Please prepare your Research Report in English or Japanese within three to ten pages including this page. The contents should include:

7. Background of Research

The atmospheric environment has experienced significant changes in the climate and air quality over the last decades, including significant increases in the levels of tropospheric ozone near the Earth surface and altered precipitation patterns in wide regions of the Earth.

Plants absorb ozone during gas exchange with the atmosphere, and, thus, increased levels of ozone can induce significant biological stress in plants which is reflected to suppressed photosynthesis and inhibited growth and productivity, effects that are similar to those of drought. Therefore, ozone pollution and drought pose a threat for seedlings planted in the framework of reforestation programs.

On the other hand, reforestation seedlings are commonly fertilized during their initial cultivation in nursery conditions. However, nutrient abundance in plant tissues may mediate plant symbioses with root mycorrhizae (which are known to enhance plant tolerance) with a potential for enhancing plant susceptibility to stressors such as ozone and drought.

Japanese larch [*Larix kaempferi* (Lamb.) Carr.] is an ectomycorrhizal conifer widely planted in wide forested areas of Japan. However, it was found to be sensitive to changing environmental conditions such as elevated ozone.

The purpose of this study was to assess the effect of ectomycorrhizae (ECM) on container-grown Japanese larch seedlings and how it depends upon soil fertility under ambient and elevated ozone levels or drought. We hypothesized that ECM can enhance the tolerance of plants against abiotic stress, which would suppress physiology and inhibit growth of plants due to lower photosynthetic efficiency, but the protection would depend on the soil fertility because plants would establish less symbioses with ECM when they can access directly abundant nutrients.

8. Research methodology

This study incorporated two independent experiments. In the first year, the interactive effects of ozone, soil fertilization and ECM were tested. In the second year, the interactive effects of drought, soil fertilization and ECM were tested.

The first year experiment was performed at the Sapporo Experimental Forest of Hokkaido University, Sapporo, Japan.

Seeds of Japanese larch were placed on wet paper filter in Petri dishes in room conditions for germination. In spring, 180 well-germinated seeds were transplanted into 180 pots filled with coco peat and irrigated until soil run-off. Plants were kept in a glasshouse of Hokkaido University, with open windows and uncontrolled environment.

The day after plantation, each plant was treated with 1 g (low fertilization, hereafter LF) or 2 g (high fertilization, hereafter HF) slow-release fertilizer [Osmocote Exact Standard 8-9M (15-9-11+2MgO+TE)].

In summer, ECM treatments were carried out. Inoculum source was obtained from freshly excavated root systems of Japanese larch saplings maintained in Sapporo Experimental Forest. Tap water in two buckets was infected with ECM by agitating ECM-infected roots. The water of the one bucket was subsequently sterilized by boiling. After sterilization, cold tap water was added to each bucket. The glasshouse was separated into two similar sides by closing a glass door in the middle. Half of the plants from each fertilization treatment were moved to the other half of the glasshouse. Plants in the one half of the glasshouse received 100 ml of sterilized water each (hereafter, non-ECM). Plants in the other half received 100 ml of non-sterilized water each (ECM).

Two weeks later, damping off symptoms were observed in seedlings of both ECM treatments. Seedlings with such symptoms were moved to a field with no nearby vegetation, and, all the remaining non-ECM plants were treated with a fungicide (during night). ECM-plants were treated with only water. This treatment was repeated approximately nine days later. Following the same methodology, some days later, non-ECM plants were sprayed again with a different fungicide. This treatment was repeated some days later.

The experimental design in the glasshouse was fully randomized, and the position of the plants was rotated on a weekly basis.

On August 2, all the plants were transferred outside from the greenhouse under moderate shadow, for adaptation to field conditions, and transferred to the Free Air Ozone-Concentration Enrichment (FACE) system on August 11. Plants were not irrigated manually for all the duration of ozone treatments.

The ozone treatments were conducted in the FACE system of Hokkaido University, Sapporo, Japan. The treatments were ambient ozone (AOZ) and elevated ozone (EOZ). For EOZ, ozone-enrichment was performed on a daily basis during the daytime, during the period August 18 to October 28. To increase the experimental robustness by accounting for environmental variability, two sub-plots were created in each FACE plot by placing 3-4 pots/plants per fertilizer and ECM treatments on a plastic base (completely randomized); one base was placed at the south side of each plot while the other base was placed at the north side of each plot.

The plants in each ozone treatment were rotated among the experimental units, every approximately ten days. Ambient ozone levels were recorded continuously using an ultraviolet absorption ozone analyzer. The average daily 10-hour (08:00-18:00) over the treatment period was ≈39 ppb. In the three EOZ plots the average daily 10-hour (07:00-17:00) ozone level over the treatment period was ≈65 ppb.

Gas exchange measurements were taken in situ using Licor gas exchange systems from 8 to 10 October. Stomatal conductance (Gs_{390}), photosynthetic rate (A_{390}) and transpiration rate (E_{390}) were determined at 390 μmol mol^{-1} CO_2, 60 ±5 % relative air humidity, and 1500 μmol m^{-2} s^{-1} light. The leaf temperature was maintained at 25 °C. After recording Gs_{390} and A_{390}, the light was set to 100 μmol m^{-2} s^{-1}, and Gs_{390} and A_{390} were recorded again 15 minutes later. The response of Gs_{390} and A_{390} to light was calculated as the percentage of change in the values of Gs_{390} and A_{390} recorded at 100 μmol m^{-2} s^{-1} (Gs_{100} and A_{100}) from those recorded at 1500 μmol m^{-2} s^{-1} (Gs_{1500} and A_{1500}). Gas exchange and was measured in randomly-selected sunlit mature needles, and the projected area of the measured needles was estimated after scanning the needles. Gas exchange measurements were taken during morning hours and completed by 11:00 JST.

Growth measurements were made on each plant on November 2. The stem height was measured as the distance from the soil surface to the top of the stem. The plant height was measured as the distance from the soil surface to the top of the crown. The height at which crown starts (from the soil surface) was also measured, and the depth of the crown was estimated by subtracting the height at which crown starts from the plant height. The crown span was measured by taking two crosswise measurements (the two widest points of the crown) using a measuring tape with 1-mm scaling. The stem basal diameter was also measured by taking two cross measurements per plant.

The seedlings were harvested on December 7, and separated into leaves, shoots and stem. Roots were washed gently to remove all the soil particles, and their length was measured. Samples were air-dried at 75 °C to constant mass. The number of buds was counted for each plant too. The dry matter of each stem (with branches) and of the foliage was measured for each plant. Shoot dry matter was calculated by summing the foliage dry matter and stem dry matter per plant.

At the end of the experiment, root analysis was carried out only for seedlings grown in the presence of LF to confirm the effectiveness of the ECM treatment. Therefore, 3 plants were analyzed for each ozone treatment for the ECM-treated plants and 4 plants were analyzed for each ozone treatment for the non-ECM-treated plants. The plants of each treatment had similar height. The root traits a) total number of root tips with fully formed mycorrhizae (with a perfect mantle, permitting categoryization into morphological types), b) total number of non-mycorrhizal roots, c) total number of root tips with forming mycorrhiza (i.e. incomplete mycorrhizae with no mantle) and d) total number of with roots with no root tip or aerial cut of root tip were counted for each plant, using an electronic stereoscope connected with a computer. From these, the total number of roots was calculated as the sum of the four measured root traits. The total number of root tips with fully formed mycorrhizae was 0 for all the measurements.

DNA of the ITS1 ~ ITS2 regions was analyzed. The basic local alignment search tool (BLAST) at the GenBank database of NCBI was utilized for identification of the symbiotic microorganisms.

Until the end of the experiment (November), all the seedlings that showed damping off symptoms in August were dead. The number of plants died due to damping off disease/condition was counted, and the survival rate of plants in each fertilization treatment was calculated as Nalive/Ntotal×100 where Nalive is the number of alive/healthy plants and Ntotal is the initial total number of healthy plants prior to damping off.

After the harvest, the dried foliage was pooled per one or two plants so as to give a composite sample for analysis and grounded into fine powder. The same was done for the roots. These samples were digested by HNO_3 using 100ml PP tubes and a thermal unit at 105 °C. The digested residue were diluted with 2% HNO_3 and filtrated with a 0.45μm Teflon membrane filter. The Al, B, Ca, Fe, K, Mg, Mn, Na, P, and Zn concentrations in the samples were determined by inductively coupled plasma mass spectrometry.

The second year experiment was performed at the Hokkaido Branch of Forestry and Forest Products Research Institute (FFPRI), Sapporo, Japan, following the similar methodology with the first year experiment. However, in this experiment drought was applied instead of ozone and LF was 0 g instead of 1 g (HF was 2 g as in the first year experiment). In this experiment, plants were given 70 mL of water twice a day, two times a week until drought treatments initiation. After plants were transplanted in big pots, water treatments began in October. Half of the plants were given 50 mL of water and half 500 mL of water per week. Gas exchange was measured before water treatments, 1 week after drought (after re-irrigation), 4 weeks after drought (after re-irrigation) and, finally, 5 weeks after drought (after-irrigation). Predawn water potential was also measured using a PMS pressure chamber at the end of the treatments. At the end of the treatments, growth, biomass, and leaf nutrient contents were also measured as explained above.

9. Results/impacts

Note: As much as possible, describe the contents and results of your research in a manner that is easily understandable to a non-specialist in your field. Provide a concrete description if (1) papers related to your work have been published in major academic journals, (2) particularly outstanding research results were achieved, or (3) patent applications have been made or other tangible outcomes achieved through the research.

In the first year experiment, we found that soil fertilization drove plant nutrient dynamics, as reflected in their contents in leaves and roots, and enhanced seedling growth. Low nutrient availability protected seedlings against damping off disease by increasing their survival rate. EOZ negatively affected gas exchange but had limited effects on growth and no effects on biomass production. Ozone was a less important stressor in this experiment where seedlings growth and productivity was restricted by factors other than ozone (soil and microorganisms). However, the results suggest potential ecological risks due to ozone-induced enhanced susceptibility to other stressors via changes in plant stem form. In contrast to our expectations, ECM treatment did not enhance tolerance of plants against elevated O_3, albeit it effectively increased the number of mycorrhiza-forming root tips. However, this is upon practical limitations arising from infection of plants with disease and other factors that might have affected the establishment of plant-microbe symbioses imbedding the formation of complete mycorrhizal root tips (e.g. small plant size). We also found that B and Na had an important role in stress responses. This experiment suggests that elevated ozone can have negative ecological effects on Japanese larch even if plants are grown in harsh environment that limits plant growth and productivity.

In the second year experiment, we found that drought reduced stomatal conductance to ¼ across treatments. In high water, ECM increased stomatal conductance by 140% in high fertilization. In low water, plants in low fertilization could keep 2-fold conductance across irrigation events than plants in high fertilization. In low water, plants in high fertilization could keep 160% higher conductance across irrigation events when treated with ECM. Overall, in low fertilization, there was a trend towards negative effects by ECM. Water limitation led to 140% lower net water potential, and, in high water, stomata of ECM-treated plants in low fertilization were unresponsive. We also found a delayed effect of drought on plants grown in low fertilization compared to high fertilization (same with conductance). Overall, photosynthesis was three times higher in high water than in low water, and, 130% higher in ECM-treated plants in high fertilization and high water than in other treatments in high water. Putting these into context, water limitation decreased water potential and thus conductance and photosynthesis. ECM treatment had a positive effect on gas exchange under drought when plants were grown in nutrient-abundant soil, however, ECM treatment tended to have a negative effect on gas exchange under drought when plants were grown in nutrient-poor soil.

This research will offer a remarkable perspective for applicability into the practice by incorporating fertilization-biostimuli optimum cost-benefit relations. The first research paper of the first year experiment is submitted into an SCI journal.

10. Research Presentations during the period of the fellowship (Name of the conference, title, place, date)

i. The 130th Annual Meeting of the Japanese Forest Society, Interactive effects of ectomycorrhizae, fertilization and drought on growth and physiology of container-grown larch, Niigata, Japan, 20-23 March 2019.

ii. International Symposium on Forests and Health and Forum for Under-Forestry Economic Industry, Novel methodologies for forest health and ecological services sustainability, Harbin, P.R. China, 2-5 August 2018.

iii. International Symposium on Forests and Health and Forum for Under-Forestry Economic Industry, Japanese larch seedlings grown in containers under the interaction of nutrient availability and ozone, Harbin, P.R. China, 2-5 August 2018.

iv. International Symposium on Forests and Health and Forum for Under-Forestry Economic Industry, The new generation Free Air Controlled Exposure (FACE) system for exposing communities of deciduous tree species to ozone in Japan, Harbin, P.R. China, 2-5 August 2018.

v. International Conference on Ozone and Plant Ecosystems, Japanese larch seedlings grown in containers, inoculated with ectomycorrhizal fungi, and exposed to ozone, Florence. Italy, 21-25 May 2018.

vi. Preconference workshop "Mediterranean & Middle East Air Pollution In A Changing Climate", "Climate Change in the Mediterranean and the Middle East: Challenges and Solutions", Container-grown Japanese larch seedlings treated with fertilizer, inoculated with ectomycorrhizal fungi, and exposed to ozone-enriched atmosphere, Nicosia, Cyprus, 16-17 May 2018.

vii. Preconference workshop "Mediterranean & Middle East Air Pollution In A Changing Climate", "Climate Change in the Mediterranean and the Middle East: Challenges and Solutions", The new generation Free Air Controlled Exposure (FACE) system for exposing communities of deciduous tree species to O_3 in Japan, Nicosia, Cyprus, 16-17 May 2018.

viii. The 65th Annual Meeting of the Ecological Society of Japan, Responses of container-grown Japanese larch to ectomycorrhizal inoculation, fertilization and ozone, Sapporo, Japan, 14-18 March 2018.

ix. The 28th IUFRO conference for Specialists in Air Pollution and Climate Change Impacts on Forest Ecosystems: "Actions for Sustainable Forest Ecosystems under Air Pollution and Climate Change", Promoting abiotic stress tolerance of container-grown seedlings by inoculating ectomycorrhizal fungi, Tokyo, Japan, 22-26 October 2017.

x. 3rd Asian Air Pollution Workshop (AAPW), Ozone effects on Japanese larch seedlings grown in containers under low or high fertilization and inoculated with ectomycorrhizal fungi, Tokyo, Japan, 20-22 October 2017.

xi. IUFRO 125th Anniversary Congress, The new generation Free Air Controlled Exposure (FACE) system for exposing communities of deciduous tree species to O_3 in Japan, Freiburg, Germany, 18-22 September 2017.

11. A list of paper published during or after the period of the fellowship, and the names of the journals in which they appeared (Please fill in the format below). Attach a copy of each article if available.

Author(s)	Title	Name of Journal	Volume	Page	Date	Note
Agathokleous, E., Kitao, M., Harayama, H.	On the non-monotonic, hormetic photoprotective response of plants to stress	Dose-Respons e	DOI: 10.1177/ 1559325 8198384		2019	In Press
Agathokleous, E., Belz, R.G., Kitao, M., Koike, T., Calabrese, E.J	Does the root to shoot ratio show a hormetic response to stress? An ecological and environmental perspective	Journal of Forestry Research	DOI: 10.1007 /s11676-018-086		2019	In Press
Agathokleous, E., Calabrese, E.J.	Hormesis can enhance agricultural sustainability in a changing world	Global Food Security	20	150-155	2019	
Agathokleous, E., Kitao, M., Calabrese, E.J.	Hormesis: A compelling platform for sophisticated plant science	Trends in Plant Science	24	28-37	2019	
Agathokleous, E., WaiLi, Y., Ntatsi, G., Konno, K., Saitanis, C.J.,	Effects of ozone and ammonium sulfate on cauliflower: emphasis on the interaction between	Science of the Total Environment	659	995-100 7	2019	
Kitao, M., Kitaoka, S., Harayama, H., Agathokleous, E., Han, Q., Uemura, A., Furuya, N., Ishibashi, S.	Sustained growth reduction in forest-floor seedlings of Sakhalin fir associated with the previous-year springtime photodamage after a winter cutting of canopy trees	European Journal of Forestry Research	138	143-150	2019	
Agathokleous, E., Anav, A., Araminiene, V., De Marco, A., Domingos, M., Kitao, M., Koike, T., Manning, W.J., Paoletti, E., Saitanis, C.J., Sicard, P., Vitale, M., Wang, W., Calabrese, E.J.	Commentary: EPA's proposed expansion of dose-response analysis is a positive step towards improving its ecological risk assessment	Environmenta l Pollution	246	566-57	2019	
Calabrese, E.J.C., Agathokleous, E., Kozumbo, W.J., Stanek, E.J.III., Leonard, D.	Estimating the range of the maximum hormetic stimulatory response	Environmenta l Research	170	337-343	2019	
Agathokleous, E., Kitao, M., Calabrese, E.J.	New insights into the role of melatonin in plants and animals	Chemico-Biol ogical Interactions	299	163-167	2019	

Araminiene, V., Sicard, P., Anav, A., Agathokleous, E., Stakėnas, V., De Marco A., Varnagirytė-Kabašinskienė, V., Paoletti, E., Girgždienė, R.	Trends and inter-relationships of ground-level ozone metrics and forest health in Lithuania	Science of the Total Environment	658	1265-1277	2019	
Calabrese, E.J., Agathokleous, E.	Building biological shields via hormesis	Trends in Pharmacological Sciences	40	8-10	2019	
Agathokleous, E., Kitao, M., Calabrese, E.J.	Hormetic dose responses induced by lanthanum in plants	Environmental Pollution	244	332-341	2019	
Agathokleous, E., Kitao, M., Harayama, H., Calabrese, E.J.	Temperature-induced hormesis in plants	Journal of Forestry Research	30	13-20	2019	
Agathokleous, E., Belz, R.G., Calatayud, V., De Marco, A., Hoshika, Y., Kitao, M., Saitanis, C.J., Sicard, P., Paoletti, E., Calabrese, E.J.	Predicting the effect of ozone on vegetation via the linear non-threshold (LNT), threshold and hormetic dose-response models	Science of the Total Environment	649	61-741	2019	
Kita, K., Kon, H., Ishizuka, W., Agathokleous, E., Kuromaru, M.	Survival rate and shoot growth of grafted Dahurian larch (*Larix gmelinii var. japonica*): a comparison between Japanese larch (*L. kaempferi*) and F_1 hybrid larch (L. gmelinii var. japonica × L. kaempferi) rootstocks	Silvae genetica	67	111-116	2018	
Abu ElEla S.A., Agathokleous, E., Ghazawy, N.A., Amin, T.R., ElSayed, Q.M., Koike, T.	Enzyme activity modification in adult beetles (*Agelastica coerulea*) inhabiting birch trees in an ozone-enriched atmosphere	Environmental Science and Pollution Research	25	32675–32683	2018	
Sicard, P., Agathokleous, E., Araminiene, V., Carrari, E., Hoshika, Y., De Marco, A., Paoletti, E.	Should we see urban trees as effective solutions to reduce increasing ozone levels in cities?	Environmental Pollution	243	163-176	2018	
Agathokleous, E., Kitao, M., Calabrese, E.J.	Human and veterinary pharmaceuticals induce hormesis in plants: scientific and regulatory issues and an environmental perspective	Environment International	120	489-495	2018	

Agathokleous, E., Kitao, M., Calabrese, E.J.	Biphasic effect of abscisic acid on plants: a hormetic viewpoint	Botany	96	637-642	2018	
Agathokleous, E., Kitao, M., Calabrese, E.J.	Environmental hormesis and its fundamental biological basis: Rewriting the history of toxicology	Environmenta l Research	165	274-278	2018	
Agathokleous, E., Kitao, M., Calabrese, E.J.	Emission of volatile organic compounds from plants shows a biphasic pattern within an hormetic context	Environmenta l Pollution	239	318-321	2018	
Agathokleous, E., Kitao, M., Qingnan, C., Saitanis, C.J., Paoletti, E., Manning, W.J., Watanabe, T., Koike, T.	Effects of ozone (O3) and ethylenediurea (EDU) on the ecological stoichiometry of a willow grown in a free-air exposure system	Environmenta l Pollution	238	663-676	2018	
Agathokleous, E., Kitao, M., Calabrese, E.J.	The rare earth element (REE) lanthanum (La) induces hormesis in plants	Environmenta l Pollution	238	1044-10 47	2018	
Agathokleous, E., Kitao, M.	Ethylenediurea (EDU) induces hormesis in plants	Dose-Respons e	16	2	2018	
Kitao, M., Harayama, H., Han, Q., Agathokleous, E., Uemura, A., Furuya, N., Ishibashi, S.	Springtime photoinhibition constrains regeneration of forest floor seedlings of Abies sachalinensis after a removal of canopy trees during winter.	Scientific Reports	8	6310	2018	
Abu ElEla S.A., Agathokleous, E., Koike, T.	Growth and nutrition of *Agelastica coerulea* (Coleoptera: Chrysomelidae) larvae changed when fed with leaves obtained from an O_3-enriched atmosphere	Environmenta l Science and Pollution Research	25	13186-1 3194	2018	
Kitao, M., Kitaoka, S., Harayama, H., Tobita, H., Agathokleous, E., Utsugi, H.	Canopy nitrogen distribution is optimized to prevent photoinhibition throughout the canopy during sun flecks	Scientific Reports	8	503	2018	
Agathokleous, E.	Environmental hormesis, a fundamental non-monotonic biological phenomenon with implications in ecotoxicology and environmental safety	Ecotoxicology and Environmenta l Safety	148	042-105 3	2018	
Agathokleous, E., Paoletti, E., Manning, W.J., Kitao, M., Saitanis, C.J., Koike, T.	High doses of ethylenediurea (EDU) as soil drenches did not increase leaf N content or cause phytotoxicity in willow grown in fertile soil	Ecotoxicology and Environmenta l Safety	147	574-584	2018	

Wang, X., Agathokleous, E., Qu, L., Fujita, S., Watanabe, M., Tamai, Y., Mao, Q., Koyama, A., Koike, T.	Effects of simulated nitrogen deposition on ectomycorrhizae community structure in hybrid larch and its parents grown in volcanic ash soil: the role of phosphorous	Science of the Total Environment	618	905-915	2018	
Agathokleous, E., Kitao, M., Kinose, Y.	A review study on O_3 phytotoxicity metrics for setting critical levels in Asia	Asian Journal of Atmospheric Environment	12	1-1	2018	
Sugai, T., Kam, D.-G., Agathokleous, E., Watanabe, M., Kita, K., Koike, T.	Growth and photosynthetic response of two larches exposed to O3 mixing ratios ranging from preindustrial to near future	Photosynthetica	56	901-910	2018	
Agathokleous, E., Sakikawa, T., Abu ElEla S.A., Mochizuki, T., Nakamura, M., Watanabe, M., Kawamura, K., Koike, T.	Ozone alters the feeding behavior of the leaf beetle *Agelastica coerulea* (Coleoptera: Chrysomelidae) into leaves of Japanese white birch (*Betula platyphylla var. japonica*)	Environmental Science and Pollution Research	24	17577–17583	2017	
Agathokleous, E.	Perspectives for elucidating the ethylenediurea (EDU) mode of action for protection against O_3 phytotoxicity	Ecotoxicology and Environmental Safety	142	530-537	2017	

12. Awards during the period of the fellowship (Name of the award, Institution, date etc.)

i. Forests 2019 Travel Award for postdocs, mdpi, 02/28/2019
ii. Outstanding Reviewer for the journals: Chemico-Biological Interactions, Biomedicine & Pharmacotherapy, Ecotoxicology and Environmental Safety, Elsevier, 12/31/2018 (please note the date represents the entire year due to different dates for different journals)
iii. Recognized Reviewer for the journals: Life Sciences in Space Research, Journal of Plant Physiology, Elsevier, 12/31/2018 (please note the date represents the entire year due to different dates for different journals)
iv. Top 1% of reviewers in Plant & Animal Science and in Environment/Ecology, Publons, 09/12/2018
v. Agronomy Travel Award for Junior Investigator, mdpi, 06/16/2018
vi. Outstanding New Investigator Award, International Dose-Response Society, 04/18/2018
vii. Top 1% of reviewers in Environmental Science and in Agricultural and Biological Sciences, Publons, 09/12/2017