergence was delayed to days 13-16 for light treatment and days 17-20 for dark treatment. While the longest form of callus is without added of PGR, to be exact at 25 DAI in light treatment and 27 DAI in dark treatment (Fig. 1).

Callus color: The treatment of 2,4-D and BAP PGR for light treatment resulted in a variety of callus colors (brownish white, greenish white and others) (Table 2). Observations at 20 days after induction, explants without additional and additional of low-dose 2,4-D single or combined with low-dose BAP (0, 0.5 ppm), both with light and dark treatment did not show callus formation.

Explants without the addition of 2,4-D with the addition of BAP 1 ppm produced white callus in light treatment meanwhile dark treatment did not produce callus. Addition of 1-2 ppm 2,4-D produced variety of callus colors which varied from white, yellowish white, greenish white (Fig. 2).

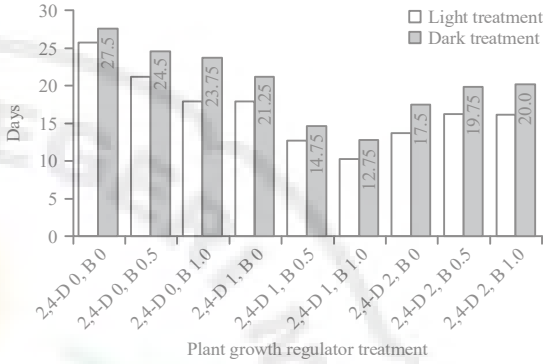

Fig. 1: Average time of callus formed in dark and light treatment
2,4-D: 2,4 dichlorophenoxyacetic acid, B: Benzyl amino purin

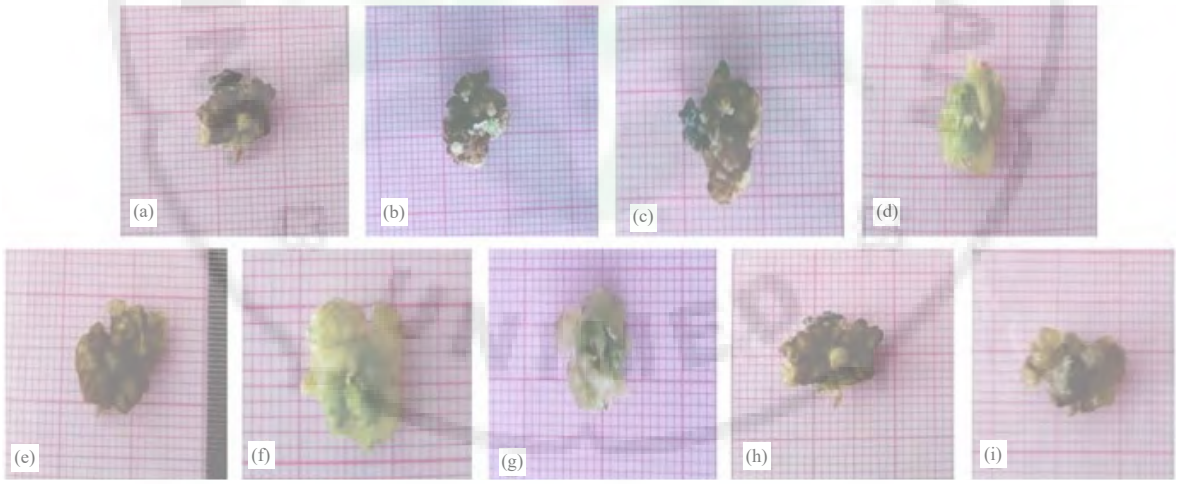

Fig. 2(a-i): Performance of callus at 35 DAI in a row starting from treatment, (a) 2,4-D 0 ppm and BAP 0 ppm, (b) 2,4-D 0 ppm and BAP 0.5 ppm, (c) 2,4-D 0 ppm and BAP 1 ppm, (d) 2,4-D 1 ppm and BAP 0 ppm, (e) 2,4-D-1 ppm and BAP 0.5 ppm, (f) 2,4-D 1 ppm and BAP 1 ppm, (g) 2,4-D 2 ppm and BAP 0 ppm, (h) 2,4-D 2 ppm and BAP 0.5 ppm and (i) 2,4-D 2 ppm and BAP 1 ppm

Table 2: Color of callus age 20 and 35 days after induction for light and dark treatment

PGR treatments		Color of callus (20 DAI)		Color of callus (35 DAI)	
2,4-D (ppm)	BAP (ppm)	Light treatment	Dark treatment	Light treatment	Dark treatment
0	0.0	Callus hasn't appeared yet	Callus hasn't appeared yet	Brown (1)	Brown (1)
0	0.5	Callus hasn't appeared yet	Callus hasn't appeared yet	Brownish yellow (2)	Brownish white (3)
0	1.0	Brownish white (3)	Callus hasn't appeared yet	Brown (1)	Brownish white (3)
1	0.0	Greenish white (7)	Callus hasn't appeared yet	Brownish green (5)	Brownish green (5)
1	0.5	Greenish white (7)	Greenish white (7)	Brownish green (5)	Brownish green (5)
1	1.0	Greenish white (7)	Greenish white (7)	Yellowish green (6)	Yellowish green (6)
2	0.0	Yellowish white (3)	Yellowish white (3)	Brown (1)	Brown (1)
2	0.5	Yellowish white	Yellowish white	Brown	Brownish yellow
2	1.0	Brownish white	Yellowish white	Brown	Greenish white

Callus biomass: Based on the results of the analysis of variance, 2,4-D PGR addition had huge effect on callus biomass for light treatment and dark but BAP in the light and dark treatment and the interaction of 2,4-D and BAP for light and dark treatment have no gave effect. The highest callus biomass was produced from the treatment of 2,4-D 1 ppm and BAP 1 ppm in the light and dark treatment that is 3.32 and 2.94 g. While the lowest callus biomass was 1.67 g (light treatment) and 1.46 g (dark treatment) from the treatment of 2,4-D 0 ppm and BAP 0 ppm (Fig. 3).

The Duncan's Multiple Range Test (DMRT) results showed that the average callus biomass was not different. It is seen that in both treatments (light and dark), the heaviest biomass is 3.32 g (bright) and 2.94 g (dark), the results of 2,4-D 1 ppm treatment and BAP 1 ppm. The lightest callus biomass is the result of 2,4-D 0 ppm and BAP 0 ppm, which is 1.67 g from light and 1.46 from dark treatment (Fig. 3).

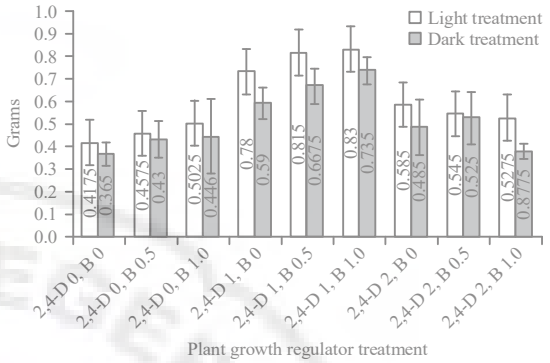

Fig. 3: Average callus biomass (grams) for light and dark treatment
2,4-D: 2,4 dichlorophenoxyacetic acid, B: Benzyl amino purin

Callus texture: The formed callus texture is differentiated into callus with friable texture and compact texture callus (Fig. 4). Friable callus is characterized by an easily separated callus texture, compact callus is in the form of a solid lump which is difficult to separate. Based on the observation of PGR 2,4-D and BAP treatment, it produced 2 types of callus texture, namely compact and friable (Fig. 2). Light and dark treatment shows that the most dominant texture is compact callus texture. Friable texture callus is generally found in 2,4-D PGR treatment with a concentration of 1 ppm both in light and dark treatments. The results of observations carried out in this study indicate that 2,4-D added to the media has an effect on the appearance of callus texture (Table 1).

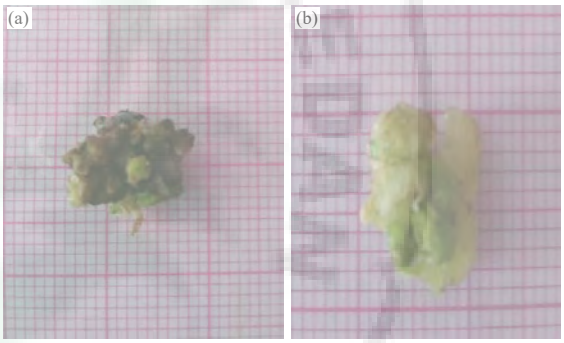

Fig. 4(a-b): Color and texture of callus, (a) Brown (2,4-D 0 ppm+BAP 1 ppm and (b) Yellowish green (2,4-D 1 ppm+BAP 1 ppm) and compact callus texture

Height stack of callus: The treatment of 2,4-D 2 ppm and BAP 0 ppm produced the highest callus stack which was 1.7 cm. The lowest callus stack height is the result of 2,4-D 0 ppm treatment and 0 ppm BAP with a stack height of 1.28 cm. Dark treatment, 2,4-D 0 ppm and 0 ppm BAP produced the highest callus stack height of 1.7 cm. The lowest callus height was treatment of 2,4-D 0 ppm and BAP 0 ppm in the light treatment, with a stack height of 1.28 cm callus (Fig. 5).

Results of analysis of variance, treatment of PGR 2,4-D; BAP; the interaction of 2,4-D and BAP on the height of the callus stack with light and dark treatment did not have effect.

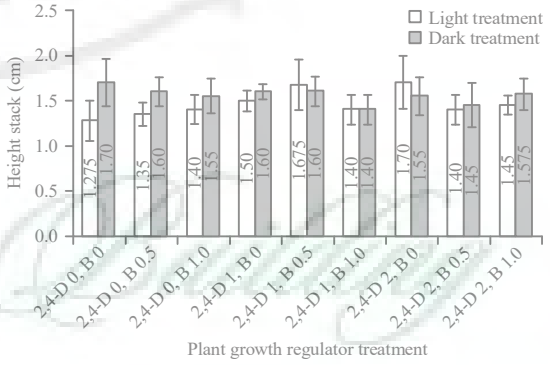

Fig. 5: Average height stack (cm) of callus for light and dark treatment
2,4-D: 2,4 dichlorophenoxyacetic acid, B: Benzyl amino purin

Callus surface area: According to the results of analysis of variance analysis, the addition of 2,4-D affected the surface area of callus both in light and dark treatment (Table 3, 4). Meanwhile, the BAP treatment did not affect the surface area of callus in both light and dark treatment. The interaction 2,4-D and BAP did not affect callus biomass for light or dark treatments (Table 4). The 2,4-D 1 ppm and BAP 0.5 ppm treatment produced the highest callus surface area of 0.95 cm

Table 3: Analysis of variance (ANOVA) effect of interaction between 2,4-D and BAP on the callus surface area of 35 DAI at light treatment

Main effect	Degree of freedom	Sum squared	Middle squared	F-count	F-table (0.05)	F-table (0.01)
Main effect						
2,4-D treatment	2	0.22	0.11	4.23*	3.35	5.49
BAP treatment	2	0.06	0.03	1.15^{tn}	3.35	5.49
Interaction of 2 factors						
2,4-D, BAP	4	0.13	0.032	1.23^{tn}	2.73	4.11
Error	27	0.70	0.026			
Total	35					

2,4-D treatment is significant, while for BAP treatment and interaction between 2,4-D and BAP is not significant, ^{tn}Not significantly different, *Significantly different

Table 4: Analysis of variance (ANOVA) effect of interaction between 2,4-D and BAP towards the surface area of callus of 35 DAI at dark treatment

Variants effect	Degree of freedom	Sum squared	Middle squared	F-count	F-table (0.05)	F-table (0.01)
Main effect						
2,4-D treatment	2	0.11	0.06	3.55*	3.35	5.49
BAP treatment	2	0.03	0.015	0.97^{tn}	3.35	5.49
Interaction of 2 factors						
2,4-D, BAP	4	0.16	0.04	1.29^{tn}	2.73	4.11
Error	27	0.83	0.031			
Total	35					

2,4 D treatment is significant, while for BAP Treatment and interaction between 2,4 D and BAP is not significant, ^{tn}Not significantly different, *Significantly different

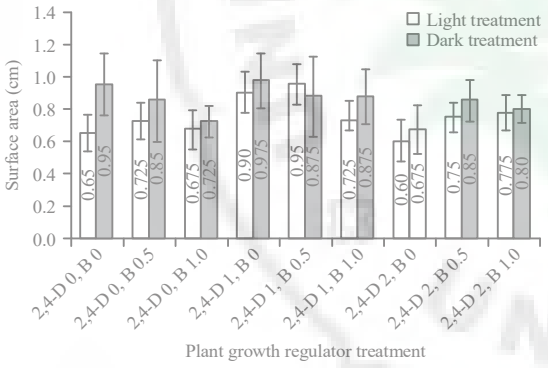

Fig. 6: Average callus surface area (cm^2) in dark and light treatment

2,4-D: 2,4 dichlorophenoxyacetic acid, B: Benzyl amino purin

(light) and PGR 2,4-D ppm and BAP 0 ppm, resulting 0.98 cm (dark) callus surface area. While the lowest surface area of callus is 0.6 cm (light) and 0.68 cm (dark) the results of 2,4-D 2 ppm and BAP 0 ppm (Fig. 6).

DISCUSSION

The data shows that, explants are generally able to form callus, except explants that were not given 2,4-D and BAP. The PGR is absolutely necessary for good callus formation, in this research was 2,4-D and BAP which will effect on increasing the percentage of explants that are able to form callus, callus appearance and acceleration of callus time formed. Callus formed in explants is formed due to the presence of openings on the tissue and response to hormones or growth regulators. The appearance of callus in the injured part is thought to be due to the stimulation of the tissue in the explants to cover the wound. One of the main characteristic of plant cells is having high plasticity for cell differentiation. Ikeuchi *et al.*[9] said, plants produce unorganized cell masses, such as callus or tumors, in response to pressure, such as wounds or pathogenic infections.

Dalila *et al.*[10] said the addition of 2,4-D and kinetin on basic MS medium gave better results of callus compared to using MS medium added with sucrose or phytagel. This is characterized by high frequency of callus induction, the callus produced is friable, beige color and grows intensively. Anita and Kumari[11] states auxiliary as IAA and IBA were not effective for callus induction in all explants tested but 2,4-D was very effective for inducing callus with sources of petiole, leaf, cotyledon and hypocotyl of *Rauvolfia tetraphylla* L., while cytokines singularly it cannot induce this plant's callus. This statement is in line with the statement of Chakraborty *et al.*[12] which resulted the maximum callus obtained on MS medium with addition of a combination of 2,4-D (0.5 mg L^{-1}) and kinetin 0.2 mg L^{-1}.

Based on the this research, the concentration of 2,4-D, BAP gave an effect on the time of callus formed. 2,4-D is a PGR that is most often used in callus culture because of its stable activity to stimulate cell multiplication, suppress organogenesis and maintain callus growth. This strong and optimal 2,4-D activity is caused by carboxyl groups separated by carbon and oxygen[11]. Each growth regulator has an influence on the induction of pineapple callus. Yifter *et al.*[13]

stated that MS media that supplemented with BAP 2 ppm and NAA 1 ppm in *Sesamum indicum* L. Hirhir variety was the best composition for accelerating time to grow this plant.

In this study, the addition of 2,4-D and BAP which is getting higher, causing the delayment of callus being formed. It appears that the optimum concentration for Sipahutar pineapple callus formation is 1 ppm 2,4-D with 1 ppm BAP. It can be understand that too high the concentration of auxin and cytokine PGR in cells, causing cells to keep on racing to make elongation and stretching. This activity takes place repeatedly without giving the cell a chance to do normal, so in the end it will cause no expression of the normal callus formation process. This study also in line with Tahir *et al.*[14] which explains that the addition of 2,4-D 3.5 mg L^{-1} gives the best effect in the formation of the callus sugarcane then the growth of callus decreases with the addition of 2,4-D above 3.5 mg L^{-1} and Mostafiz and Wagiran[15], the formation of rice callus shows better as the addition of concentration 2, 4-D but declining growth if exceeding 3 mg L^{-1}.

The dark treatment did not show a positive effect for accelerating formation of callus. As the latest study known that auxin works maximally on dark situations. Most likely there is another factor that affected the delayment of forming the callus in the dark treatment. Auxin works optimally in dark conditions and will be disturbed if there is light. From the results of this study there may be other factors that affect the formation of callus. Harahap[2] stated the ratio of auxin and cytokinin in the cell will determine the direction of induction in the tissue. If inside the cell, the auxin:cytokinin ratio is 1:1 so the tendency that occurs is callus formation. From this statement, the possibility is not only light factor which inhibits the formation of callus but there are other factors, in this case for example the balance of 2,4-D and BAP in the cell has not reached the desired ratio to form callus in the treated explants. Light in general is not giving strong effect for callus growth[2]. However, light affects the cell metabolism and effectiveness of PGR in the media. Light can damage auxin and can also cause the transfer of auxin in a direction away from light[16]. Tissue culture method in dark conditions is one of the way to make auxin effective in order to accelerate callus formation.

In vitro plant culture growth is not always hampered by the presence of light, whereas light is actually needed for optimal results. George and Sherrington[3] stated that in most cultures, cells will be able to do division in light conditions with the presence of external auxin in the media. In this study 2,4-D PGR was very effective for inducing callus of Sipahutar pineapple. As the literatures stated that IAA and IBA are not effective for inducing callus but 2,4-D is more effective for inducing callus with sources of petiole, leaf, cotyledonary leaf, hypocotyl explants[11].

The speed of growth that occurs in explants is due to the proper interaction between endogenous hormones explants and exogenous hormones given. This is reinforced by Urfiana[17] and Maciel *et al.*[5], stating that the interaction and balance of PGR given to the media and endogenously produced by plants determines the direction of development of a culture, Wahyuni *et al.*[18] say the interaction and the balance between each plant growth regulator which provided to the medium and produced by the plant cells indigenously determinated the direction of the culture development, this also in line with research from Chakraborty *et al.*[13] that stated BAP treatment alone was not all suitable for induction of callus.

The emergence of callus obstructed and also the emergence of the brown callus in treatment without 2,4-D and low dose either in single or combined with BAP, indicating that there is no addition of auxin PGR in the treatment of both light and dark treatments will inhibit callus growth and affect the color of the callus to brown, as well as the addition of sugar. Harahap and Solim[19] stated that the high content of sugar and carbohydrates in the medium can spur the occurrence of browning. The addition and increasement in the dose of 2,4-D and BAP both with light and dark treatment, will delay the change in callus color to brown.

Growth is characterized by one of which is increasing weight, so that measurements of callus biomass can represent variable callus growth originating from explants. According to Wahyuni *et al.*[18] said the fresh weight is an increase in the callus fresh weights is due to an increasing number of cells (cell division) and the increase in the cell size (cell enlargement). In conclusion the result of fresh weight is depend on the speed at which the cells divide, multiply themselves and continue with the enlargement of the callus. Through this study, it showed in order to induce callus maximally, besides 2,4-D, BAP was also needed so that the resulting callus biomass was maximal. This is in line with the statement from Harahap *et al.*[20] and Qosim[21] that BAP is needed to regulate cell division, which is characterized by increased production of number of leaves, number of segments and nodules of mangosteen callus.

2,4-D is a growth regulating agent in the auxin group which functions to boost callus induction and has ability to affect plant genetic stability. This is in accordance with the research results of the Dalila *et al.*[10], indicating that PGR 2,4-D

and kinetin with various combinations in MS Medium produced better callus compared to other basic media. Harahap[2], stated that 2,4-D is effective for forming callus because its strong activity spurred cell dedifferentiation processes, suppressing organogenesis. Tang *et al.*[22] obtained that the highest frequency of callus formation was acquired on MS medium with 0.5 mg L^{-1} BA and 3.0 mg L^{-1} 2, 4-D. The ratio between endogenous hormones explants and exogenous hormones given will determine the direction of the culture development and organ type formation[20].

Auxin affects the division, enlargement and elongation of cells. Auxin is usually applied to stimulate callus growth, cell suspension and organs and root initiation. While cytokines play a role in regulating cell division, tissue and organogenesis[23]. According to Dalila *et al.*[10], Harahap and Solim[19], that the addition of basic media without auxin as growth regulator substances or only given kinetin cannot induce callus growth. Overall showed that 2,4-D was essential for inducing callus, this is in line with the Romeida and Ganefianti[24] study, MS medium that supplemented with 1 mg L^{-1} 2,4-D produced the highest callus diameter, friable callus structure and transparent green callus and the addition of kinetin are very useful for increasing callus growth. Many researchers report that the size of the callus that being transferred to the regeneration medium also determines the success of regeneration. Callus measuring 1-2 mm is the best callus to be transferred to the regeneration medium, while callus measuring less than 1 mm will be difficult to regenerate or die[19,20]. Another study obtained, the addition of 2,-D with kinetin on MS media produced better callus than only by giving MS basic media[10].

Callus texture is one of the indicators used to assess the growth of a callus. Lizawati[25] obtained a yellowish-white, friable callus, which is characteristic of embryogenic callus, obtained from 2.5 ppm of 2,4-D treatment with the addition Tridiazuron (TDZ), this is in accordance with the results of this study. In addition, compact texture callus is a good producer of secondary metabolites. Compact callus texture is considered good because it can accumulate more secondary metabolites[26], the adding of 2,4-D around 0.25-1.00 mg L^{-1} was able to maintain the green color of explants, friable callus quality and transparent green color[23].

According to Harahap[2] friable callus is a callus that composed of long tubular cells where the structure of cells is tenuous, irregular and fragile. Dwi *et al.*[27] stated that the callus induced with cytokinin has a compact texture than the callus compared to callus that is not induced by cytokines. The compact callus texture is the effect of cytokinin and auxin which affect the water potential in cells. This causes the absorption of water from the medium into the cell to increase, so the cell becomes more rigid. 2,4-D concentrations of 1-2 ppm can produce friablely textured callus. This is in accordance with what was revealed by Dalila *et al.*[10], MS medium was added 1.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kinetin, produced friable callus textured and Khatak *et al.*[28] get result 2,4-D generate green friable callus and inclusion of BAP as a cytokinin, the days to callus induction decrease and Dharmayanti *et al.*[29] also gets the same results, namely giving a combination 2 ppm BA and 1-4 ppm 2,4-D can induce good callus formation and inhibits shoots and roots growth.

CONCLUSION

This study found that 2,4 D and BAP plant growth regulator is needed to induce callus on Sipahutar pineapple bulb. All explants can form callus, except explants without the addition of 2,4-D and BAP. The concentration of 2,4-D and BAP PGR of 1 ppm gave the best results for callus growth. Increased dose of 2,4-D and BAP causes the delayment of callus being formed. The dark treatment did not accelerated the formation of Sipahutar pineapple. This study will help the researchers to uncover the critical areas of auxin use (2,4-D) in dark and light treatments for callus induction, that many researchers were not able to explore. Thus a new theory on auxin ratio: cytokines in cells for callus induction may be arrived at.

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In Vitro Callus Induction of *Sipahutar* Pineapple (*Ananas comosus* L.) from North Sumatra Indonesia

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Running title: In Vitro Callus Induction of *Sipahutar* Pineapple

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Commented [WU1]: Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique,

Changed to

In Vitro Callus Induction of Sipahutar Pineapple (Ananas comosus L.) from North Sumatra Indonesia

Commented [WU2]: Fauziyah Harahap : The Research Team Coordinator
Sampling to the Field, Eksplan Planting, Observation
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Diky Setya Diningrat: Observation
Editing Report Draft, Preparation of Journal draft
Roedhy Poerwanto: Journal drafting
Journal editing and draft review
Nanda Eska Anugrah Nasution : Statistical data analysis
Search for literature, Journal drafting, Translate to english
Rifa Fadhilah Munifah Hasibuan: Statistical data analysis
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Abstract

Background and objectives: Sipahutar pineapple (*Ananas comosus* L.) is a indigeneus of pineapple grown in Sipahutar District, North Sumatra, Indonesia. Propagation of Sipahutar pineapple that being done traditionally is less effective, because the number of seeds that produced is very limited and requires a long time. Propagation through in vitro culture is an alternative solution to solve this problem. It is necessary to add Plant Growth Regulator (PGR) to stimulate callus formation in Sipahutar pineapple explants (*Ananas comosus* L.). Callus induction of pineapple from Sipahutar was carried out by PGR treatment on MS medium. The purpose of this study was to determine the effect MS medium treatment with added Dichlorophenoxy acetic acid (2,4-D) and Benzyl Amino Purin (BAP) PGR on Sipahutar pineapple callus formation (*Ananas comosus* L.) with light and dark treatment. **Materials and methods:** This callus induction research used a *Completely Randomized Design* (CRD) with 2 factors, the first factor was treatment 2,4-D (0, 1, 2) ppm. The second factor is BAP (0, 0.5, 1) ppm. Nine combinations of treatments are obtained. Each combination of treatments is treated in both light and dark conditions. The parameters of this study were the percentage (%) of explants that formed callus, the time of callus formed, callus texture, callus biomass, callus surface height and callus surface area. Data were analyzed with two-way Anava, followed by Duncan Multiple Rate Test (DMRT). **Conclusion:** The study showed that the interaction between 2,4-D and BAP significantly affected the time of callus formed, but 2,4-D and BAP did not significantly affect callus biomass, callus surface height, and callus surface area. All explants can form callus, except explants without the addition of 2,4-D and BAP. The callus formed on 10 Days After Induction (DAI) and 12 DAI with the treatment of light and dark. The color of the produced callus were white, yellowish white, greenish white, brown, brownish yellow, brownish white, brownish green, yellowish green, greenish white. The callus formed is generally compact textures, except for explants which by giving 1 ppm 2,4-D produce friable callus.

Keywords: 2,4-D, BAP, in vitro, pineapple, callus

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Introduction

Sipahutar pineapple is a indegeneus, local pineapple that is famous for its sweet taste, watery, large and yellow skin color[1]. This pineapple has long been cultivated, has prospects, has the potential to be developed. *Sipahutar* Pineapple provides good prospects, to help increase agricultural production, especially for food crop needs. Efforts to develop *Sipahutar* pineapple plant continue to be carried out, especially in the supply of seeds.

Usually farmers grow pineapple traditionally. At present, *Sipahutar* Pineapple has been planted like a pineapple plantation, but the supply of seeds has always been a big problem[1]. In order to acheieve large scale development, traditional propagation is not effective, because the number of seedlings produced is measly and takes long time. Propagation through tissue culture is an alternative technique for solving this problem[1,2,3], specifically using callus culture. Callus is a collection of amorphous cell masses which divide continuously, composed by parenchymal cells which bonds are very tenuous[2,3]. Callus culture aims to obtain callus from grown explants

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on a culture medium continuously, by in vitro technique, one of the methods to develop of reproducible of plantlets through callus because it was the most suitable material used for genetic transformation in plant[4], incuction of somatic embryogenesis[5]. Callus culture is important to do with various purposes including to study cell metabolism and differentiation, cell morphogenesis, somaclonal variation, genetic transformation, secondary metabolite production[6]. In this study, callus culture was carried out to obtain the best combination of media and to produce the best callus that could be regenerated, becoming a source of explants that would eventually be produced in large numbers of *Sipahutar* pineapple plantlets.

In terms of inducing callus, growing media are needed, generally using Murashige and Skoog (MS) media, PGR is combined with basic media. The most commonly used compounds for callus induction is 2,4-Dichlorophenoxyacetatic acid (2,4-D), Naphthalene Acetate Acid (NAA), Indol Acetate Acid (IAA), Indol Butyric Acid (IBA). Amin[7]states that there is an effect of pineapple callus growth of 75% by adding 2,4-D of PGR 2.0 mg/l, the combination between 2.4-D 2.0 mg/l and BAP 2.0 mg/l showed an effect of 95% callus growth.

This study aims to determine the effect of: (1) PGR 2,4-D, (2) PGR BAP, (3) Combination of PGR 2,4-D and BAP, (4) Dark and light treatment, on induction of *Sipahutar* pineapple callus (*Ananas comosus* L.).

Materials And Methods

This research was conducted at YAHDI Tissue Culture Laboratory, Perum Pelabuhan Jl. Lambung No. 16 Tanah 600 Medan Marelan, Medan, and Universitas Negeri Medan Biology Laboratory, for 8 months from March - October 2018. Tools that being used in this study were standard tissue culture tools. The material used in this study are: in vitro *Sipahutar* pineapple, Murashige and Skoog (MS) media, PGR 2,4-D, PGR BAP, alcohol, 0.1 N HCl, 0.1 N NaOH, sterile aquadest, detergent, chlorox.

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Sterilization and Making the Media:

All tools sterilized using an autoclave, at 121°C for 1 hour at a pressure of 17.5 psi. Everything is according to the amount listed in the composition of making 1 Liter MS media, all ingredients are mixed. 2,4-D and BAP were added according to the treatment.

Callus Induction

The plant material was used as of this study was 1 cm in vitro *Sipahutar* pineapple bulb. This study was carried out in *Completely Randomized Design* (CRD) with 9 treatment combinations. This study used MS basic media with added PGR, namely (2,4-Dichlorophenoxy acetatic acid (0, 1, 2 ppm) and Benzyl amino purine (0, 0.5 , 1 ppm), with 4

replications, therefore there are 36 experimental units. All combinations of treatment are placed in both dark and light treatment, hence experimental units are 72 bottles.

Callus induction was carried out in a laminar air flow cabinet (LAFC) using in vitro *Sipahutar* pineapple bulb. In vitro shoots are taken, placed on petridish, in vitro leaves are removed. Buds cut into 1 cm size for each treatment media according to the concentration that has been made.

Maintenance was carried out by placing bottles filled with explants on culture racks at a temperature range of 22^{O} C for 36 bottles of light treatment by application of flourescent light of 3000-3200 lux in a 16 h photoperiod, and 36 bottles closed using black cloth as a dark treatment. These samples were incubated, maintained at 24^{o}C by regulating the room air conditioner in the culture room.

Observation parameters:

The Percentage of Explans that Formed Callus

Explants forming callus were observed from the first day after induction to 35th day of observation. The percentage of explans that formed callus calculated by the formula: $\%\ explants\ form\ callus = \frac{Jumlah\ eksplan\ yang\ membentuk\ kalus}{Jumlah\ eksplan\ seluruhnya} \times 100\%$

The time of the Calus formation

The time of the calus formation, characterized by the emergence of irregular amorphous cells, were observed from the first day after induction to 35th day of observation.

Callus biomass

Callus biomass measurements in Sipahutar pineapple explants on light and dark treatments were carried out after 35 days after induction (DAI). The callus was removed from the culture bottle and weighed using a digital scale.

Callus color

The color of callus was observed after the formation of callus, 20th day and 35th day. Determination of callus color was based on Andaryani[8] with the researchers modifications, namely: brown (1), brownish yellow (2), brownish white (3), greenish white (4), brownish green (5), yellowish green (6), whitish green (7), green (8).

Callus Texture

Callus texture was observed 35 days after induction. Characterized by a compact and friable callus texture. Friable callus is marked by the form of callus that is easily separated. While the compact callus is marked by the callus that is not easily separated.

Callus height stack

Callus stack height was measured after 35 days after induction. Callus was removed from the culture bottle and measured using millimeter paper and a ruler.

Callus surface area.

The callus surface area was measured after 35 days after induction. Callus was removed from the culture bottle and measured using millimeter paper.

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Data analysis technique

This research uses factorial completely randomized design model and analysis with factorial ANOVA, the formula: $Yijk = \mu + \alpha i + \beta j + (\alpha\beta)\, ij + \varepsilon ijk$

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Description:
Yijk = observations on the k test, which received 2,4D treatment the-i and BAP treatment the -j
μ = middle value
αi = the effect of 2,4D concentration on the i level
βj = the effect of BAP concentration at the j level
(αβ) ij = the effect of the interaction of 2,4D treatment at the i-level and the BAP-j treatment
Eijk = the effect of the error with 2,4D treatment at the i level and BPA treatment at the j level at the k-replication
If the hypothesis testing obtained significantly different, then proceed with the Duncan Multiple Range Test (DMRT)

Result

Percentage of Explants Forming Callus

Both light and dark treatment, all explants (100%) formed callus. Only callus treated with MS media without the addition of PGR formed callus of 75%, the rest of the explants were able to form callus (Table 1). The highest percentage of explants forming callus came from the treatment of MS media with an additional 1 ppm 2,4 D and 0.5 ppm BAP. The treatment of 1 ppm 2,4-D and 1 ppm BAP was also able to induce rapid and good callus formation.

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Time of callus formed

From Table 1, it can be obtained that the combination treatment of MS medium with the addition of 1 ppm PGR 2,4-D and 1 ppm BAP was able to induce the fastest callus at 10 Days After Induction (DAI) in the light treatment and 12 DAI in the dark treatment.

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only the important main findings

With the same treatment of PGR 2,4-D, which is 1 ppm and addition of the lower concentration of BAP (0.5 ppm) causing delayement to form callus, that is on day 12 in the light treatment and day 14 in dark treatment. Increasing 2,4-D concentration to 2 ppm with combination of 0, 0.5 and 1 ppm BAP was not able to accelerate in forming callus, callus emergence was delayed to days 13 to 16 for light treatment and days 17 to 20 for dark treatment.

While the longest form of callus is without added of PGR, to be exact at 25 DAI in light treatment and 27 DAI in dark treatment. For more details, see Figure 1.

Callus Color

The treatment of 2,4-D and BAP PGR for light treatment resulted in a variety of callus colors (brownish white, greenish white and others , can be seen in Table 2). Observations at 20 days after induction, explants without additional and additional of low-dose 2,4-D single or combined with low-dose BAP (0, 0.5 ppm), both with light and dark treatment, did not show callus formation.

Explants without the addition of 2,4-D with the addition of BAP 1 ppm produced white callus in light treatment meanwhile dark treatment did not produce callus. Addition of 1 to 2 ppm 2,4-D produced variety of callus colors which varied from white, yellowish white, greenish white (Figure 2).

Callus Biomass

Based on the results of the analysis of variance, 2,4-D PGR addition had huge effect on callus biomass for light treatment and dark, but BAP in the light and dark treatment and the interaction of 2,4-D and BAP for light and dark treatment have no gave effect. The highest callus biomass was produced from the treatment of 2,4-D 1 ppm and BAP 1 ppm in the light and dark treatment that is 3.32 grams and 2.94 grams. While the lowest callus biomass was 1.67 grams (light treatment) and 1.46 grams (dark treatment) from the treatment of 2,4-D 0 ppm and BAP 0 ppm. For more details, see in Figure 3.

The Duncan's Multiple Range Test (DMRT) results showed that the average callus biomass was not different. It is seen that in both treatments (light and dark), the heaviest biomass is 3.32 grams (bright) and 2.94 grams (dark), the results of 2,4-D 1 ppm treatment and BAP 1 ppm. The lightest callus biomass is the result of 2,4-D 0 ppm and BAP 0 ppm, which is 1.67 grams from light and 1.46 from dark treatment (Figure 3).

Callus Texture

The formed callus texture is differentiated into callus with friable texture and compact texture callus (Figure 4). Friable callus is characterized by an easily separated callus texture, compact callus is in the form of a solid lump which is difficult to separate. Based on the observation of PGR 2,4-D and BAP treatment, it produced 2 types of callus texture, namely compact and friable (Figure 2). Light and dark treatment shows that the most dominant texture is compact callus texture. Friable texture callus is generally found in 2,4-D PGR treatment with a concentration of 1 ppm both in light and dark treatments. The results of observations carried

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out in this study indicate that 2,4-D added to the media has an effect on the appearance of callus texture (Table 1).

Height Stack of Callus

The treatment of 2,4-D 2 ppm and BAP 0 ppm produced the highest callus stack which was 1.7 cm. The lowest callus stack height is the result of 2,4-D 0 ppm treatment and 0 ppm BAP with a stack height of 1.28 cm. Dark treatment, 2,4-D 0 ppm and 0 ppm BAP produced the highest callus stack height of 1.7 cm. The lowest callus height was treatment of 2,4-D 0 ppm and BAP 0 ppm in the light treatment, with a stack height of 1.28 cm callus (Figure 5).

Results of analysis of variance, treatment of PGR 2,4-D; BAP; the interaction of 2,4-D and BAP on the height of the callus stack with light and dark treatment did not have effect.

Callus Surface Area

According to the results of analysis of variance analysis, the addition of 2,4-D affected the surface area of callus both in light and dark treatment (Table 3, 4). Meanwhile, the BAP treatment did not affect the surface area of callus in both light and dark treatment. The interaction 2,4-D and BAP did not affect callus biomass for light or dark treatments (Table 4). The 2,4-D 1 ppm and BAP 0.5 ppm treatment produced the highest callus surface area of 0.95 cm (light), and PGR 2,4-D ppm and BAP 0 ppm, resulting 0.98 cm (dark) callus surface area. While the lowest surface area of callus is 0.6 cm (light) and 0.68 cm (dark) the results of 2,4-D 2 ppm and BAP 0 ppm (Figure 6).

Discussion

The data shows that, explants are generally able to form callus, except explants that were not given 2.4 D and BAP. PGR is absolutely necessary for good callus formation, in this research was 2,4-D and BAP which will effect on increasing the percentage of explants that are able to form callus, callus appearance and acceleration of callus time formed. Callus formed in explants is formed due to the presence of openings on the tissue and response to hormones or growth regulators. The appearance of callus in the injured part is thought to be due to the stimulation of the tissue in the explants to cover the wound. One of the main characteristic of plant cells is having high plasticity for cell differentiation. Ikeuchi[9] said, plants produce unorganized cell masses, such as callus or tumors, in response to pressure, such as wounds or pathogenic infections.

Dalila[10] said the addition of 2,4-D and kinetin on basic MS medium gave better results of callus compared to using MS medium added with sucrose or phytagel. This is characterized by high frequency of callus induction, the callus produced is friable, beige color and grows intensively. Anita and Kumari[11] states auxiliary as IAA and IBA were not effective for callus

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induction in all explants tested, but 2,4-D was very effective for inducing callus with sources of petiole, leaf, cotyledon and hypocotyl of *Rauvolfia tetraphylla* L., while cytokines singularly it cannot induce this plant's callus. This statement is in line with the statement of Chakraborty et al[12] which resulted the maximum callus obtained on MS medium with addition of a combination of 2,4-D (0.5 mgl-1) and kinetin 0.2 mgl^{-1}.

Based on the this research, the concentration of 2,4-D, BAP gave an effect on the time of callus formed. 2,4-D is a PGR that is most often used in callus culture because of its stable activity to stimulate cell multiplication, suppress organogenesis and maintain callus growth. This strong and optimal 2,4-D activity is caused by carboxyl groups separated by carbon and oxygen[11]. Each growth regulator has an influence on the induction of pineapple callus. Yifter[13] stated that MS media that supplemented with BAP 2 ppm and NAA 1 ppm in *Sesamum indicum* L. Hirhir variety was the best composition for accelerating time to grow this plant.

In this study, the addition of 2,4-D and BAP which is getting higher, causing the delayement of callus being formed. It appears that the optimum concentration for Sipahutar pineapple callus formation is 1 ppm 2,4-D with 1 ppm BAP. It can be understand that too high the concentration of auxin and cytokin PGR in cells, causing cells to keep on racing to make elongation and stretching. This activity takes place repeatedly without giving the cell a chance to do normal, so in the end it will cause no expression of the normal callus formation process. This study also in line with Tahir et al[14] which explains that the addition of 2,4-D 3.5 mg/L gives the best effect in the formation of the callus Sugarcane then the growth of callus decreases with the addition of 2,4-D above 3.5 mg/L and Mostafiz and Wagiran[15] , the formation of rice callus shows better as the addition of concentration 2, 4-D but declining growth if exceeding 3 mg/L.

The dark treatment did not show a positive effect for accelerating formation of callus. As the latest study known that auxin works maximally on dark situations. Most likely there is another factor that affected the delayment of forming the calus in the dark treatment. Auxin works optimally in dark conditions and will be disturbed if there is light. From the results of this study there may be other factors that affect the formation of callus. Harahap[2] stated the ratio of auxin and cytokinin in the cell will determine the direction of induction in the tissue. If inside the cell, the auxin:cytokinin ratio is 1:1 so the tendency that occurs is callus formation. From this statement, the possibility is not only light factor which inhibits the formation of callus, but there are other factors, in this case for example the balance of 2,4-D and BAP in the cell has not reached the desired ratio to form callus in the treated explants. Light in general is not giving strong effect for callus growth[2]. However, light affects the cell metabolism and

effectiveness of PGR in the media. Light can damage auxin and can also cause the transfer of auxin in a direction away from light[16]. Tissue culture method in dark conditions is one of the way to make auxin effective in order to accelerate callus formation.

In vitro plant culture growth is not always hampered by the presence of light, whereas light is actually needed for optimal results. George and Sherrington[3] stated that in most cultures, cells will be able to do division in light conditions with the presence of external auxin in the media. In this study 2,4-D PGR was very effective for inducing callus of Sipahutar pineapple. As the literatures stated that IAA and IBA are not effective for inducing callus, but 2,4-D is more effective for inducing callus with sources of petiole, leaf, cotylodenary leaf, hypocotyl explants[11].

The speed of growth that occurs in explants is due to the proper interaction between endogenous hormones explants and exogenous hormones given. This is reinforced by Urfiana[17], and Maciel et al [5], stating that the interaction and balance of PGR given to the media and endogenously produced by plants determines the direction of development of a culture, Wahyuni et al[18] say the interaction and the balance between each plant growth regulator which provided to the medium and produced by the plant cells indegenously determinated the direction of the culture development, this also in line with research from Chakraborty et al[13] that stated BAP treatment alone was not all suitable for induction of callus.

The emergence of callus obstructed and also the emergence of the brown callus in treatment without 2,4-D and low dose either in single or combined with BAP, indicating that there is no addition of auxin PGR in the treatment of both light and dark treatments will inhibit callus growth and affect the color of the callus to brown, as well as the addition of sugar. Harahap and Solim[19] stated that the high content of sugar and carbohydrates in the medium can spur the occurrence of browning. The addition and increasement in the dose of 2,4-D and BAP both with light and dark treatment, will delay the change in callus color to brown.

Growth is characterized by one of which is increasing weight, so that measurements of callus biomass can represent variable callus growth originating from explants. According to Wahyuni et al[18] said the fresh weight is an increase in the callus fresh weights is due to an increasing number of cells (cell devision) and the increase in the cell size (cell enlargement). In conclusion the result of fresh weight is depend on the speed at which the cells divide, multiply themselves and continue with the enlargement of the callus. Through this study, it showed in order to induce callus maximally, besides 2,4-D, BAP was also needed so that the resulting callus biomass was maximal. This is in line with the statement from Harahap et al.[20]

and Qosim[21] that BAP is needed to regulate cell division, which is characterized by increased production of number of leaves, number of segments and nodules of mangosteen callus.

2,4-D is a growth regulating agent in the auxin group which functions to boost callus induction and has ability to affect plant genetic stability. This is in accordance with the research results of the Dalila et al.[10] , indicating that PGR 2,4-D and kinetin with various combinations in MS Medium produced better callus compared to other basic media. Harahap[2], stated that 2,4-D is effective for forming callus because its strong activity spurred cell dediferentiation processes, suppressing organogenesis. Tang et al[22] obtained that the highest frequency of callus formation was acquired on MS medium with 0.5 mg/l BA and 3.0 mg/l 2, 4-D. The ratio between endogenous hormones explants and exogenous hormones given will determine the direction of the culture development and organ type formation[20].

Auxin affects the division, enlargement and elongation of cells. Auxin is usually applied to stimulate callus growth, cell suspension and organs and root initiation. While cytokines play a role in regulating cell division, tissue and organogenesis[23]. According to Dalila et al.[10], Harahap and Solim[19], that the addition of basic media without auxin as growth regulator substances or only given kinetin cannot induce callus growth. Overall showed that 2,4-D was essential for inducing callus, this is in line with the Romeida et al[24] study, MS medium that supplemented with 1 mg/l 2,4-D produced the highest callus diameter, friable callus structure and transparent green callus and the addition of kinetin are very useful for increasing callus growth. Many researchers report that the size of the callus that being transferred to the regeneration medium also determines the success of regeneration. Callus measuring 1-2 mm is the best callus to be transferred to the regeneration medium, while callus measuring less than 1 mm will be difficult to regenerate or die[19,20]. Another study obtained, the addition of 2,-D with kinetin on MS media produced better callus than only by giving MS basic media[10]

Callus texture is one of the indicators used to assess the growth of a callus. Lizawati[25] obtained a yellowish-white, friable callus, which is characteristic of embryogenic callus, obtained from 2.5 ppm of 2,4-D treatment with the addition Tridiazuron (TDZ), this is in accordance with the results of this study. In addition, compact texture callus is a good producer of secondary metabolites. Compact callus texture is considered good because it can accumulate more secondary metabolites[26], the adding of 2,4-D around 0.25 to 1.00 mg L^{-1} was able to maintain the green color of explants, friable callus quality and transparent green color[23].

According to Harahap[2] friable callus is a callus that composed of long tubular cells where the structure of cells is tenuous, irregular and fragile. Dwi[27] stated that the callus induced with cytokinin has a compact texture than the callus compared to callus that is not induced by

cytokines. The compact callus texture is the effect of cytokinin and auxin which affect the water potential in cells. This causes the absorption of water from the medium into the cell to increase, so the cell becomes more rigid. 2,4-D concentrations of 1 ppm to 2 ppm can produce friablely textured callus. This is in accordance with what was revealed by Dalila et al[10], MS medium was added 1.5 mg/l 2,4-D and 0.5 mg/l Kinetin, produced friable callus textured and, Khatak et al[28] get result 2,4-D generate green friable callus and inclusion of BAP as a cytokinin, the days to callus induction decrease and Dharmayanti et al[29] also gets the same results, namely giving a combination 2 ppm BA and 1-4 ppm 2,4-D can induce good callus formation and inhibits shoots and roots growth.

Conclusion

This study found that 2,4 D and BAP plant growth regulator is needed to induce callus on *Sipahutar* pineapple bulb. All explants can form callus, except explants without the addition of 2,4-D and BAP. The concentration of 2,4-D and BAP PGR of 1 ppm gave the best results for callus growth. Increased dose of 2,4-D and BAP causes the delayement of callus being formed. The dark treatment did not accelerated the formation of *Sipahutar* pineapple. This study will help the researchers to uncover the critical areas of auxin use (2,4-D) in dark and light treatments for callus induction, that many researchers were not able to explore. Thus a new theory on auxin ratio: cytokines in cells for callus induction may be arrived at.

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Acknowledgement

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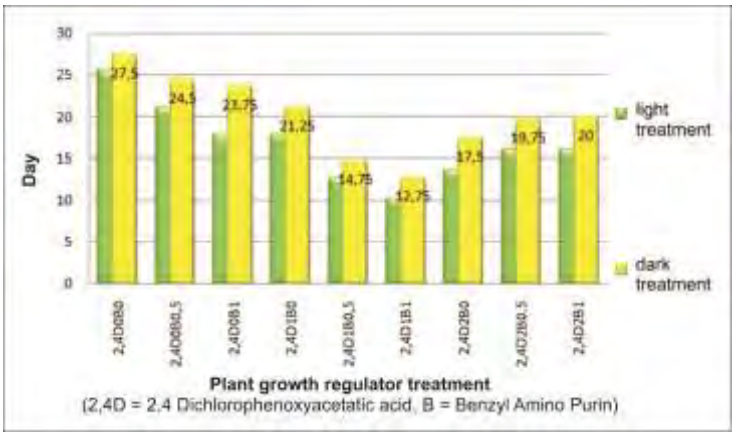

Figure 1. Average time of callus formed in dark and light treatment

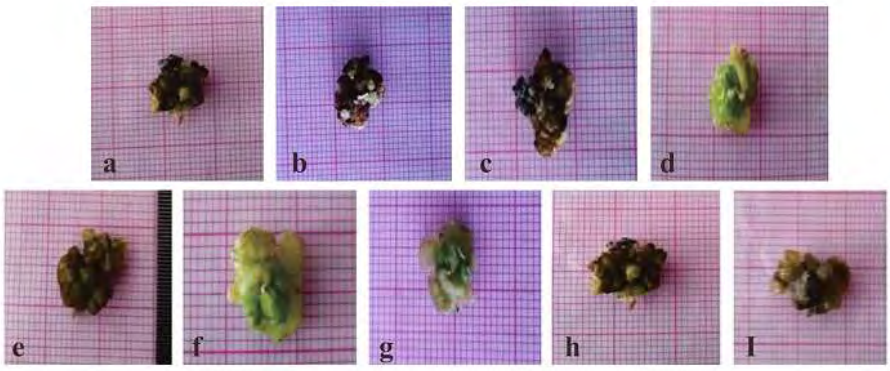

Figure 2. Performance of callus at 35 DAI in a row starting from treatment (a) 2,4-D 0 ppm and BAP 0 ppm, (b) 2,4-D 0 ppm and BAP 0.5 ppm (c) 2,4-D 0 ppm and BAP 1 ppm, (d) 2,4-D 1 ppm and BAP 0 ppm, (e) 2,4-D 1 ppm and BAP 0.5 ppm, (f) 2,4-D 1 ppm and BAP 1 ppm, (g) 2,4-D 2 ppm and BAP 0 ppm, (h) 2,4-D 2 ppm and BAP 0.5 ppm, (i) 2,4-D 2 ppm and BAP 1 ppm

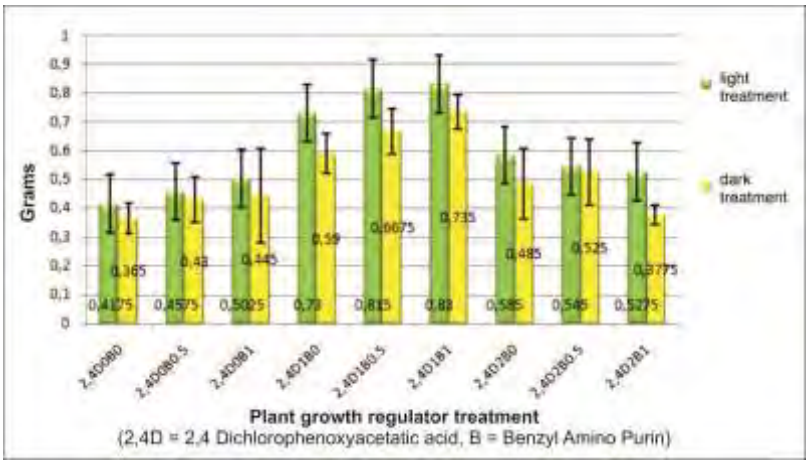

Figure 3. Average callus biomass (grams) for light and dark treatment

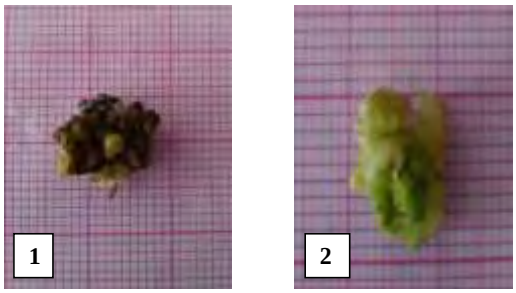

Figure 4. Color and texture of callus: 1. Brown (2,4-D 0 ppm + BAP 1 ppm, 2) Yellowish green (2,4-D 1 ppm + BAP 1 ppm) and compact callus texture.

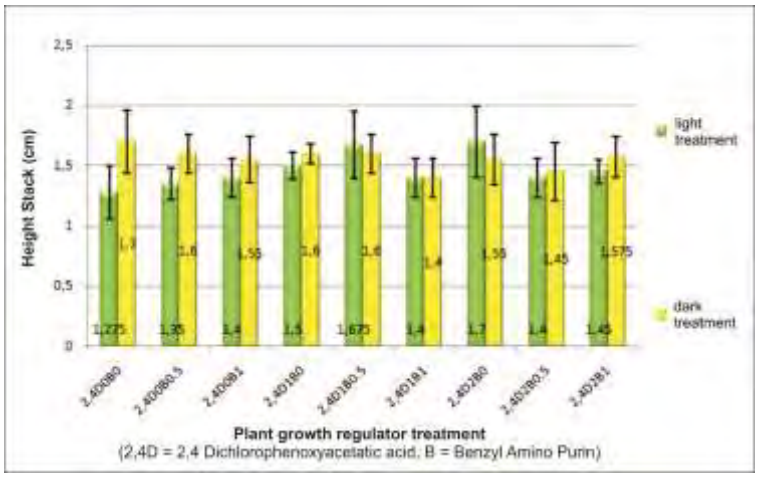

Figure 5. Average height stack (cm) of callus for light and dark treatment

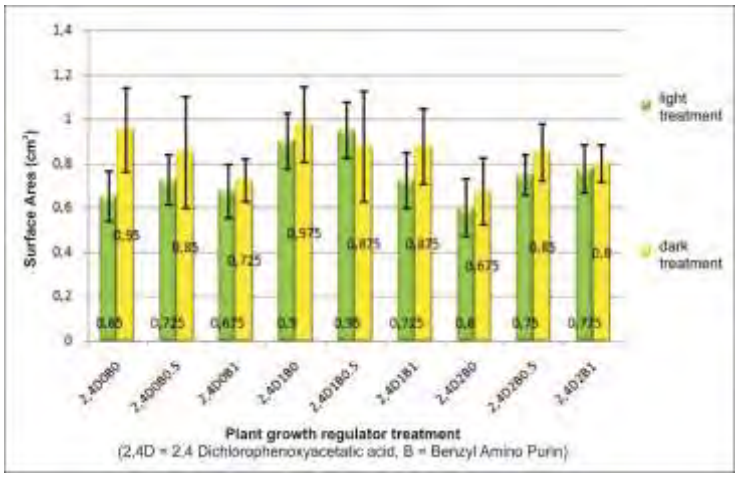

Figure 6. Average callus surface area (cm^2) in dark and light treatment

Table 1. Percentage of explants forming callus and time of callus formation for light and dark treatment

PGR Treatment		Explant forming callus (%)		Callus Texture	
2,4-D (ppm)	BAP (ppm)	Light Treatment	Dark Treatment	Light Treatment	Dark Treatment
0	0	75	75	Compact	Compact
0	0.5	100	100	Compact	Compact
0	1	100	100	Compact	Friable
1	0	100	100	Compact	Friable
1	0.5	100	100	Friable	Friable
1	1	100	100	Friable	Friable
2	0	100	100	Compact	Compact
2	0.5	100	100	Friable	Compact
2	1	100	100	Compact	Compact

Table 2. Color of callus age 20 and 35 days after induction for light and dark treatment

PGR		Color of Callus 20 DAI		Color of Callus 35 DAI	
2,4-D (ppm)	BAP (ppm)	Light Treatment	Dark Treatment	Light Treatment	Dark Treatment
0	0	Callus hasn't appeared yet	Callus hasn't appeared yet	Brown (1)	Brown (1)
0	0.5	Callus hasn't appeared yet	Callus hasn't appeared yet	Brownish yellow (2)	Brownish white (3)
0	1	Brownish white (3)	Callus hasn't appeared yet	Brown (1)	Brownish white (3)
1	0	Greenish white (7)	Callus hasn't appeared yet	Brownish green (5)	Brownish green (5)
1	0.5	Greenish white (7)	Greenish white (7)	Brownish green (5)	Brownish green (5)
1	1	Greenish white (7)	Greenish white (7)	Yellowish green (6)	Yellowish green (6)
2	0	Yellowish white (3)	Yellowish white (3)	Brown (1)	Brown (1)
2	0.5	Yellowish white	Yellowish white	Brown	Brownish yellow
2	1	Brownish white	Yellowish white	Brown	Greenish white

Commented [WU26]: data presentation has been improved, data repetition has been removed

Table 3. Analysis of variance (ANAVA) The Effect of interaction between 2,4-D and BAP on the callus surface area of 35 DAI at light treatment

Main effect	Degree of freedom	Sum Squared	Middle Squared	F count	F table 0,05	F table 0,01
			Main effect			
2,4D Treatment	2	0,22	0,11	4,23*	3,35	5,49
BAP Treatment	2	0,06	0,03	1,15^{tn}	3,35	5,49
			Interaction of 2 factors			
2,4D,BAP	4	0,13	0,032	1,23^{tn}	2,73	4,11
Error	27	0,70	0,026			
Total	35					

Note: 2,4 D Treatment is significant, while for BAP Treatment and interaction betweeen 2,4 D and BAP is not significant, tn: not significantly different, **: differs very real,*: significantly different

Table 4. Analysis of variance (ANAVA) Effect of interaction between 2,4-D and BAP towards the surface area of callus of 35 DAI at dark treatment

Variants Effect	Degree of freedom	Sum Squared	Middle Squared	F count	F table 0,05	F table 0,01
			Main effect			
2,4-D Treatment	2	0,11	0,06	3,55*	3,35	5,49
BAP Treatment	2	0,03	0,015	0,97^{tn}	3,35	5,49
			Interaction of 2 factors			
2,4-D,BAP	4	0,16	0,04	1,29^{tn}	2,73	4,11
Error	27	0,83	0,031			
Total	35					

Note: 2,4 D Treatment is significant, while for BAP Treatment and interaction betweeen 2,4 D and BAP is not significant, tn: not significantly different, **: differs very real, *: significantly different

97358-PJBS-ANSI Research Article

Final Decision: Reconsider for Evaluation after Modifications and Clarifications

Reference your article entitled "Callus Induction of Pineapple (Ananas comosus L.) from Sipahutar North Sumatra Indonesia through In Vitro Technique" submitted for publication to Pakistan Journal of Biological Sciences.

Author is suggested to incorporate the following recommended modifications in paper and resubmit for further evaluation. My decision is based on the following reason(s):

MAJOR comments in support of the decision

Main Title and Running Title:

1. The main title of the article captures the reader attention .The title of current study is not so attractive. It must be more specific and attractive (but within 17 words) so rewrite it.

   In Vitro Callus Induction of Sipahutar Pineapple (Ananas comosus L.) from North Sumatra Indonesia

2. Besides main title, short title/ running title is also necessary to fulfill journal's criteria. So provide a running title of 7-9 words and it must be the punch line of main title.

   In Vitro Callus Induction of *Sipahutar* Pineapple

Author information:

1. Do provide the active contact number of corresponding author in manuscript.

   +6281376817918

2. Provide the contribution of each author in the working and processing of this research work under thea separate heading of "Authors contribution" either at the start or end of the manuscript.

No	Nama	Contribution
1	Fauziyah Harahap	The Research Team Coordinator Sampling to the Field Eksplan Planting, Observation Report Drafting, Journal Preparation and editing.
2	Diky Setya Diningrat	Observation Editing Report Draft, Preparation of Journal draft

3	Roedhy Poerwanto	Journal drafting Journal editing and draft review
4	Nanda Eska Anugrah Nasution	Statistical data analysis Search for literature Journal drafting Translate to english
5	Rifa Fadhilah Munifah Hasibuan	Statistical data analysis Search for literature Translate to english

Abstract:

1. Abstract of the manuscript should be structured into separate sections according to the journals criteria background and objective (context and purpose of the study); materials and methods (how the study was performed and which statistical analysis was being used); Results (the main findings); Conclusion (core outcomes of the study).

Already equipped, all components are already in the abstract. Background and objectives, Materials and methods, Conclusion

Introduction: without

1. "Introduction" is inappropriate and not properly set a foundation to understand the research problem so rewrite it only within 1 and half page as:

- First of all present the background studies about the topic in a manner that set a foundation to understand the research problem with proper reference citations.
- Provide the rationale behind the study and main objective of this work (in 3-4 lines) in its last portion reference citation.
- The introduction has been improved, according to the input provided

Materials and Methods:

1. "Materials and Methods" is inappropriate even author has not mentioned that how the parameters were calculated. Following amendments are required in it:

   Remove all extra and unnecessary information from it. And rewrite it with proper subheadings.

Mention the location and total time duration of research work with specific months and year in the start of "Materials and Methods".

- ✓ Please clearly state that which parameters were calculated in this study and provide the complete adopted methodology of the study (i.e. how the parameters were calculated?) in a very concise form and also provide reference source of that adopted methodology (if it is extracted from somewhere else).
- ✓ Please clearly mention that which statistical analysis (mean±SD, ANOVA or Regression etc) was being used in this study.

✓ Already written down :
✓ location and total time duration of research work with specific months and year
✓ how parameters are observed and calculated are written
✓ Observation parameters: The Percentage of Explans that Formed Callus
✓ which parameters were calculated in this study
✓ statistical analysis used are written

✓

Results:

1. "Results" of the study are lengthy and inappropriate. Rewrite it with following amendments:
   - ➢ Rewrite the "Results" in summarize form with proper subheadings and remove all extra general and unnecessary information from it.
   - ➢ Cite all tables and figures in their respective descriptions in ascending order.
   - ➢ Rewrite the theoretical descriptions of all tables and figures in a concise form and make them completely synchronized with respective tables and figures.
   - ➢ Don't repeat the all information of tables and figures in their theoretical description; provide only the important main findings of the study and avoid over explanation.
   - ➢ Do provide only the direct information (which is easily predictable in tables and figures) and remove all indirect information (which is not easily understandable from tables and figures) because indirect values may cause confusion to the reader.

- all extra general and unnecessary information has been removed, only the important main findings
- results have been summarized, removing unnecessary information, only the important main findings
- contents have been synchronized with images and tables
    - results have been summarized, removing unnecessary information, only the important main findings

- tables and figures have been cited
- tables and figures have been cited, contents have been synchronized with images and tables
- results have been summarized, removing unnecessary information

2. We are confused about figures. Are these figures showing data repetition of tables? If yes then please remove all these figures which are showing data repetition because data repetition is not allowed.

tables and figures have been cited, information already in the picture is not repeated in the table, vice versa

Discussion:

1. "Discussion" is poorly written. Rewrite it only within 2 pages with following suggestions:

- In the start discuss your own results in 2 -3 lines only.
- Correlation of your results with previous literature is essential. So co-relate this study with at least 7 recent previous publications either in support or in contradiction for justification of results.
- Add 2-3 lines about future recommendation or implications of research in last portion.

- The discussion has been rewritten,
- has been fixed according to instructions.
- Already tried to abbreviate the review.
- but because of the many parameters observed, researchers find it more difficult to shorten them

Grammar:

1. There are grammatical and spelling mistakes. Avoid using personal pronounces and the use of “we” and “our” in the article. Use third person pronoun and past tense in the write up of the article. Reread this article for typographical, grammar and spelling errors.

   Grammer has been improved.
   personal pronounces and not use of “we” and “our” in this article .

Significance Statement:

1. The “Significance Statement” is missing. Provide this Statement either at the start or end of the manuscript under separate heading of significance statement. **State this statement as follows:** “This study discovered the ---- that can be beneficial for ---- and **last sentence of this statement could be as:** this study will help the researchers to uncover the critical areas of ---- that many researchers were not able to explore. Thus a new theory on ----- may be arrived at.

   Significance Statement: already revised

References:

1. Author is requested to exclude the old dated back references because such references are only acceptable in “Materials and Methods” in the determination of parameter.

2. According to the Journal’s format provide the DOI or URL of each reference in the reference list. Moreover the formatting of some references in reference list is not according to the journal format. Please visit the link below and set the reference list accordingly.

   unneeded literature has been discarded

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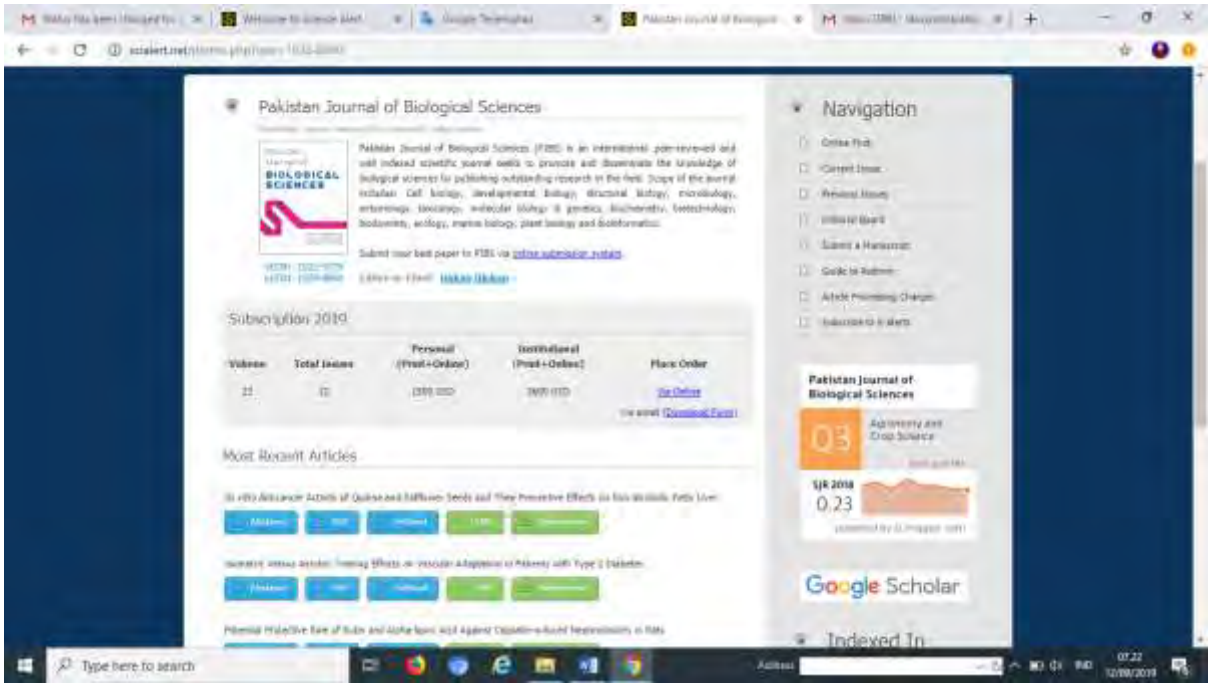
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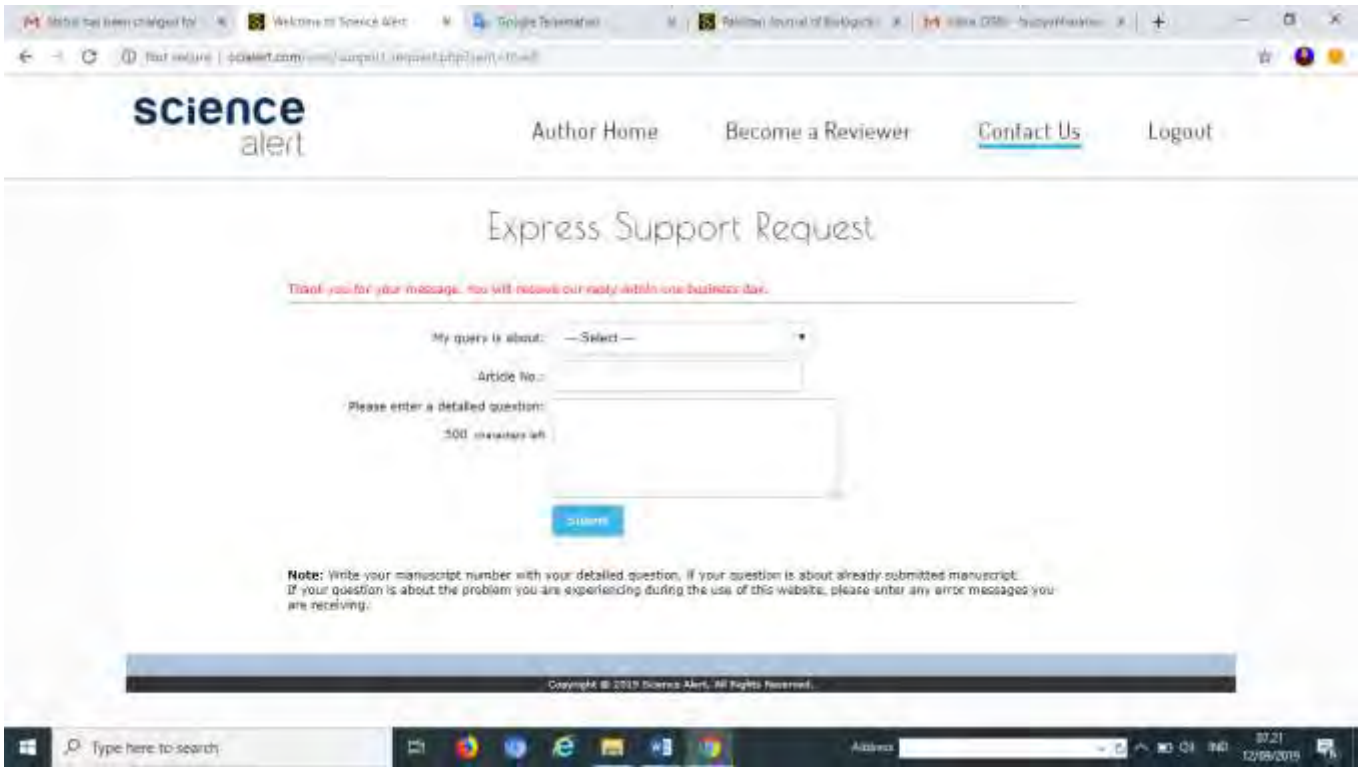
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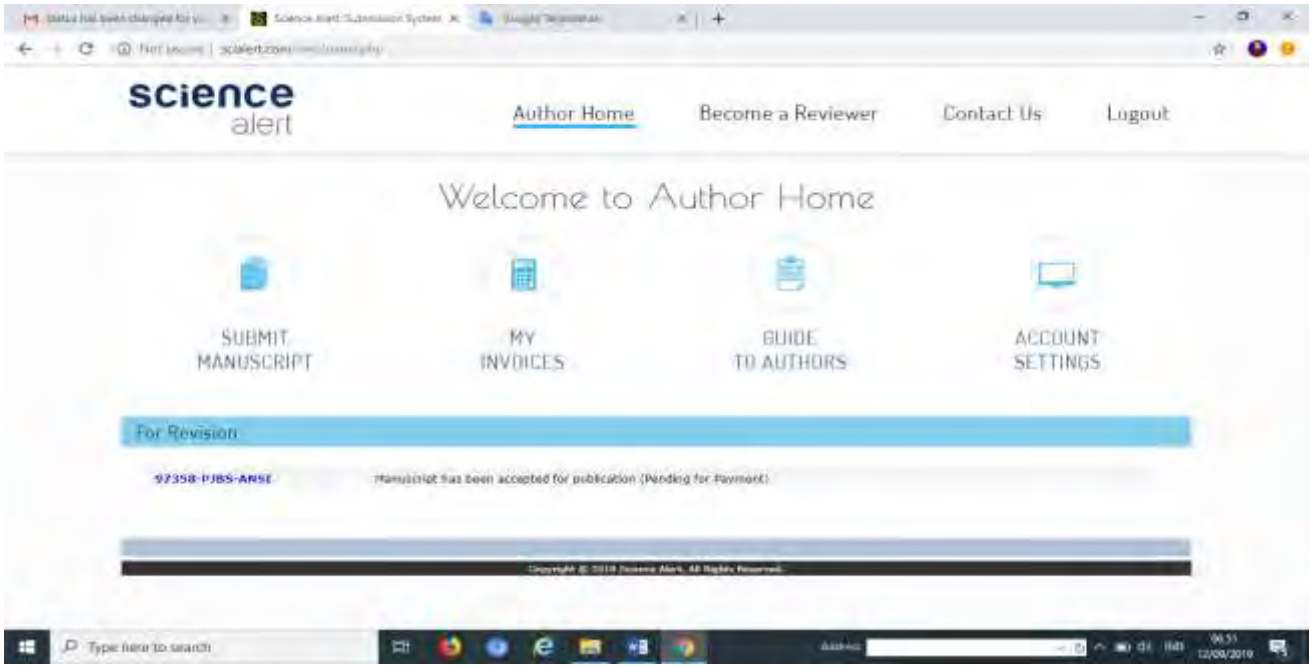
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