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GROWTH RESPONSE AND LEAF CHLOROPHYLL CONCENTRATION OF ROMAINE LETTUCE PLANTS (*Lactuca sativa* L. var. *longifolia*) APPLIED WITH MYKOPLUS UNDER VARYING SALINE CONDITIONS

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ABSTRACT – Salinity stress in agricultural lands negatively affects plant's yield and income of farmers. Our study investigated the effects of MykoPlus biofertilizer on the growth response (leaf number, root and shoot length, and plant biomass), photosynthetic activity (chlorophyll concentration), and leaf appearance of Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) subjected to varying saline concentrations in the soil. The 28 day-old lettuce plants without and with MykoPlus biofertilizer application prior to transplanting were grown in saline soil (0 mM [0 dS m^{-1}], 100 mM [11.55 dS m^{-1}], 200 mM [22.46 dS m^{-1}] and 300 mM [31.72 dS m^{-1}] NaCl) for 19 days. Results showed that MykoPlus-applied plants produced higher chlorophyll concentration (4.59 to 9.94 SPAD values) with reduced leaf senescence (less yellowing and browning) and more number of leaves (0.8 to 1.3 leaves), produced longer shoots (10.04 to 30.05 mm) and roots (15.91 to 24.46 mm), and heavier plant biomass (0.03 to 0.06 g) than the plants without MykoPlus under salinity stress condition (up to 300 mM NaCl). Salinity in the soil reduced the lettuce growth regardless of biofertilizer treatments and salinity stress level. Leaf production, shoot and root length, plant biomass, chlorophyll content and leaf appearance were reduced with increasing level of salinity at 33 days after transplanting. The promotive effect of MykoPlus on the growth and photosynthetic activity of salinity-stressed lettuce plants was due to the plant growth promoting microorganisms contained in the biofertilizer. Results indicate that MykoPlus application is an effective way of mitigating the deleterious effects of salinity stress on Romaine lettuce plants.

Keywords: growth response, lettuce, microorganisms, MykoPlus, plant growth promoting, salinity stress

INTRODUCTION

Salinization is one of the important soil degradation problems in the Philippines where about 0.4 million hectares are considered to be salinization-prone coastal lands in the country. Low-lying coastal areas in the Philippines experienced soil salinization due to salt water intrusion and the use of saline water for irrigation (Asio et al. 2009). Soil salinization is a widespread constraint to rice production in the

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Philippines where about 70,000 hectares of rice production area are potentially affected by saline water intrusion (PhilRice, 2001). In the country, lettuce plants were mostly planted in the uplands of Cordillera Administrative Region and Northern Mindanao, and these are only minimally planted in near coastal areas. However with the scarcity of agricultural lands in the country due to the conversion of agricultural lands for non-agricultural purposes (OECD, 2017), the utilization of the near coastal areas for producing crops such as lettuce can be an option.

Soil salinity is one of the major problems of coastal, arid, and semi-arid areas which negatively affect crop production. Salinity-stressed plants have reduced growth which resulted in reduced crop yield. Plants can be glycophytes wherein most of the vegetables specifically leafy vegetables cannot tolerate high salinity in the soil. Soil salinity negatively affects the plant's nutrition, morphology, and physiological processes. Plants under salinity stress absorb more of the sodium than potassium resulting to non-availability of potassium due to competition with sodium ions in the binding sites (Rus et al. 2001). Reduced absorption of plants in phosphorus caused by soil salinity is due to the precipitation of phosphorus with calcium, magnesium, and zinc ions (Azcon-Aguilar et al. 1979). Moreover, salinity stress can cause reduction on the shoot growth of the growing tissues, stunted growth of the leaves and stem, reduced leaf production, osmotic inhibition of water uptake by roots, and reduction in photosynthetic activity of the plants (Mahajan and Tuteja, 2005). With prolonged exposure of the plants to salinity stress, the plant could experience yellowing and browning of the leaves resulting to death. Salt tolerant plants, on the other hand, show minimal reduction in growth at a concentration of 300 mM NaCl or more (Tester and Davenport, 2003). Optimal growth can be achieved even at 50 mM to 250 mM NaCl in many dicotyledonous halophytes (Flowers et al. 1986), whereas monocotyledonous halophytes generally grow optimally in a low NaCl concentration (50 mM NaCl or less) (Glenn et al. 1999).

Based on the idea that plant-growth promoting microorganisms have important roles in enhancing plant growth (Han and Lee, 2005; Saravanakumar and Samiyappan, 2007; Yue et al. 2007; Rokhzadi et al. 2008; Yildirim et al. 2008; Kohler et al. 2009; Engamberdieva et al. 2011) especially the beneficial effect on root and shoot growth in stressed environment such as saline soils, the National Institute of Molecular Biology and Biotechnology (BIOTECH) of the University of the Philippines Los Baños (UPLB) developed MykoPlus, a locally available biofertilizer. MykoPlus contains multi-strain and multi-species of mycorrhizal fungi, nitrogen fixers, phosphorous solubilizers, growth hormone secretors, and others (Pagcaliwagan, 2015; Sarian, 2018). MykoPlus aids in better water and nutrient absorption of plants in the soil and the microorganisms contained in the biofertilizer provides additional nutrients through biological nitrogen fixation, phosphorus solubilization and growth hormone secretion (Perez, 2019), hence, enhance crop growth and yield (DOST, 2016; UP, 2016). Plant growth promoting microorganisms such as mycorrhizal fungi and plant-growth promoting rhizobacteria (PGPR) were reported to produce microbial biofilm that enhances the growth and productivity of crops, regulate plant nutrition and enhanced the production of phytohormones and antioxidant enzymes (Rekadwad and Khobragade, 2017). Several studies have elucidated the beneficial effects of mycorrhizal fungi association in plants under salinity stress. Mycorrhizal application in alleviating salinity stress was reported in strawberry cultivars (Fan et al. 2011), maize (Sheng et al. 2008; Sheng et al. 2009), lettuce (Ruiz-Lozano et al. 1996; Cantrell and Linderman, 2001; Al-Karaki et al. 2001; Zuccarini, 2007; Kohler et al. 2009; Aroca et al. 2013), onion (Cantrell and Linderman, 2001), cotton (Tian et al., 2004), and *Sesbania sp*. (Giri and Mukerji, 2004).

Species from *Asteraceae* family have different salt tolerant capacities. In a study by Pasternak et al. (1986) in Iceberg lettuce (*Lactuca sativa* L.), growth and quality was not affected at 4.4 dS m^{-1} water salinity. Meanwhile, Jerusalem artichoke (*Helianthus tuberosus*) and Globe artichoke (*Cynara scolymus*) were both moderately salt tolerant plants with a threshold electrical conductivity (EC_e) of 8.33 dS m^{-1} (Newton et al. 1991) and 4.9 dS m^{-1} (Graifenberg et al. 1993), respectively. Romaine lettuce is a high value leafy vegetable and is considered to be a relatively salt-sensitive vegetable (Barassi et al. 2006) especially during the early seedling stage and at reproductive stage (Shannon and Grieve, 1999) and are moderately sensitive at a threshold EC_e of 1.33 dS m^{-1} (Maas and Hoffman, 1977). Sensitivity of lettuce to soil salinity differ in cultivars where Romaine types are more salt tolerant than with the Iceberg types (Shannon and Grieve, 1999). Romaine lettuce is utilized in the study for the aforementioned qualities.

This study aimed to investigate the growth responses and leaf chlorophyll concentration of lettuce plants by using the Mykoplus biofertilizer as a pre-conditioning treatment before exposure to varying salt concentrations in the soil. The study was conducted on August 2017 to November 2017 at the Institute of Biological Sciences (IBS), UPLB which aimed to improve the growth of salinity-stressed Romaine lettuce plants through the application of MykoPlus biofertilizer.

MATERIALS AND METHODS

Establishment of experimental pots with varying saline concentrations

The experiment was laid out employing the Split Plot Design in Completely Randomized Design with 3 replications. Each treatment has 10 sample plants per replication. The lettuce plants were applied with MykoPlus biofertilizer (without MykoPlus and with MykoPlus) and were exposed to different levels of salinity (0 mM NaCl, 100 mM NaCl, 200 mM NaCl, and 300 mM NaCl). The leaf number, root and shoot length, plant dry weights, Soil Plant Analysis Development (SPAD) index, and leaf color and appearance were determined at 33 days after transplanting (DAT).

'Green Towers' Romaine lettuce seeds (Condor, Allied Botanical Corporation, Philippines) were separately germinated in seedling trays filled with soil media without MykoPlus and MykoPlus-applied soil media for 28 days. The light textured soil media (pH 6.11; 2.67 dS m^{-1} EC; 3.70% OM; 42.90 ppm Available P; and 1,235.41 ppm Available K) composed of 1:1 garden soil and coconut coir dust mixture was sterilized through oven drying in 100^{o}C for 1 hour (Aroca et al. 2013). The lettuce seedlings (0.16 g whole plant fresh weight (FW), 0.04 g root FW, 0.12 g shoot FW, 40.29 mm shoots, 32.81 mm roots, with 4 true leaves) were transplanted in polybags filled with 300 g of sterilized soil media. The plants were acclimatized for 13 days in the greenhouse of IBS, UPLB, Laguna (latitude: N14^{o}09'58.5", longitude: E121^{o}14'22.5", elevation: 47.9 masl) before salinity stress imposition.

MykoPlus-treated seedlings were drenched with Mykoplus biofertilizer solution (16.67 g l^{-1}) during sowing, at 7 and 14 days after sowing (DAS). A total of 30 pots per treatment were subjected to salinity stress. To impose salinity stress in the plants, the lettuce seedlings were irrigated with 25 mL of varying salt solutions (0 mM, 100 mM, 200 mM, and 300 mM NaCl) with EC values of 0 dS m^{-1}, 11.55 dS m^{-1}, 22.46 dS m^{-1}, and 31.72 dS m^{-1}, respectively (Eutech® Con 2700 Electrical Conductivity Meter, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) at 14, 21, and 28 DAT. The plants were grown under greenhouse condition with 74.2 µmol $m^{-2}s^{-1}$ Photosynthetically Active Radiation (PAR) (Li-Cor LI-189® Light Meter, Li-Cor Biosciences, Lincoln, NE, USA), mid-day temperature range of 30.19^{o}C - 30.8^{o}C and 84.5% relative humidity. All the lettuce plants were applied with 5 g l^{-1} of complete fertilizer (14-14-14) after salinity stress imposition. The plants were also irrigated with 30 mL tap water once a week.

Growth Parameters: leaf number, root and shoot length (mm), and plant biomass (g)

Leaves per plant that are green, fully developed and expanded, and are not dried were counted. Root and shoot were measured from the base of the plant to the tip of the longest root and leaf, respectively, using a vernier caliper. The lettuce plants were oven dried at 70ºC for 48 hours and dry weights (shoot, roots, whole plant) were gathered using an analytical balance (Shimadzu AUW220®, Shimadzu Corporation, Japan).

Leaf chlorophyll concentration: chlorophyll reading and color appearance index

SPAD index was measured using a chlorophyll meter (SPAD-502 Plus® Chlorophyll Meter, Konica Minolta, Inc., Japan). Measurement was taken at the second youngest fully expanded leaf (Aroca et al. 2013) where the SPAD meter was clipped on the lamina away from the midrib. Three measurements/readings were taken on both sides of the leaf where the sensor/receptor of the SPAD meter was facing the adaxial side of the leaf. Leaf color and appearance of the salinity-stressed lettuce plants was determined at 33 DAT using a set of criteria (Fig. 1).

Rate	Description	
5	The whole plant is green; absence of necrotic spots in the young leaves; absence of leaf drying in the older leaves	
4	Older leaves are yellow green, yellow or pale; young leaves are still green; absence of necrotic spots in the young leaves; absence of leaf drying in the older leaves	
3	Older leaves are brown; young leaves are light green; absence of necrotic spots in the young leaves; and older leaves dried	
2	Older leaves are brown; young leaves are light green; presence of necrotic spots in the young leaves; older leaves dried	
1	The whole plant turned yellow to pale; all the leaves dried	

Figure 1. Leaf color and appearance rating

Statistical analysis

Data were subjected to Analysis of Variance (ANOVA) using the Statistical Tool for Agricultural Research 2.0.1, 2013 (International Rice Research Institute, Los Baños, Laguna, Philippines). Statistical differences between the lettuce plants without MykoPlus and with MykoPlus application under different levels of salinity stress were determined using the Least Significant Difference (LSD) test at 0.05% level of significance.

RESULTS AND DISCUSSION

Growth response (leaf number; root and shoot length; shoot, root, and whole plant dry weights) of Romaine lettuce applied with MykoPlus under varying saline concentrations

More leaves were produced in salinity-stressed Romaine lettuce plants applied with MykoPlus biofertilizer where the MykoPlus-applied lettuce plants produced 0.8 to 1.3 more leaves than the lettuce plants without MykoPlus regardless of salinity stress level (Fig. 2a). The positive effect of MykoPlus application in leaf production of salinity-stressed lettuce plants resulted to more area for photosynthesis that enhances the growth. Longer roots and shoots were observed in MykoPlus-applied lettuce plants under different levels of salinity stress. With increasing NaCl concentration up to 300 mM, roots of MykoPlus-applied lettuce plants were 15.91 mm to 24.46 mm longer than the lettuce plants without MykoPlus application (Fig. 2b). Moreover, shoots of MykoPlus-applied lettuce plants are 30.05 mm and 23.96 mm longer than the lettuce plants without MykoPlus grown in saline soils with 200 mM and 300 mM NaCl concentrations, respectively (Fig. 2c). Increased root and shoot growth can be due to the beneficial effects of the mycorrhizal fungi (Bowles et al. 2016), nitrogen fixers (Islam et al. 2013), and growth hormone secretors (Tailor and Joshi, 2014) contained in the biofertilizer. Beneficial effect of MykoPlus in terms of root and shoot growth is evident even up to 300 mM NaCl. For shoot to root ratio, MykoPlus-applied lettuce plants exposed to different levels of salinity stress produced longer shoots than roots, hence high shoot:root ratio. Heavier biomass was observed in lettuce plants applied with MykoPlus biofertilizer grown in saline soils. MykoPlus-applied lettuce plants produced 0.03 g to 0.04 g heavier shoots, 0.01 g heavier roots, and 0.04 g to 0.06 g heavier whole plant biomass as compared to lettuce plants without MykoPlus grown under different levels of salinity stress (Fig. 2d, 2e, and 2f). Moreover, the biomass of the lettuce plants without MykoPlus decreased with increasing levels of salinity stress. In a study by Kim et al. (2008), plant dry weight and height of lettuce plants were reduced at salinity levels greater than 100 mM NaCl.

The promotive effect of MykoPlus application in the leaf production of MykoPlus-applied lettuce plants grown in saline soil was evident at 100 mM and 200 mM NaCl concentrations. Root length was significantly improved by MykoPlus application in lettuce plants grown in all salinity stress level. Shoot length was also significantly enhanced in MykoPlus-applied lettuce plants grown in saline soils with 200 mM and 300 mM NaCl concentrations. Moreover, with increasing levels of salinity up to 300 mM NaCl, MykoPlus-applied lettuce plants produced heavier shoots, roots, and whole plant biomass than the lettuce plants without MykoPlus. On the other hand, leaf production, shoot length, and shoot dry weight of MykoPlus-applied lettuce plants were comparable to lettuce plants without MykoPlus grown in non-saline soil (Fig. 2).

Leaf chlorophyll concentration of Romaine lettuce applied with MykoPlus under varying saline concentrations

More than 4.49 to 9.94 increase in the SPAD reading or chlorophyll content (SPAD index) was observed in MykoPlus-applied lettuce plants and it remained to be high even at the highest level ofsalinity stress (300 mM NaCl) (Fig. 4a). MykoPlus application in the lettuce plants significantly increased the chlorophyll content of the leaves regardless of salinity stress level. The beneficial microorganisms contained in the biofertilizer may possibly promote enhanced photosynthetic activity and greener leaves in salinity-stressed lettuce plants. Improved chlorophyll content of salinity stressed-plants was reported by Cantrell and Linderman (2001) where vesicular-arbuscular mycorrhizal (VAM) fungi-treated lettuce plants produced greener leaves even at the highest level of salt. Sannazzaro et al. (2006) also reported the positive effect in the chlorophyll content of Mycorrhizal application in plants in response to salinity stress.

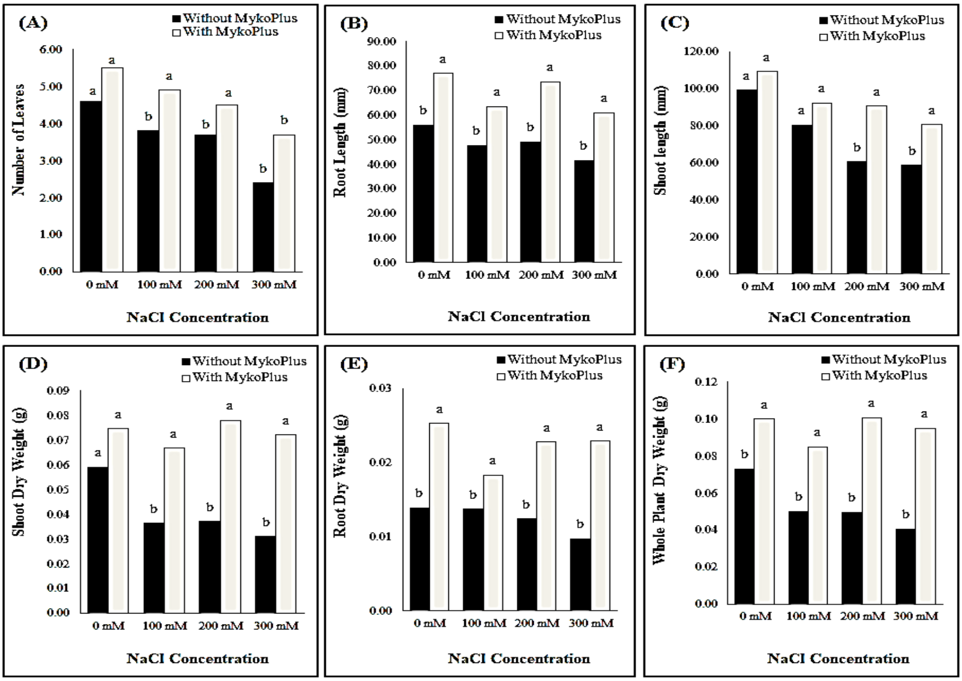

Figure 2. Effects of MykoPlus biofertilizer application on the number of leaves (*A*), root length (*B*), shoot length (*C*), shoot dry weight (*D*), root dry weight (*E*), whole plant dry weight (F) of Romaine lettuce (*Lactuca sativa L. var. longifolia*) plants under different levels of salinity stress. Growth parameters were taken at 33 DAT. DAT means days after transplanting. Means with the same letter are not significantly different at 0.05 level (LSD).

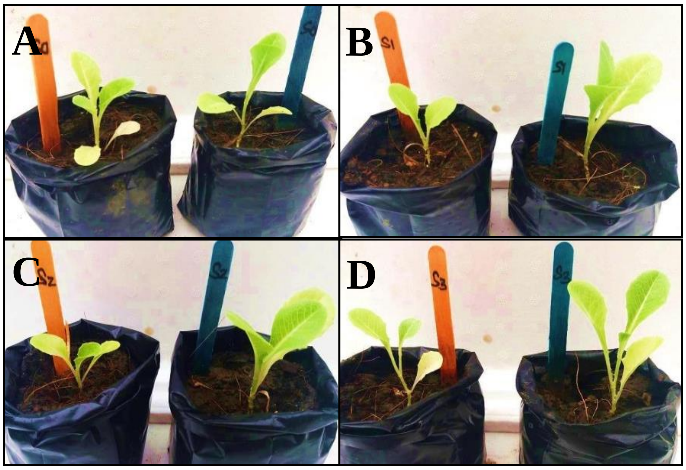

Figure 3. Representative samples of MykoPlus-applied Romaine lettuce plants grown in saline soils. Photos were taken after three applications of salt solution. Romaine lettuce plants without MykoPlus (*orange stick*) and with MykoPlus application (*blue stick*) were exposed to different levels of salinity stress by applying 0 mM NaCl (*A*), 100 mM NaCl (*B*), 200 mM NaCl (*C*), and 300 mM NaCl (*D*) thrice at 7 days interval.

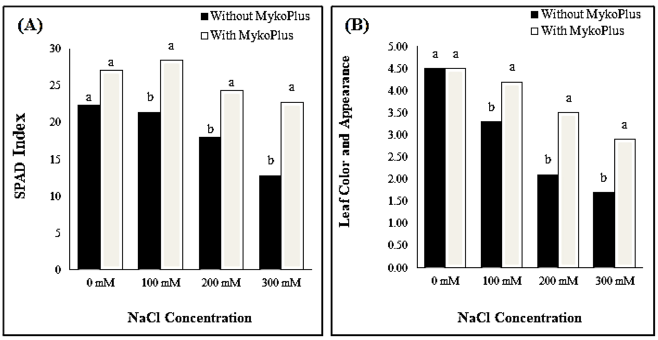

Figure 4. Effects of MykoPlus biofertilizer application on the SPAD index (*A*) and leaf color and appearance index (*B*) at 33 DAT of Romaine lettuce (*Lactuca sativa L. var. longifolia*) plants under different levels of salinity stress. SPAD index and leaf color and appearance were taken at 33 DAT. DAT means days after transplanting. Means with the same letter are not significantly different at 0.05 level (LSD).

The leaf color and appearance of the plants were determined at 33 DAT. The application of MykoPlus biofertilizer in the lettuce plants reduced leaf senescence or the incidence of yellowing and browning of the leaves (Fig. 4b). MykoPlus-applied lettuce plants developed less yellowing and necrosis in the young leaves and minimal leaf drying of the older leaves. The greenness and appearance of the leaves were enhanced by MykoPlus application at different levels of salinity. The reduced leaf senescence effect of MykoPlus biofertilizer in salinity-stressed lettuce plants became visible even if the plants were grown in high salinity (300 mM NaCl). Improved chlorophyll content was due to the reduced leaf senescence of the MykoPlus-applied lettuce plants planted in saline soils. The beneficial microorganisms from MykoPlus such as mycorrhizal fungi, nitrogen fixers, phosphorus solubilizers, growth hormone secretors, and others may have contributed to the greening of the leaves of the lettuce plants under salinity stress by providing proper growth conditions such as increased water absorption, increased nitrogen and phosphorus content of the soil (Perez, 2019), and providing growth hormones such as auxin and cytokinin (Allen et al. 1980; Barker and Tagu, 2000; Tailor and Joshi, 2014). However, with increasing level of salinity, the leaves drastically lost its green pigment. Similar result was reported by Kim et al. (2008) where the greenness of the lettuce decreased with increased NaCl concentrations. Yellowing, browning, and drying of the leaves and decreased chlorophyll content would result to low photosynthetic rate in plants, hence, less food production of plants resulting to decreased yield. Sheng et al. (2008) mentioned the reduction of chlorophyll content under salinity stress due to inhibition of the enzyme required for the biosynthesis of the chlorophyll molecules which Parida and Das (2005) correlate with the loss of pigments.

CONCLUSION AND RECOMMENDATIONS

The growth response and leaf chlorophyll concentration of the Romaine lettuce plants grown in saline soil were significantly enhanced by MykoPlus application. Under salinity stress conditions up to 300 mM NaCl, MykoPlus application significantly increased the chlorophyll content (high SPAD values), number of leaves, shoot and root length, and plant weights; and reduced leaf senescence (yellowing and browning of the leaves). The lettuce plants without MykoPlus application exhibited increased leaf senescence (accelerated yellowing, occurrence of necrotic spots, browning and drying of the leaves); reduced chlorophyll content (low SPAD values) and leaf production per plant; decreased shoot length, root length, and plant biomass with increasing levels of salinity. No statistically significant interaction effects were observed between MykoPlus and salinity stress level. It is recommended to extend the observation period towards the marketable stage of lettuce which is at 45 to 55 DAT and to apply MykoPlus biofertilizer to Romaine lettuce prior to transplanting in saline soil to alleviate the adverse effect of salinity stress.

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STATEMENT OF AUTHORSHIP

Both authors conceptualized and designed the study and prepared the manuscript. The first author prepared the initial write-up, performed the experiment, data gathering, and data analysis while the second author reviewed, edited, and finalized the manuscript.

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