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Copper sulphate treatment induces *Heterozostera* seed germination and improves seedling growth rates

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ABSTRACT

Evidence for seed germination and/or seedling recruitment in wild populations of the Southern Hemisphere seagrass *H. nigricaulis* are lacking. Additionally, seeds of *H. nigricaulis*, have proven extremely difficult to store and germinate in laboratories, even when using techniques for germination proven successful in other Zosteraceae. Prior studies reveal Zosteraceae seed and seedling failure may be correlated with oomycete infections. Copper sulphate treatments can reduce oomycete and other seagrass infections in laboratory tests. Here, we tested whether copper sulphate seed treatments promote germination and seedling growth in *H. nigricaulis*. We found treatment with 2.0 ppm copper sulphate solution induced significant seed germination and led to 3 times more photosynthesizing *H. nigricaulis* seedlings after 3 months. Thus, in addition to reducing disease, copper sulphate appears to be a novel cue for *H. nigricaulis* germination and improves seedling development and success in *H. nigricaulis*. This discovery will improve our opportunities to overcome biogeochemical germination cues and bottlenecks for Zosteraceae and improve seed-based seagrass restoration strategies where copper sulphate treatment is available to pre-treat and cue germination and promote seedling development and growth.

1. Introduction

Seagrasses, such as Zosteraceae, are marine flowering plants forming expansive ‘meadows’ in shallow coastal areas of every continent except Antarctica (Green and Short, 2003). Zosteraceae reproduce asexually (e.g., through rhizome and shoot extensions) and/or sexually (e.g., through generation of hydrophilic male and female flowering structures, including pollen and seed). Like other plants, Zosteraceae are known to variably invest energetic resources (Obeso, 2002) for vegetative (Smith et al., 2016a) and sexual (Phillips and Backman, 1983; Smith et al., 2016b) reproduction strategies. For instance, some Zosteraceae populations depend entirely upon seed production to maintain annual meadows (Hootsmans et al., 1987; Meling-López and Ibarra-Obando, 1999), while other large populations persist without any evidence of flowering (Smith et al., 2018).

Laboratory experiments reveal *Zostera marina* germination rates can be as high as 100% in some instances (Hootsmans et al., 1987),

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where evidence of seed germination in *Heterozostera* are quite rare and with no observations of seed-based recovery following in-situ disturbances, despite efforts to look for them (Smith et al., 2016b). Further, controlled germination trials report no greater than 20% germination success for *H. nigricaulis* seed, despite collection, storage and processing under environmental conditions that were otherwise favorable to Zosteraceae, including *Zostera marina* (Cumming et al., 2017). Thus, to store and reliably germinate enough seed to restore lost *H. nigricaulis* meadows, bottlenecks and mechanisms underlying *H. nigricaulis* seed germination and seed viability require additional research.

Bottlenecks to successful sexual reproduction may hit different life-stage transitions, and are critical for understanding limitations for seed-based recovery (Statton et al., 2017). Temperature, salinity and burial depth have been implicated as drivers of success in Zosteraceae seed storage and germination trials (Moore et al., 1993; Brenchley and Probert, 1998; Abe et al., 2008; Jarvis and Moore, 2014; Xu et al., 2016; Cumming et al., 2017; Xu et al., 2021), however potential roles for microbiome and chemical treatments in inhibition or promotion of seed germination and storage have not been well-studied for Zosteraceae.

Microbial pathogens of seagrass, such as *Labyrinthula,* are reported from every seagrass meadow examined (Sullivan et al., 2013; Martin et al., 2016). Pathogens of Zosteraceae capable of causing seed and seedling mortality in both sexual and non-sexual life stages, have been observed in *Zostera noltei* and *Zostera marina* (Govers et al., 2016, Govers et al., 2017).

Copper sulphate is a commonly studied biotoxin, where the impact of copper sulphate dosage on seed germinations for aquatic Alismatales demonstrated little impact to germination rates and shoot and root elongation (Muller et al., 2001). Copper sulphate treatment is suggested as an anti-microbial treatment for improving seed storage (Xu et al., 2019) as it inhibits the growth of multiple oomycete pathogens (*Phytophthora* and *Halophytophthora)* in *Zostera* (Govers et al., 2017). The deleterious effect of pathogens on *Zostera* restoration and conservation occur because of restricted growth and development of sexual propagules (Govers et al., 2016). Basic knowledge of host-pathogen interactions in *H. nigricaulis* is essential and only beginning (Sullivan et al., 2017; Trevathan-Tackett et al., 2018). Presence and potential impacts of infectious pathogens on *H. nigricaulis* seed storage, viability, germination and growth in remain unstudied.

Seagrass meadows, are vulnerable, yet critical for production of valuable fisheries (Unsworth et al., 2018) and other ecosystem services (Mtwana Nordlund et al., 2016). As seagrasses are declining globally (Waycott et al., 2009), pathways for large-scale recovery are urgently needed (Orth et al., 2020). Transplanting clonal ramets, while effective for restoration (Katwijk et al., 2016), can also be detrimental to donor beds, labor-intensive and expensive (Fishman et al., 2004). Thus, seed-based restoration has emerged as a preferred method for cost-efficient, labor-friendly and effective recovery of large areas of seagrass, while also reducing genetic bottlenecks (Xu et al., 2020). Success of large-scale of seed-based *Zostera marina* restoration has been demonstrated in coastal bays (Orth et al., 2006; Unsworth et al., 2019), sometimes with very minimal effort (Pickerell et al., 2005).

The objective of our study is to explore whether copper sulphate treatments can improve seed germination and support structural growth and development of *H. nigricaulis* seedlings*,* as needed for advancing research, including large-scale seagrass aquaculture and restoration efforts. We measure impacts of copper sulphate treatment on *H. nigricaulis* germination rates and growth, and determine whether seeds with disease or decay symptoms can be linked to the presence of oomycete seed pathogens known to impact seed viability and dormancy of seagrass.

2. Methods and materials

2.1. Seed collection and storage

Heterozostera seeds were collected from flowering *H. nigricaulis* plants in the field and transported to the Victorian Marine Science Consortium in Queenscliff, Victoria for long-term seed storage and germination tests. The flowering shoots were collected over 3 collections days between November to December 2016 from Port Phillip Bay (38°08’34.2"S 144°25’04.7"E), near Point Henry in Geelong, Victoria. Reproductive shoots were kept in large (1000 L) aerated plastic tubs with flow-through ambient seawater and light regimes to allow spathes to mature and seed to drop out of the leaves naturally (Infantes and Moksnes, 2018). In January 2017, upon maturation, the shoots were removed from the plastic tubs and seawater was sieved to 0.45 μm to collect dropped seeds and remnant spathes. Seeds and spathes were then stored in an aerated 1 L plastic tub with continuous flow-through seawater until seed trials commenced (approximately 2 weeks).

2.2. Sterile seawater (SSW)

A single point collection of ambient seawater was made from the seawater intake system (operated by the Victorian Fisheries Authority) at the Victorian Marine Science Consortium. Ambient salinity at the time of collection was measured at 40 SSU. Ambient seawater was then sterilized in a covered 5 L polyethylene capped pitcher and autoclaved at 121 °C for 20 min (Parsons 2013). Following sterilization, the sterilized water was kept refrigerated at a constant temperature of 6 °C prior to use in the trials.

2.3. Copper sulphate ($CuSO_4$) solutions

Copper treatment solutions were prepared by dissolving copper sulphate ($CuSO_4$) into sterile seawater (SSW) and creating two stock treatment solutions (Govers et al., 2017). The first solution was a low-copper treatment, prepared as a solution of 0.2 ppm $CuSO_4$ and a high-copper treatment prepared as a 2.0 ppm solution of $CuSO_4$. In addition, a control SSW treatment was set up in which no copper sulphate was added.

2.4. Stages of germination and growth

Seed germination and seedling development in our experiments were scored based on five observable stages of growth (Fig. 2) (Xu et al., 2016). We modified the *Zostera marina* germination model presented by Xu. et al. to account for 5 distinct stages of germination commonly observed in *H. nigricaulis*.

2.5. Graduated salinity

Given previous success of freshwater pulses on *H. nigricaulis* germination (Cumming et al., 2017), we germinate *Heterozostera* seeds in fresh water and then gradually reintroduced them to saline conditions to provide a control group for germination and growth measurements. Germination trials commenced by placing 96 seeds in individual 1 cm round wells with deionized water (0 salinity). They were checked weekly for a break in dormancy. Once a break in dormancy was observed (Stage 1) seeds with hypocotyl extension (Stage 2) were transferred to vials of seawater with salinity of 20. Upon further evidence of survival, including cotyledonary growth and elongation (Stage 2), germinated seeds were transferred to SSW with salinity of 40 and placed in a window with ambient light and kept at 20–21 °C. Germination and growth characteristics, including stages of development, stem length (cm), and seedling mortality (fouled), were recorded over 12 weeks.

2.6. Presence of seed pathogens and impacts on viability

To test for a potential correlation of seed and seedling pathogens in reduced germination success, *H. nigricaulis* seed with visible signs of distress (seed failure/death, fouling and/or presence of disease symptoms) were opportunistically sampled from various storage and germination conditions (Table 1) and sent to the National Plant Protection Services lab in the Netherlands (NVWA). The lab tested for the presence of known oomycete pathogens and for seed viability.

All seeds tested for disease presence were sampled directly from ongoing laboratory storage vials and placed in 1.0 ml sealed plastic vials filled with 0.5 ml of sterilized seawater, then immediately shipped to the Dutch NVWA for pathogenic testing according to the methods described in Govers et al. (2016). The lab individually tested *H. nigricaulis* seeds for the presence of known agents of pathogenic seagrass diseases, including *Phytophthora* spp. and *Halophytophthora* spp. The laboratory also assessed the viability of the seeds by scoring seed germination up to 4 weeks after incubation on ParpH, according to the method described in Govers et al. (2016).

2.7. Experimental design

To test the effect of copper concentration on seed dormancy, germination and growth we randomly allocated 16 containers, each with 30 seeds, to one of two seawater treatments: (1) high copper (2.0 ppm) or (2) no copper/control (0.0 ppm). Stock treatment

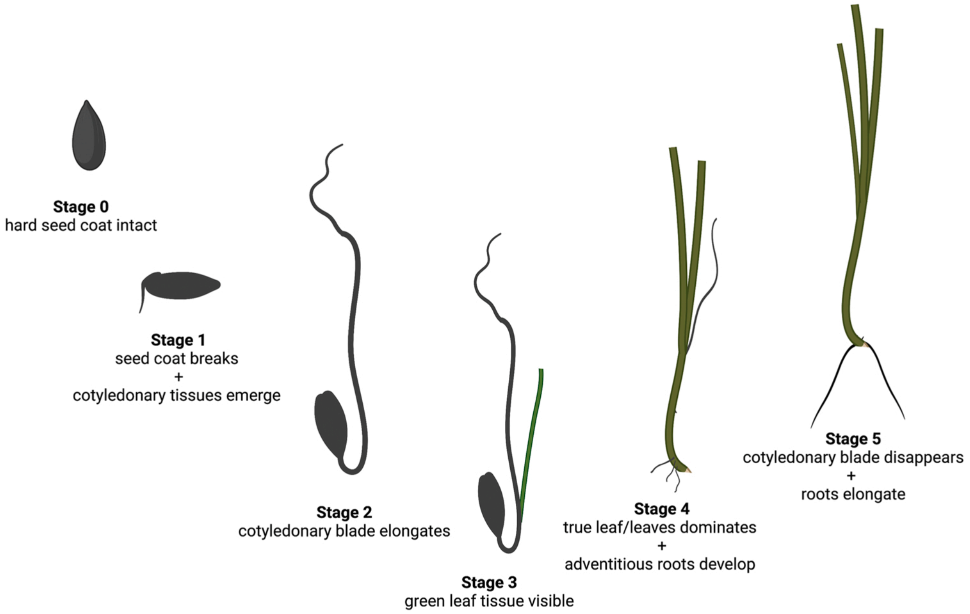

Fig. 1. FIVE STAGES OF HETEROZOSTERA NIGRICAULIS GERMINATION AND SEEDLING DEVELOPMENT. Intact *Heterozostera nigricaulis* seed (Stage 0) germinates (Stage 1), elongates (Stage 2), develops initial photosynthetic tissues and adventitious roots (Stage 3), then ingpresent two or more complete photosynthesizing blades (Stage 4), until finally, the roots elongate and the cotyledonary blade can no longer be observed (Stage 5) and the seedling has transformed into a non-reproducing adult.

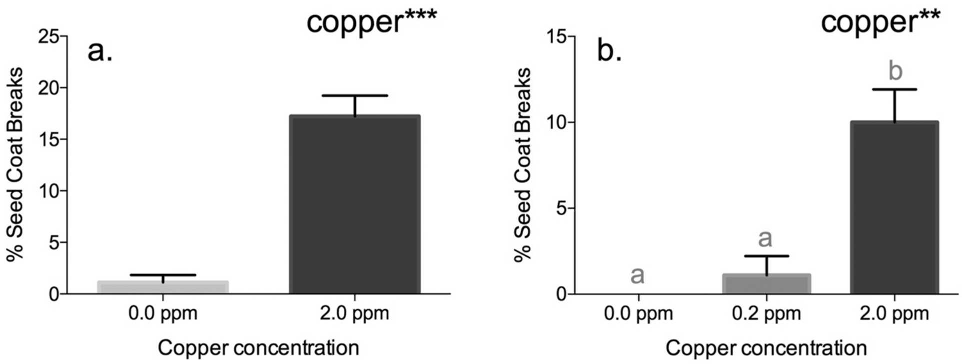

Fig. 2. COPPER GERMINATIONS SUMMARY. Bar graphs of the mean percent of seeds in each replicate that broke dormancy (Seed Coat Breaks) following 12-weeks immersion in one of three copper sulphate treatments (0.0 ppm, 0.2 ppm, and 2.0 ppm). A significant effect of high levels of copper sulphate on breaking seed dormancy in *Heterozostera* seed was detected across replicate studies (Error bars represents 1 standard error from the mean).

Table 1

SEED PATHOLOGY AND VIABILITY. A total of 72 seeds from Port Phillip Bay *Heterozostera nigricaulis* were opportunistically sub-sampled from ongoing storage treatments. Samples were targeted to test for potential presence of seagrass pathogens and for seed viability. Collections targeted symptomatic material (fouled, discolored, cessation of germination etc.).

Seeds (#)	Plant Material	Temperature (C)	Salinity	Light Condition
12	seedlings	20–21	0–20	Ambient
20	seeds	20–21	40	Ambient
20	seeds	6	40	Dark
20	2-year-old seeds	6	40	Dark

solutions were used to fill each 25 ml capped polystyrene cylindrical container. The replicate containers were stored for 12 weeks without aeration, under ambient daylight and relatively stable ambient air temperatures of 20–21 °C. Following the incubation period, we evaluated germination (Fig. 1) and recorded whether the seed developed cotyledonary blade and adventitious root growth and photosynthesizing (true/green) leaf tissues. We also noted any fouling, discoloration, and seedling mortality.

Following robust germination results from seed stored in high copper concentrations, the study was broadened to include a moderate concentration of copper sulphate solution (0.2 ppm). To test the effects of high, medium and no copper concentration gradients on seed dormancy and germination, we repeated observations of germination rates across treatments after 12 weeks. In all, 270 seeds were equally distributed between three levels of copper sulphate solution treatment (0.00 ppm, 0.20 ppm and 2.0 ppm). Replicate treatment and control experimental units consisted of labeled and capped 25 ml cylindrical polystyrene containers containing 30 seeds immersed in the copper sulphate or control treatment solutions. All units were stored under ambient daylight and temperatures (20–21 °C) to mimic and hold constant existing field conditions. Stages of germination (Fig. 1), length of cotyledonary blades, and presence of photosynthesizing leaf tissues were recorded for replicates in each treatment.

To test the effect of the copper seed treatments on subsequent seedling success, we opportunistically observed seedling development for 20 seeds that germinated (Stage 1) in the high copper treatments and 2 seeds that germinated (Stage 1) in the control treatment (Table 2). This was accomplished by transferring each germinated seed from the experimental unit to an 10 ml glass vial and surrounded them with ambient sterile seawater (SSW). Following transfer, seeds were returned to the window of ambient light and controlled temperature (20–21 °C), where germination and seedling morphology (according to Xu et al., 2016) could be observed over a 12-week period. At the same time, seeds that broke dormancy (Stage 1) in the graduated salinity treatment were also placed in 10 ml glass vials with SSW and seedling stage and total length were recorded for each seedling after 12 weeks. Distilled water was added to test vials as needed to maintain salinity. This allowed for growth characteristics of seedlings germinated in control/ambient salinity to be observed and recorded compared to seeds germinated with freshwater pulses and copper sulphate treatments.

Throughout germination experiments, several seeds broke dormancy (Stage 1) and then failed to grow, quickly browning. Fouled

Table 2

DATA SUMMARY OF SEEDLING DEVELOPMENT RECORDED ACROSS TREATMENTS AFTER 12 WEEKS.

Treatment Name	Germinated (Stage 1) # (%)	Adult (Stage 5) # (%)
Control	2 /90 (1.1)	0/90 (0)
Graduated Salinity	49 /96 (51)	0/96 (0)
Copper (0.2 ppm)	1/90 (0.01)	0/90 (0)
Copper (2.0 ppm)	20/90 (22)	6/90 (7)

seeds often appeared overgrown with fungal or bacterial growth. The germinated seeds became discolored, presenting tissues that appeared light brown to black, and sometimes fouling with what appeared to be black or white fungi or both. Since the symptoms observed in failed *H. nigricaulis* seed were like diseases described by the pathogenic protists *Phytophthora* and *Halophytophthora* (Sullivan et al., 2017, Govers et al., 2017), all 12 symptomatic seeds that broke dormancy in high copper treatment experiment then failed to grow and develop were sampled for further disease testing. Pathogen assays were completed ad hoc to test failed *H. nigricaulis* seeds for viability and presence of known Zosteraceae seed and seedling pathogens.

2.8. Statistical analyses

Variable effects of copper sulphate concentrations on *Heterozostera* seed germination and seedling development were analyzed. The significance of a high concentration copper treatment on germination percentages was analyzed using Welch's *t*-test for independent samples with unequal variance (for testing difference in the mean for independent samples with equal sample sizes and unequal variances). Germination rates including the subsequent low and high copper treatment experiment were analyzed through a one-way analysis of variance (ANOVA) and supported by pairwise testing with the Tukey-Kramer test of highly significant differences. The significance of copper seed treatments on germinated seedling length after 12 weeks was also analyzed through ANOVA and supported with the Tukey-Kramer test.

3. Results

3.1. Copper treatment induced break in H. nigricaulis seed dormancy

The high copper sulphate solution had a surprising and significant effect on the mean number of *H. nigricaulis* seeds observed breaking dormancy (Fig. 2). In all, an average of 5.16/30 (17.2%) seeds per replicate ($n = 6$, SEM +/- 0.60) broke dormancy (germinated) over a 4-week period in the high copper sulphate solution treatment (2.0 ppm). In comparison, a break in dormancy of seeds under the control treatment averaged only 0.33/30 seeds (1.1%) ($n = 6$, SEM +/- 0.21). Further, the two seeds from the control treatments that did break dormancy promptly became fouled and seedling failure was recorded. A high copper sulphate treatment (2.0 ppm) significantly increased the proportion of *H. nigricaulis* seeds that broke dormancy (Welch's t-test, $P < 0.001$). This germination percentage rate was comparable to the germination percentage rates achieved by other researchers using freshwater pulses for 12 h, but which rarely occur in the field.

One-way analysis of variance in mean germination rates of seeds placed in the subsequent copper sulphate gradient treatments and control experiments continued to demonstrate a significant increase in probability that a seed would break dormancy under high copper sulphate solution treatments (F=18.249, df=2, $P = 0.0028$). Additionally, hypocotyls of seeds treated with high copper (2.0 ppm) solution were significantly more likely to grow following germination (f=63.9906, df=2, $P = 0.0001$). Lastly, seeds treated with 2.0 ppm copper sulphate were significantly more likely to develop green leaf tissues over the same 12-week sampling period (f=12, df=2, $P = 0.008$). The effects of low copper sulphate (0.2 ppm) and control (0.0 ppm) treatments were not significantly different.

3.2. Seedling development correlated to copper sulphate treatment

No cotyledonary or photosynthesizing tissues developed from either of the 2 seeds that broke dormancy from the control experiment, though the sample size was not comparable to the number that germinated from the high copper treatment.

Fifteen of the 20 (75%) copper sulphate treated seeds continued to develop cotyledonary tissues following a break in dormancy and transfer to the grow chambers. However, successful seedling maturation rates remained extremely low overall, as only 6 out of the 20 seeds (15%) that broke dormancy and germinated in high copper treatments went on to develop green photosynthesizing tissues, or true leaves in our growth chambers. Further, these 6 seedlings that developed green leaves represent only 2.5% of the total seeds treated with the high copper sulphate solution. All the seedlings that successfully developed leaves were from seeds treated in 2.0 ppm copper sulphate solutions, suggesting efforts to improve this method could be useful for increasing seed success for restoration. After 12 weeks, germinated seedlings in the copper treatment ranged in maximum length from 0.2 to 1.2 cm, with an average length of 0.375 cm. In comparison, seeds in the graduated salinity treatments (no addition of copper sulphate) had an average germination rate of 51% ($n = 96$) and eight of these continued to grow with a mean cotyledonary length of 0.55 cm (ranging from 0.2 to 1.0 cm). However, while germination rates and average cotyledonary blade lengths were greater, no seeds that broke dormancy in the graduated salinity or control treatments presented photosynthesizing leaf tissues after 12 weeks.

3.3. Microbial and chemical germination cues inconclusive

Despite appearing symptomatic, seeds that broke dormancy and failed to thrive remained 90% viable and with no detectable oomycetes found in any of the seeds sent to the lab ($n = 72$).

4. Conclusions

Here we present a novel method for inducing germination of seed in a southern hemisphere seagrass, *H. nigricaulis*. While exploring methods for successful germination and growth, high copper sulphate treatment (2.0 ppm for 4 weeks) had a strong positive effect on seed germination (%) rates and was coupled with a positive effect of copper sulphate pre-treatment on subsequent seedling growth and development. Even though this treatment has been shown to successfully prohibit pathogenic organisms from infecting Zosteraceae populations, treating seeds in copper sulphate solutions of 2.0 ppm or greater may also induce germination in *Heterozostera*,. This discovery provides a novel germination cue that may be used to improve seed-based restoration success of *H. nigricaulis*.

Prior attempts to break dormancy of *H. nigricaulis* seed in Australia required time intensive manual scarification and additions of periodic brackish or fresh water in shallow dishes that were kept under lower temperatures without aeration (~17–21 °C). Overall, germination from manual scarification and freshwater pulse techniques resulted in less than 20% germination (Cumming et al., 2017). Our work demonstrates for the first time that treatment of seagrass seeds with copper solutions in ambient temperature and salinity, and with no scarification, will induce comparable seed germination, while requiring significantly less manual effort to process for out planting. Additionally, seed germination induced by copper sulphate concurrently stimulated an increase in seedling growth and maturation of seedlings into photosynthesizing young plants over a 12-week period.

Our experiments did not reveal the mechanisms behind the positive effects of high copper sulphate treatment on *H. nigricaulis* seed germination under ambient salinity and temperature. However, observed results may be related to the well-known inhibitory effects of copper on microbial activity. Copper-based compounds are lethal to fungi, oomycetes and algae and are commonly used as fungicides due to the lethal effects on sporangia and chlamydospores of these organisms (reviewed in Govers et al., 2017). Similarly, bacteria may be inhibited or even killed due to toxic effects of copper on a cellular level (Thurman et al., 1989). Epiphytic bacteria or protists on the seed coat of *H. nigricaulis* may be a cause of inhibition in seed germination, though to our knowledge no inventory of seagrass seed-associated microbes in *H. nigricaulis* has been published.

Our copper sulphate treatment may have also altered chemical processes enhancing dormancy in seeds, potentially by affecting the epi- and endophytic microbial community that promote or protect dormancy and/or provide essential micronutrients, such as soluble copper, which could be a requirement for germination. However, our results are not conclusive and further exploration of seed germination mechanisms is urgently needed to resolve these topics in seagrasses, and for poorly germinating species, such as *Heterozostera nigricaulis*.

Testing for presence and pathogenicity of known pathogens in Zosteraceae more generally is required to better understand the role disease may have in the autoecology and lifecycle of seagrass seed globally (Sullivan et al., 2017). Finding no trace of known disease agents in Zosteraceae seed tested from temperate regions of the southern hemisphere was unexpected given the global occurrence of known pathogens, including *Labyrinthula*, Phytomyxids, *Phytophthora* and *Halophytophthora* where they have been researched in the northern hemisphere (reviewed in Sullivan et al., 2017). However, we cannot conclude that this demonstrates these oomycete pathogens are not present in the southern hemisphere, or in southeastern Australia in particular, because we sampled from a narrow region and sample transport prior to analysis, including anoxia may have affected the survival of these pathogens on their way to Europe. Thus, the inability to detect omnipresent disease agents in samples from symptomatic southern hemisphere seed also necessitates more research. Other physiological conditions that may be inhibiting growth or seedling success broadly need more investigation.

5. Summary

Here we report a novel pathway for *H. nigricaulis* germination, which may be relevant to other Zosteraceae seed collection and storage efforts for restoration. This information may be used to better understand natural and human induced cues to germination in Zosteraceae, which can be useful for restoration ecologists looking to effectively restore seagrass meadows from seed, particularly *H. nigricaulis*. Through identification of a novel cue for seed germination, we were able to demonstrate a negative effect of high concentrations of copper sulphate on seed storage and positive effects for early germination stages, in both ambient salinity and temperature. These results present a significant advancement in our understanding of germination cues for *H. nigricaulis* seed, despite not knowing the exact mechanism(s) of germination. Further investigations into the mechanisms of copper-induced seed germination, the potential for copper sulphate treatments to promote resilience through disease control and abatement, and its ability to enhance seagrass seedling germination and development are urgently needed given the little evidence of seedling recruitment in-situ, and low germination rates for *H. nigricaulis* and other seagrasses *ex-situ*.

CRediT authorship contribution statement

BS conceived and designed the research; BS, LG performed experiments, BS, LG, and MK analyzed the data; BS, LG, MK contributed materials and analysis tools; BS wrote the manuscript; BS, LG, MK edited the manuscript and figures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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