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Culture initiation of *Avicennia marina* from Indonesia using two different culture media

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ABSTRACT

The grey mangrove population faces overexploitation, pollution, and global warming. Traditional propagation is challenging due to the recalcitrant seeds. The issues with *Avicennia marina* tissue culture are the browning of the explants and endophytes, which can affect the explants at any growing phase. This study aimed to produce and maintain A. *marina* explants until they developed into healthy plantlets. MS medium and WPM were used along with 0.4% PPM. Improving the survival, 400, 450, and 450 ppm PVP was also applied. The treatment using MS medium and WPM were compared, and the media enriched with 0.5 ppm BA and 1 ppm kinetin and no cytokinins were compared. The result showed that WPM+400 ppm PVP was better than MS medium (in any kind of treatment) in inducing shoots in A. *marina* (27%), as well as longer survival in *in vitro* conditions. In the MS media, all explants died after week 11, whereas in the WPM medium, three explants survived until week 16. In addition, most of the explants cultured in WPM exhibited no contamination until week 8, whereas in all treatments using MS media, there was contamination in the same week observed.

KEYWORDS: Murashige & Skoog (MS), Plant Preservative Mixture (PPM), Polyvinylpyrrolidone (PVP), Woody Plant Medium (WPM)

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INTRODUCTION

Grey mangrove (*Avicennia marina*) is a type of mangrove that can be found throughout Southeast Asia, including Indonesia (Kusmana, 2014; Flora & Fauna Web, 2022). A. *marina* is a type of mangrove that lives in the further reaches of the coastline. This mangrove is also among the most common in tropical coastal wetlands (Budiadi *et al.*, 2022). The pneumatophores of this mangrove form a massive aboveground aerial root complex (Al-Khayat & Alatalo, 2021) which aids in preventing abrasion (Herison *et al.*, 2023). Furthermore, mangrove pneumatophores help to keep the coastline environment healthy (Hao *et al.*, 2015). For example, they provide food and refuge for large herbivorous animals (Hao *et al.*, 2021) which benefits the entire food chain. This helps to sustain the great biodiversity.

Overexploitation, pollution, (Triest *et al.*, 2021) and global warming (Martínez-Díaz & Reef, 2023) have all contributed to the decline in the population of A. *marina* over the last few decades. A. *marina* lumber is a highly valuable supply of furniture and other human necessities (Triest *et al.*, 2021), while its fruits are frequently used as sources of food and medicine (Yang *et al.*, 2018). The increase in pollutants in the estuary harmed A. *marina* survival because they can degrade soil quality (Münzel *et al.*, 2023). Global warming has caused significant damage to the A. *marina* population because it rapidly alters the climate and temperature (Martínez-Díaz & Reef, 2023), making A. *marina* unsuitable for such an environment.

Because of the recalcitrant seeds (Purnobasuki & Utami, 2017), A. *marina* is difficult to propagate. For example, high-quality seeds thrive exclusively in arid climates; in humid climates, they are more prone to deterioration. This is because the entire seed is made of soft, fleshy tissue (Everett, 1994). One way to quickly grow plants is through tissue culture. On the other hand, knowledge regarding A. *marina* propagation in vitro is scarce. Most recent studies published in Al-Bahrany and Al-Khayri (2003) and Alatar *et al.* (2015) claimed that the high proportion of browning is one of the challenges in the nodes culture of A. *marina*. Our earlier research revealed that there was an additional endophyte-related issue in addition to browning. Microbes are known as endophytes residing inside

plants (Gouda *et al.*, 2016). After being cultivated *in vitro*, the endophytes emerge from the plant and contaminate plant tissue culture.

Browning in explants can be addressed in a few different ways. It is a common procedure to utilize activated charcoal (Sipayung *et al.*, 2018). In plant tissue culture, other antioxidants such as ascorbic acid (AA) (Ndakidemi *et al.*, 2014) and polyvinylpyrrolidone (PVP) (Taghizadeh & Dastjerdi, 2021) are also utilized. Activated charcoal was less successful in reducing the browning percentage in A. *marina* in vitro culture, according to our earlier research. Therefore, PVP was employed in this investigation to regulate browning.

In plant tissue culture, endophytic bacteria and fungi are a frequent source of issues. Certain plants, primarily woody ones, produce more endophytes than herbaceous ones (Ganley *et al.*, 2004). It is essential to remove endophytes from plant tissue culture to guarantee the explants' survival. Among the woody plants, A. *marina* demonstrates the same trait. Plant preservative mixture (PPM) was therefore also utilized in this investigation. Producing healthy A. *marina* plantlets for subsequent micropropagation was the primary objective of this study.

MATERIALS AND METHODS

Explants Preparation

The nodes obtained from six-month-old *Avicennia marina* seedlings from Wonorejo Mangrove Ecotourism in Surabaya, Indonesia (Figure 1). Node explants that were between 0.5 and 1.5 cm in size (one node explant) were used. After that, the explants were immersed for two hours in fungicide and bactericide (Nordox™), which has 50% copper (Cu) as the active ingredient. The explants were removed after two hours and given a ten-minute rinse under running water.

After moving to laminar airflow, the prepared explants were twice rinsed with sterile distilled water. Following a 30-second 70% ethanol wash, the explants were promptly rinsed three times with sterile distilled water. Subsequently, the explants were shaken for 20 minutes while immersed in a 25% NaOCl+Tween 20 solution (3 drops/100 mL). The explants were once again washed with sterile distilled water after completion. Following a two-minute shaking period, the explants were once more immersed in a 0.1% $HgCl_2$ solution. The explants were promptly washed three times with sterile distilled water after two minutes. The explants were then immersed for an hour in 1,500 mg/L of citric acid and 100 mg/L of ascorbic acid, respectively. The

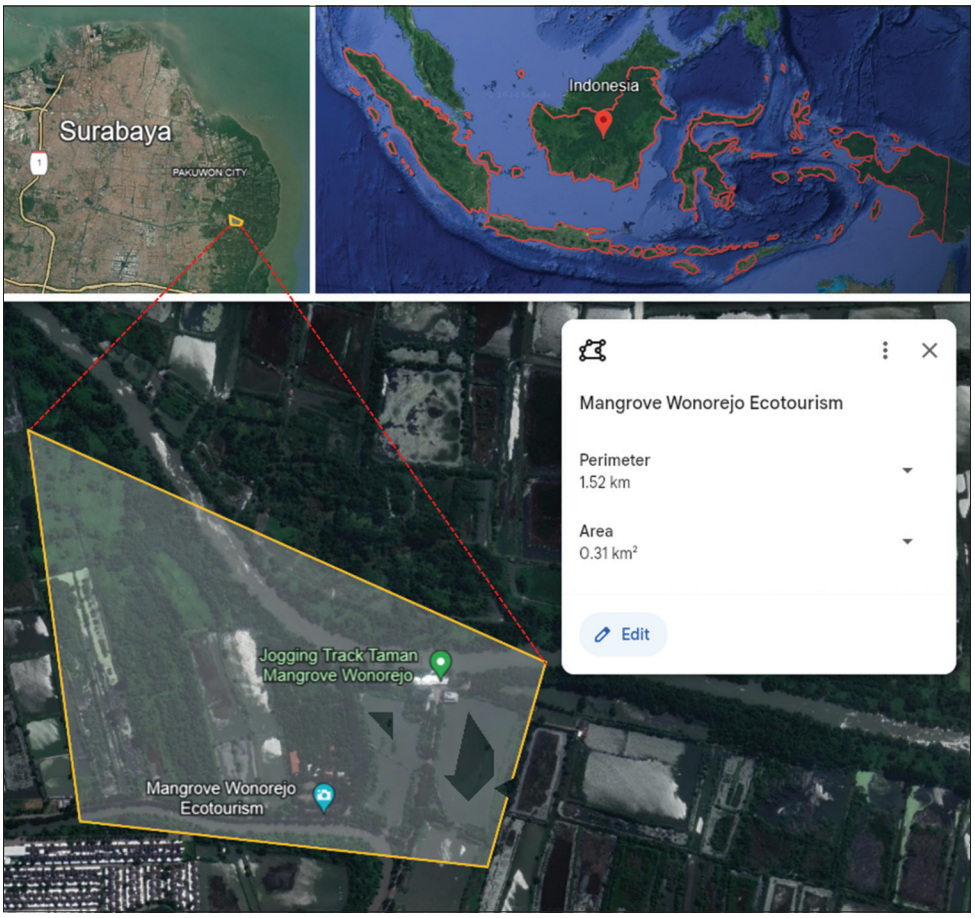

Figure 1: Original location of sample mangrove plants *A. marina* at the Mangrove Wonorejo Ecotourism, Surabaya, East Java, Indonesia (Source: Google Earth, 2024)

purpose of this soaking is to lessen the quantity of phenolic that builds up as a result of the sterilizing procedure. After finishing, the explants are removed and immersed in sterile distilled water to carry out the planting procedure.

Media Preparation

Instant MS basal medium with vitamins (M519, Phytotech Labs™) and Woody Plant Media (WPM) (Lloyd & McCown Woody Plant Basal Medium with Vitamins, L449, Phytotech Labs™) were used in this investigation. One litre of MS media required 4.43 g of instant MS media and 20 g of sucrose. Every liter of WPM required 0.53 g of WPM powder and 20 g of sugar. To reduce the endophyte growth, 0.4% plant preservative mixture (PPM™, Plant Cell Technology) was applied to each treatment. We are interested in learning how different PVP concentrations - 400, 450, and 500 ppm - affect the reduction of browning levels and bud induction. Additionally, three different types of media with the addition of 0.5 ppm BA and 1 ppm kinetin (AL-Bahrany & Al-Khayri, 2003) at the same PVP dosage were made. Following the mixing of all the ingredients, the pH was adjusted to 5.8. Solidifying the media, 8 g of agar was added to 1 L media solution. Subsequently, the media was brought to a boil and transferred into culture bottles. After that, the medium was autoclaved for 20 minutes to sterilize it.

Reassuring endophyte content inside the cultured explants, Plate Count Agar (PCA, Merck™) was made by suspending 17.5 g powder in 1 L of distilled water. The solution was brought to boil with stirring and then autoclaved for 15 minutes. After the sterilization, the PCA solution was left to warm. The warm solution was poured aseptically into sterile Petri dishes.

Culturing and Incubator Condition

Each node was aseptically cultured in a culture container, and there were 13-16 samples per treatment. Some 8 weeks old explants that survive but not showing any sign of shoot, were cut longitudinally. The cut explants were cultured with the cut parts placed facing the media.

The incubator room's settings included 23 to 25 °C, 60% relative humidity, 1000-1300 lux of light intensity, and 16 hours of light and 8 hours of darkness photoperiod. There was an eight-week incubation period.

Data Collection

Data were collected after 8 weeks of incubation. Data collection included browning and contamination emergence, shoot production, also dead explants and survival rate.

RESULTS AND DISCUSSION

Role of PVP in *A. marina in vitro* Growth and Reducing Browning

The addition of 400 ppm PVP (Table 1) is most likely to support shoot initiation in the MS medium and WPM by percentages of 8% and 27%, respectively. This finding is also shown on Figure 2 as a comparison between WPM+400 ppm PVP and MS+400 ppm PVP+Cytokinins. Chen *et al.* (2022) confirmed that the addition of PVP on the media increases the germination rate of peonies. According to some published research, browning of 'Hongyang' kiwi fruit (Chai *et al.*, 2018), *Saccharum officinarum* (Shimelis *et al.*, 2015), and *Paeonia lactiflora* Pall (Chen *et al.*, 2022) can be reduced when PVP concentrations were applied to up to 500 ppm. The use of 1000 ppm PVP in reducing browning in the callus culture of mangroves *Heritiera fomes* and *Bruguiera gymnorhiza* was reported in 2015 (Kader *et al.*, 2015). This claim has been confirmed by experiment results showing that browning can be decreased in conditions with increased PVP concentrations (Table 1). However, because endophytic microorganisms directly interact with explant metabolism, the goal of employing PVP to minimize the oxidation reaction from browning cannot be seen correctly.

The length and quantity of induced shoots increased when the PVP concentration was raised in *Abrus precatorius* plantlets (Perveen *et al.*, 2013). This result also supported these findings where 500 ppm PVP was better in suppressing browning (19%) in A. *marina* whereas MS media were employed together with Cytokinins. The same result did not apply to explants cultured on WPM. PVP concentrations over 120 ppm, however, actually decreased the quantity and length of generated shoots. Consequently, adding more PVP at higher concentrations

Table 1: Contaminated, Browning, and Dead Explants at the 8th Week of Culture

Variable	No. of Explants	Browning (%)	Contaminated (%)	Dead (%)	Survival (%)	Induced Shoots (%)
WPM, PVP 400	15	73 (11/15)	0	73 (11/15)	27 (4/15)	27 (4/15)
WPM, PVP 400, Cytokinins	15	87 (13/15)	0	87 (13/15)	7 (2/15)	0
WPM, PVP 450	15	87 (13/15)	0	87 (13/15)	13 (2/15)	7 (1/15)
WPM, PVP 450, Cytokinins	15	60 (9/15)	27 (4/15)	87 (13/15)	13 (2/15)	0
WPM, PVP 500	15	87 (13/15)	7 (1/15)	93 (14/15)	7 (1/15)	7 (1/15)
WPM, PVP 500, Cytokinins	15	87 (13/15)	0	87 (13/15)	13 (2/15)	0
MS, PVP 400	14	71 (10/14)	29 (4/14)	100 (14/14)	0	0
MS, PVP 400, Cytokinins	13	46 (6/13)	31 (4/13)	77 (10/13)	23 (3/13)	8 (1/13)
MS, PVP 450	15	73 (11/15)	20 (3/15)	93 (14/15)	7 (1/15)	0
MS, PVP 450, Cytokinins	15	53 (8/15)	40 (6/15)	93 (14/15)	7 (1/15)	7 (1/15)
MS, PVP 500	15	47 (7/15)	47 (7/15)	93 (14/15)	7 (1/15)	0
MS, PVP 500, Cytokinins	16	19 (3/16)	75 (12/16)	94 (15/16)	6 (1/16)	0

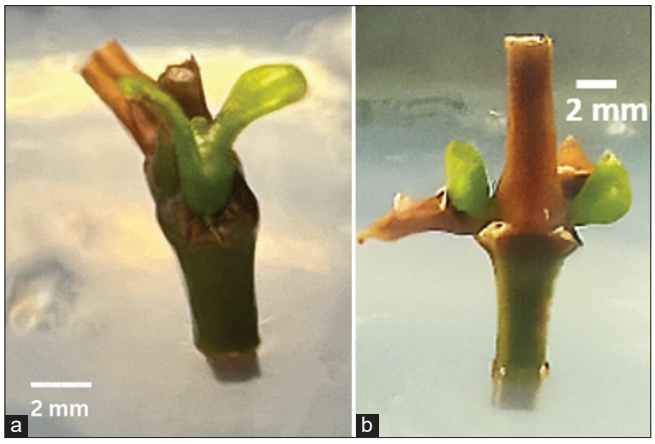

Figure 2: Axillary shoots 7 weeks after culture. a) WPM+400 ppm PVP and b) MS medium+400 ppm PVP+Cytokinins

(150 ppm) might prevent the stimulated shoots from growing (Perveen *et al.*, 2013). However, because a higher PVP concentration can lessen browning in comparison to a lower PVP concentration, the phytotoxic potential of phenolic compounds can also be lessened to A. *marina* explants (Table 1).

PVP is a non-toxic synthetic polymer that is water soluble, pH-stable, and temperature resistant (Koczkur *et al.*, 2015; Kurakula & Rao, 2020). German scientist Walter J. Reppe created PVP before 1939 (Haaf *et al.*, 1985; Kurakula & Rao, 2020). It was revealed in 1994 that PVP could be used to induce microcallus in grapevine protoplasts (Reustle & Natter, 1994). In 1997, PVP was used to stimulate callus production in chestnuts from the immature leaf (Abenavoli & Pennisi, 1998). Since then, a lot of in vitro culture research has been reported to use PVP for various kinds of plants, including *Rollinia mucosa* (Figueiredo *et al.*, 2001), jojoba (Prakash *et al.*, 2003), *Cryptomeria japonica* (Koguta *et al.*, 2017), *Sideritis raeseri* (Virginia & Eleni, 2019), kiwifruit (Sui *et al.*, 2020), *Tectona grandis* (Widhiastuty *et al.*, 2023) and *Thottea siliquosa* (Vrundha & Thomas, 2023). The application of PVP in mangrove propagation was reported in 2015, on two mangrove species *Heritiera fomes* and *Bruguiera gymnorhiza* (Kader *et al.*, 2015).

Comparison between WPM and MS Medium *A. marina in vitro* Growth

In comparison to MS medium, WPM is probably more effective at stopping the growth of endophytic microorganisms in A. *marina*'s node explants. Explants cultivated in WPM produced 0% contamination in week 8 for 4 out of 6 WPM treatments, as Table 1 illustrates, but all of them are contaminated in MS media. Despite the fact that contamination rates in MS media range from 19% to 73%. Although there is no evidence of any specific components in WPM that minimize contamination, the combination of plant preservative mixture (PPM) and WPM was found to be more effective for maintaining the aseptic condition of A. *marina* explants than the MS medium.

In all treatments, an enormous number of explants were dead. However, explants grown in MS media had the largest percentage of dead plants - between 93 and 94%. Only one treatment - MS+400 ppm PVP+Cytokinins - shows 77% dead explants out of all the treatments; the other treatment - MS + 400 ppm PVP - shows 100% dead explants. However, the majority of WPM treatments yielded 87% dead percentage for the explants cultivated in them; In contrast, one treatment showed 73% dead percentage (WPM+400 ppm PVP) and another treatment showed 93% dead percentage (WPM+500 ppm PVP).

WPM is higher in nitrogen and Sulfur than MS (Murashige & Skoog, 1962; McCown & Lloyd, 1981). Therefore, WPM is often used for woody plant micropropagation (Nowakowska *et al.*, 2019). Some mangrove plants were reportedly micropropagated successfully by using MS Medium or modified MS medium, including *Rhizophora annamalayana* Kathir. (Kandasamy & Chinnappan, 2013), *Excoecaria agallocha* L. (Arumugam & Panneerselvam, 2012), *Bruguiera cylindrica* L. (Vartak & Shindikar, 2008), and *Avicennia marina* (Al-Bahrany & Al-Khayri, 2003). Although WPM was reportedly used in many micropropagations of woody plants, there is still a lack of information on the application of WPM in mangrove plant micropropagation.

Started from week 12, there was only 3 survivals remained, two of them were treated with WPM+400 ppm PVP and one of them treated with WPM+500 ppm PVP (Figure 3). The result shows that WPM is better to maintain the survival and growth of A. *marina* node explants than MS media. However, the browning and wilting of the explants' part is still the issues to be tackled. The three survivals only survived until week 16.

Role of PPM in *A. marina* Micropropagation

PPM suppresses the growth of bacteria, interrupts the electron transport chain, and stops the movement of monosaccharides and amino acids from media into microbial cells by entering the microbial cell wall and disrupting numerous crucial enzymes in the citric acid cycle (George & Tripepi, 2001). The use of PPM has been reported in micropropagation of axillary buds of *Guadua angustifolia*, *Cannabis sativa*, *Petunia hybrida*, *Centella asiatica*, *Swietenia macrophylla*, *Cedrela odorata* with concentrations ranging from 0.075 to 0.5 (Jiménez *et al.*, 2006; Lata *et al.*, 2009; Miyazaki *et al.*, 2010; Moghaddam *et al.*, 2011; Flores *et al.*, 2012). However, there is no report yet about the use of PPM in micropropagation of mangrove plants through axillary buds. In this study, the tissue culture medium was incorporated with 0.4% PPM after several pre-studies on PPM concentration (Table 2). Although 0.5% PPM is 10% better than 0.4% PPM resulting in less browning and producing more survival, 0.4% PPM was chosen because the browning was less damaging than the application of 0.5% PPM on A. *marina* tissue culture as seen in Figure 4.

The Present of Endophytes and the Continuity of Browning

Some survivors (Table 1) did not successfully induce shoots. Even in the 8th week of culture, it was noted that the surviving

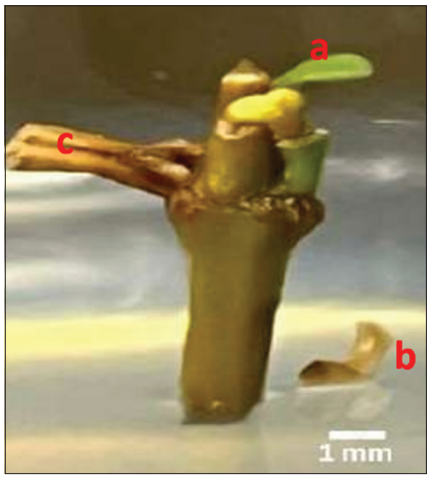

Figure 3: A survive node explant in week 12. (a-induced shoot, b-wilting shoot, c-browning on petiole)

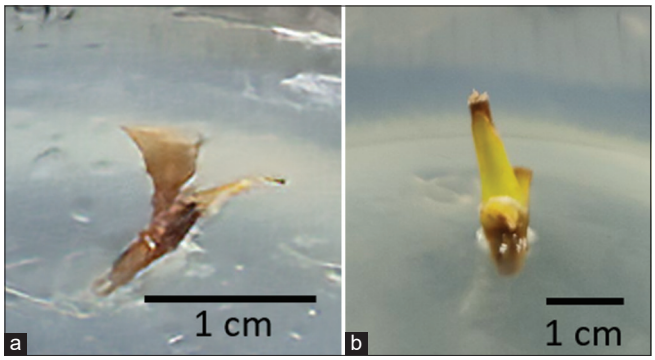

Figure 4: Comparation of two sample explants cultured on a) 0.5% PPM and b) 0.4% PPM observed in the 2nd week of culture

explants had not undergone browning and remained green in color. On plate count agar (PCA), an examination was conducted to determine whether those kinds of survival explants still included endophytes. The outcome, as seen in Figure 5, demonstrates that endophytes are proliferating inside the longitudinally sliced node explants. Since the sterile node explants have been previously in the tissue culture media for 8 weeks without visible contamination, it is reasonable to believe that endophytes are still present within the explants.

Endophytes are microbes that live inside the plant tissue without harming the host plants (Siraj *et al.*, 2023). However, in tissue culture, endophytes have adverse effects (Thomas, 2004; Volk *et al.*, 2022) including browning (Pirttilä *et al.*, 2008; Shen *et al.*, 2023). Browning could occur while certain endophytic microorganisms produce enzymes or secondary metabolites that induce oxidative reactions in plant tissues (Permadi *et al.*, 2023). Some examples of the enzymes associated with oxidative reactions produced by endophytes are peroxidase (PO) (Caverzan *et al.*, 2012; Chaudhary *et al.*, 2022), polyphenol oxidase (PPO) (Parlindo *et al.*, 2023) and β -1,3-glucanase (Prasetyawan *et al.*, 2018). Other than that, endophytes may produce phenolic compounds in plant tissue (Gautam *et al.*, 2022), leading to browning when exposed to oxygen (Amente & Chimdessa, 2021). Because endophytes need nutrients for their

Figure 5: Endophytes are present in cut node explants (8th week) cultured in PCA. Red arrow indicates the endophytes

Table 2: Pre-eliminary study on application of PPM on A. marina *in vitro* culture

PPM (%)	No. of explants	Browning (%)	Survival (%)
0.4	40	37.5	62.5
0.5	40	27.5	72.5

growth and development, plant tissue may undergo nutrient depletion that causes stress to plant cells and then browning (Oberschelp & Gonçalves, 2018). Under certain conditions, endophytes may turn pathogenic (Saikkonen *et al.*, 1998) that will induce defense mechanisms of plant tissue culture, triggering browning (Feng *et al.*, 2023).

Two research studies on A. *marina* propagation describe effective cloning using MS media (Al-Bahrany & Al-Khayri, 2003; Alatar *et al.*, 2015). However, removing endophytes from the explants through surface sterilization is not easy (Jaiswal *et al.*, 2022), because they can escape surface sterilization (Gunson & Spencer-Phillips, 1994). Despite the use of numerous sterilant agents, the findings of this experiment remain inconsistent.

The presence of endophytes within plant tissue may be impacted by the environment, such as climate and plant location (Nair & Padmavathy, 2014). From an environmental standpoint, Mangrove Wonorejo Ecotourism, the source of the samples in our research, was a heavily polluted estuary that receives water from heavily polluted rivers, such as the Surabaya river and the Jagir river (Lestari *et al.*, 2020). The biodiversity and endophytic compositions of A. *marina* residing in this area are undoubtedly different from those gathered in other locations.

Pollutants impact the diversity of endophytes within the plant (Harrison & Griffin, 2020). Additionally, there may be an increase in the prevalence of endophytic bacteria that are resistant to pollutants (Afzal *et al.*, 2019). Pollutant-resistant endophytic bacteria may potentially be more difficult to eliminate through surface sterilization compared to non-resistant bacteria. They can adapt to environmental changes to improve their chances

of survival, such as living in an environment with sterilizing chemicals (Marmion *et al.*, 2022).

CONCLUSION

WPM is more effective than MS at inducing shoots and preventing *A. marina* node explants from browning too quickly. The use of PPM and other antioxidants is critical in lowering endophytes in explants, although additional research is needed to reduce endophytes to zero. The primary causes of explant death are browning caused by endophyte activity and endophyte pathogenicity. Although healthy plantlets are not effectively produced, the current study has succeeded in extending the age of healthy plantlets.

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