



# Salt Stress Reduced the Seedling Growth of Two Larch Species Under Elevated Ozone

Tetsuto Sugai<sup>1†</sup>, Wang Yannan<sup>2†‡</sup>, Toshihiro Watanabe<sup>1\*</sup>, Fuyuki Satoh<sup>3</sup>, Laiye Qu<sup>4,5\*</sup> and Takayoshi Koike<sup>1,4</sup>

<sup>1</sup> Plant Nutrition Laboratory, Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan, <sup>2</sup> Forest Ecology and Silviculture Laboratory, Graduate School of Agriculture, Hokkaido University, Sapporo, Japan, <sup>3</sup> Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Japan, <sup>4</sup> State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Science, Chinese Academy of Sciences, Beijing, China, <sup>5</sup> University of Chinese Academy of Sciences, College of Resources and Environment, Beijing, China

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### \*Correspondence:

Toshihiro Watanabe  
nabe@chem.agr.hokudai.ac.jp  
Laiye Qu  
lyqu@rcees.ac.cn

<sup>†</sup>These authors have contributed  
equally to this work

### ‡Present address:

Wang Yannan,  
Jiangxi Academy of Forestry,  
Nanchang, China

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The effect of elevated ozone (eO<sub>3</sub>) and soil salinization with alkaline salts in northeastern (NE) China is a serious concern affecting the success of the national replanting project. As planted areas exceed 4 million hectares in China, we must consider future afforestation efforts after thinning and harvesting. Here, we investigated eO<sub>3</sub> and salt stress on Dahurian larch (*Larix gmelinii* var. *japonica*; DL) and Japanese larch (*L. kaempferi*; JL) seedlings. The seedlings were exposed to eO<sub>3</sub> (~70 nmol mol<sup>-1</sup>) and ambient ozone (aO<sub>3</sub>) (~25~40 nmol mol<sup>-1</sup>) for one growing season in an open top chamber (OTC) system with simulated salinity in NE China (alkaline salt, NaHCO<sub>3</sub>:NaCl = 9:1, 20mM Na<sup>+</sup>). The seedlings under salt-free treatment received tap water. Although the effects of eO<sub>3</sub> on DL were not significant, eO<sub>3</sub> significantly increased total dry mass and total leaf area index of JL. There was no significant reduction in total dry mass under salt stress in both species. The relationship between needle Na and other mineral contents indicated that both species maintained K contents even with excess Na contents in needles. DL showed relatively lower reduction of other mineral contents, indicating higher salt tolerance of needle element homeostasis than JL. Contrary to our hypothesis, there were no interaction effects of eO<sub>3</sub> and salt stress on both species. These results indicated that DL seedlings may be more suitable than JL seedlings as a future afforestation species under eO<sub>3</sub> levels of <70 nmol mol<sup>-1</sup> at saline soil condition.

**Keywords:** combined effect, salt stress, larch, interspecific differences, homeostasis

## INTRODUCTION

Ground-level ozone (O<sub>3</sub>) is the most harmful air pollutant to forest ecosystems on both local and global scales (Paoletti et al., 2007, 2010; Matyssek et al., 2012; Sicard and Dalstein-Richier, 2015; Sicard et al., 2016). Ozone suppresses plant growth, accelerates leaf senescence, changes carbon allocation to roots, and induces nutritional imbalances in leaves (Matyssek et al., 2012; Agathokleous et al., 2015; Shi et al., 2017). Effects of elevated O<sub>3</sub> (eO<sub>3</sub>) on forest ecosystems are a concern in northeast (NE) Asia (Koike et al., 2013; Akimoto et al., 2015). In China, the annual daily maximum O<sub>3</sub> concentration in some regions often reaches 60 nmol mol<sup>-1</sup> (Gaudel et al., 2018). Recent studies have predicted that the annual mean change in surface O<sub>3</sub> concentrations

in Asia will rise to  $\sim 8$  nmol mol<sup>-1</sup> by 2050 (Lee et al., 2015; Turnock et al., 2018). The current eO<sub>3</sub> level is high enough to suppress tree growth and degrade ecosystem health (Chen et al., 2015; Feng et al., 2015). Thus, the conservation of forests in China is an issue of increasing interest in regions that face multiple environmental stresses, such as eO<sub>3</sub>, nitrogen deposition, and drought (Reilly et al., 2007; Fang et al., 2014; Hu et al., 2015; Yuan et al., 2016).

Several regions of China have begun reforestation because of the intensive thinning and harvesting that has persisted since the Grain for Green Project began in 1999 (Zhang et al., 2000; Feng et al., 2005; Xu, 2011). However, in NE China, where forestry practices have been active, not all afforestation is well established cause to the soil salinization that occurs with low precipitation and high temperature (e.g., Feng et al., 2005; Sakai, 2012). Saline soil occupies  $\sim 99,000$  km<sup>2</sup> of China's land area (Zhang et al., 2010; Xu, 2011; Li et al., 2014). The frequency of extreme high temperatures in summer has been increasing in China over 60-fold since the 1950s (Sun et al., 2014). The relative drought conditions may increase the potential for salinization stresses (Polle and Chen, 2015). Especially in NE China, soil salinization often occurs with low precipitation and high temperature (Ma and Fu, 2006; Zhou et al., 2011; Sakai, 2012). Excess salinity causes physiological and metabolic imbalance in plants via ionic stresses (Marschner, 2012; Polle and Chen, 2015; Wungrampha et al., 2018). The effects of salinity on plants have been investigated with sodium chloride (NaCl), which are known to cause ionic imbalance and inhibit mineral ion uptake of potassium (K) and calcium (Ca) (Gerosa et al., 2014; Guidi et al., 2016; Plesa et al., 2018). Since arid and semi-arid lands contain chlorides and carbonates of several minerals, including Na, Ca, and magnesium (Mg), the severe effects of alkaline salt accumulation on plants and trees have received increased attention (Yang et al., 2008; Liu and Shi, 2010; Wang et al., 2013). For example, in the Songnen plain of Heilongjiang Province, 3.2 million ha are influenced by alkaline salt accumulation, and continues to expand by 20,000 ha per year (Wang et al., 2009). Thus, for forest conservation in NE China, we should investigate the combined stresses of eO<sub>3</sub> and salts on forest growth and health.

The combined effects of eO<sub>3</sub> and salt have largely been described in Mediterranean areas. For example, metabolic responses in pomegranate (*Punica granatum* L.) have indicated eco-physiological adaptation up to 50 mM NaCl stress, but did not mitigate O<sub>3</sub>-induced oxidative stresses (Calzone et al., 2019). Holm oaks (*Quercus ilex* L.) treated under 150 mM NaCl treatments did not show significant photosynthetic depression by eO<sub>3</sub> (Guidi et al., 2016). In addition, previous studies have reported that salt stresses mitigated negative effects of eO<sub>3</sub> via stomatal closure (Gerosa et al., 2014), and its effects were exacerbated under salt stresses (Calzone et al., 2019), or no interaction between eO<sub>3</sub> and salt stresses (Guidi et al., 2016). Thus, the views of the combined effects of eO<sub>3</sub> and salt stresses are still inconsistent.

Larch (*Larix* sp.) has a higher tolerance to harsh conditions and is widely planted throughout northern hemisphere (Gower and Richards, 1990; Zhang et al., 2000; Ryu et al., 2009; Mao et al.,

2010). For examples, Dahurian larch (*Larix gmelinii*) has a higher expectation as an afforestation species in NE China (Zhang et al., 2000; Shi et al., 2010). Japanese larch (*L. kaempferi*, JL) has been planted in the Korean peninsula and northern Japan (Lee et al., 2004; Ryu et al., 2009). The current larch forests occupy over 40 % of the total carbon growing stock in Eurasian boreal forests, including NE China (Alexeyev et al., 2000; Wang et al., 2001). Based on the Chinese policy of replantation since 1999, increased afforestation has raised concerns regarding low species diversity in forests and poor ecological functions, such as material cycling and habitat provision (Xu and Wilkes, 2004; Zhang and Zhang, 2007). Thus, the effects of eO<sub>3</sub> and salt should be investigated on *Larix* sp. (Zhang et al., 2000; Chazdon, 2008; Ryu et al., 2009) as well as exciting afforestation species, such as *Populus* sp.

The purpose of this study was to evaluate the effects of eO<sub>3</sub> and alkaline salt on Dahurian larch (DL) and Japanese larch (JL) seedlings. Koike et al. (2012) reported the O<sub>3</sub> suppressed photosynthesis rate of only DL but not of JL. While many studies have reported that O<sub>3</sub>-induce inhibition can be determined by absorbed O<sub>3</sub> amount (Matyssek et al., 2004; Hu et al., 2015), there were no significant differences in stomatal conductance between DL and JL seedlings (Koike et al., 2012). These results indicate that O<sub>3</sub> sensitivity differences between these two larch species can be caused by non-stomatal factors, which may be evaluated through PSII photochemical systems (Gielen et al., 2006; Guidi et al., 2016) and the leaf nutritional status (e.g., Barnes et al., 1995; Shang et al., 2018).

We expected that the inhibitions of eO<sub>3</sub> and salt could be evaluated via nutrient imbalance status. It was reported that the nutrient concentrations in plants, such as N and P, were increased by eO<sub>3</sub>, which may be adaptive strategy against eO<sub>3</sub> (Cao et al., 2016; Shang et al., 2018). Besides, Larch may have specific salt tolerance to the K homeostasis (Renault, 2005; Plesa et al., 2018). It was reported that NaCl treatment did not inhibit K absorption in larch saplings; rather, the saplings took up more K even with NaCl treatment compared to the control condition (Renault, 2005; Plesa et al., 2018). K homeostasis is one of the salt tolerances and it has been considered as secondary importance in many published literatures (Tester and Davenport, 2003; Marschner, 2012). There are several other mechanisms to manage excess Na contents such as the exclusion of Na and osmotic regulation by compatible solutes (Flowers, 2004; Munns et al., 2006). However, it has reported that the practical outcomes of these approaches were not effective on improving salt tolerance of plants under filed conditions (Shabala and Cuin, 2007; Wang et al., 2013; Anschutz et al., 2014).

Increasing K contents could induce the homeostasis of photosynthesis and metabolic activity, and the osmotic adjustment (Volkov et al., 2003; Shabala and Cuin, 2007). In fact, an optimal K status can be index for stress resistance when plants are exposed to abiotic stresses (Marschner, 2012; Wang et al., 2014). It was reported that a K fertilization compensated the O<sub>3</sub> effects on potted Norway spruce (*Picea abies*) seedlings although there were genetic-specific responses (Keller and Matyssek, 1990; Barnes et al., 1995), suggesting that the species with relatively higher K content may show insights for the O<sub>3</sub> tolerance. Furthermore, several studies have investigated

the mineral homeostasis under eO<sub>3</sub> (Alcántara et al., 2006; Agathokleous et al., 2018). These studies suggest that the homeostatic adjustment in leaf element content can mitigate physiological inhibitions of eO<sub>3</sub> although they focused on the homeostasis of other mineral element such as iron and P.

Here, we firstly expected that (i) O<sub>3</sub> would suppress growth of DL but not of JL as Koike et al. (2012) reported. However, we hypothesized (ii) DL is more tolerant relative to JL since it is more adapted to salty soils due to its ecological distribution (Abaimov, 2010; Mao et al., 2010; Mamet et al., 2019), where soil salinization often occurs (Shi et al., 2010; Zhou et al., 2011), and thus salt treatment would not inhibit K absorption in DL. This comparison of salt responses between the two larches may provide novel insights on the eco-physiology to understand how larch acquires the adaptive capacity to salt stress. Further, it has reported that the reduction of K content in salt-tolerant wheat was lower than in salt-sensitive wheat at combined effect of eO<sub>3</sub> and salt treatment (Zheng et al., 2014). Hence, given that DL can maintain higher K contents than JL under eO<sub>3</sub> and salt treatment, (iii) DL may show higher tolerance to the synergic effect than JL. Based on our results, we assessed the possibility of future candidate afforestation species in northeast Asian regions.

## MATERIALS AND METHODS

### Study Site and Materials

Experiments were conducted at the experimental nursery of the Sapporo Experimental Forest, Hokkaido University in northern Japan (43° 04' N, 141° 20' E 15 m a. s. l.). Two-year-old Dahurian larch (*Larix gmelinii* var. *japonica*; DL) and Japanese larch (*Larix kaempferi*; JL) seedlings were cultivated from seeds in the nursery of the Hokkaido Forestry Research Organization (HRO), Forestry Research Institute in Bibai city near Sapporo. The seedlings were transplanted into 1-L pots (Height: 114 mm, top diameter: 135 mm, bottom diameter: 95 mm) in early May 2017 before buds opened. The initial stem diameter means  $\pm$  standard deviations were  $1.13 \pm 0.23$  mm for DL and  $1.32 \pm 0.31$  mm for JL, and the height was  $8.83 \pm 1.35$  cm for DL and  $8.55 \pm 1.73$  cm for JL, respectively. Soils underneath the seedlings were removed just before transplantation, with well-weathered volcanic ash soil (Kanuma and Akadama soil, 1:1, v/v) used as cultivation soil in pots. To measure the original nutrient status of the soil,  $\sim 10$  g of soil and 25 ml ultrapure water were mixed and shaken for 1 h. The samples were poured through filter paper (110 mm mesh, ADVANTEC, Tokyo, Japan) and the filtrate was centrifuged at 2,000 G for 20 min. The supernatant fluid was mixed with 61% nitric acid and adjusted for the concentration of 2% nitric acid. Mineral elements contents of K, Ca, Mg, and Na were measured by ICP-MS (ELAN, DRC-e; Perkin Elmer, Waltham, MA, USA). The Na adsorption rate of this soil medium was also measured. The three levels of NaCl dissolved in demineralized water were loaded on the pot soil, which contained approximately 1,200 g of soil without seedlings, seven times during 3 weeks (110, 190, and 240 mmol Na in total). There were no leaks from the pot after each loading. After completing Na loading, the soil sample was obtained from the well-mixed and dried soil. Na content of the sample was measured by the same method described above.

Na adsorption rate was calculated as the difference between total loaded Na and the water extracted Na amount. Results of the original nutrient status and the Na adsorption rate were summarized in **Supplemental Data**. Until treatments started, irrigation was manually carried out in the morning once every 3 or 4 days to avoid soil drying. Balanced liquid fertilizer (200 ml, 1/2,000, v/v, HYPONEX, Japan; 36.2 mg N L<sup>-1</sup>, 142 mg P L<sup>-1</sup>, 47.1 mg K L<sup>-1</sup>) was applied once every week to ensure nutrient balance (four times in total).

### Experimental Design

Four treatments were totally established in this study: ambient O<sub>3</sub> and tap water irrigation as the control (aO<sub>3</sub>+CW), elevated O<sub>3</sub> and control water irrigation (eO<sub>3</sub>+CW), ambient O<sub>3</sub> and salt water irrigation (aO<sub>3</sub>+SW), and elevated O<sub>3</sub> and salt water irrigation (eO<sub>3</sub>+SW), respectively. All treatments were conducted from June 27 to September 25, 2017 before the winter season. The mean temperature was 19.6°C and precipitation was 127.38 mm during the experimental period. The open top chamber (OTC; 1.2  $\times$  1.2  $\times$  1.2 m) system was adopted to control the O<sub>3</sub> concentration levels in each treatment. Seedlings were grown in OTC surrounded by a polyvinyl chloride film (Noh-bi Co. Ltd., Sapporo, Japan), such that approximately 88% sunlight could be transmitted without UVB and UVC radiation. The O<sub>3</sub> concentration monitored is described in **Table 1**. More detailed descriptions of the methodology of the O<sub>3</sub> exposure system in OTCs were provided in Sugai et al. (2019). Plants in the salt treatment were watered with 200 ml of saline water. Control plants were maintained by watering with 200 ml of tap water. The applications were performed at 3- or 4-day intervals for a total of 20 times. Mixed salt composed of alkaline salt (NaHCO<sub>3</sub>) and neutral salt (NaCl) was used to imitate the realistic extant salt-alkaline soil condition of NE China (Ge and Li, 1990; Sakai, 2012). The two salts were mixed NaHCO<sub>3</sub>:NaCl = 9:1 to make 20 mM Na<sup>+</sup> saline water. To prevent salt leaching, rainfall was excluded by plastic shelters over the top of the OTC only during rainy days. During the final experimental period when the seedlings were harvested, 10 g of fresh soil samples were taken from the surface and bottom soils in the pots of all treatments to measure soil pH. The depth of soil sampling was set at approximately 2 cm from both the surface and bottom. The soil was shaken for 1 h, with 25 ml ion-exchanged water as soon as possible after sampling. The soil pH was measured by a portable pH meter (M-12, Horiba, Japan). All treatments were replicated four times with 16 OTCs, with a split-plot completely randomized design with four seedlings in each OTC (totally 128 seedlings).

### Growth, Leaf Mass Area, and Total Leaf Area Index

Sixty four seedlings for each species were harvested and washed with tap water. After washing, needles, branches, stems, and roots were separated. Each sample was oven dried at 70°C until a constant weight was achieved, and then weighed.

Mature needle leaves were sampled at the same shoot position near the crown in each seedling at the end of September 2017. Part of the needle samples was used to measure the leaf mass area

**TABLE 1** | Summary of the mean concentration at ambient (aO<sub>3</sub>) and elevated ozone (eO<sub>3</sub>) for daytime (06:00–18:00) and over 24 h in the OTC from June to September 2017, and pH in the surface and bottom soil (~2 cm layer) of Dahurian larch (DL) and Japanese larch (JL) seedlings under all treatments: ambient O<sub>3</sub> and tap water irrigation (aO<sub>3</sub>+CW) as the control, elevated O<sub>3</sub> and control water irrigation (eO<sub>3</sub>+CW), ambient O<sub>3</sub> and salt water irrigation (aO<sub>3</sub>+SW), and elevated O<sub>3</sub> and salt water irrigation (eO<sub>3</sub>+SW) in September 2017.

		O <sub>3</sub> concentration (nmol mol <sup>-1</sup> )	
		Daytime average	24 h average
aO <sub>3</sub>	June	43.99 ± 0.79	39.28 ± 0.62
	July	43.59 ± 0.82	36.99 ± 0.61
	August	40.09 ± 1.48	32.24 ± 0.94
	September	34.02 ± 1.00	27.11 ± 0.89
eO <sub>3</sub>	June	71.91 ± 1.29	50.28 ± 1.39
	July	71.13 ± 1.12	47.38 ± 1.28
	August	70.64 ± 1.44	46.60 ± 1.62
	September	70.39 ± 1.19	46.17 ± 1.87
		Soil pH	
		Surface soil	Bottom soil
DL	aO <sub>3</sub> +CW	6.37 ± 0.01	6.05 ± 0.03
	eO <sub>3</sub> +CW	6.52 ± 0.04	6.08 ± 0.03
	aO <sub>3</sub> +SW	7.96 ± 0.11	7.19 ± 0.01
	eO <sub>3</sub> +SW	8.17 ± 0.13	6.93 ± 0.01
JL	aO <sub>3</sub> +CW	6.37 ± 0.03	6.04 ± 0.04
	eO <sub>3</sub> +CW	6.37 ± 0.02	6.11 ± 0.02
	aO <sub>3</sub> +SW	7.92 ± 0.07	7.21 ± 0.07
	eO <sub>3</sub> +SW	8.10 ± 0.02	7.29 ± 0.04

All values are means ± SE.

(LMA) and the other part was used to measure the contents of several nutrient elements (see below). The projected area of fresh needles was measured by an image scanner (LIDE 200, Canon, Japan). After the projection measurement, the needle dry mass was measured by the same method as above. LMA was calculated as dry weight divided by the needle area projected.

As a species difference between two studied larches, the lower needle proportion of DL than JL has been reported (Harayama et al., 2013; Dong-Gyu et al., 2015). To evaluate these differences under eO<sub>3</sub> and salt stress, we calculated needle dry mass divided by total dry mass as the needle ratio. While larch species have both short and long needles (Gower and Richards, 1990), the ratio of mature short needles dry mass is relatively lower than the mature long needles at a seedling stage (Powell, 1988). Based on this characteristic, we estimated the leaf area at the whole plant level as a total leaf area index (TLA) by LMA of mature needle leaves times needle dry mass per a seedling.

## Maximal Photochemical Efficiency of Photosystem II

The maximal photochemical efficiency of photosystem II ( $F_v/F_m$ ) was measured with a portable fluorometer (PAM-2000, Walz, Effeltrich, Germany) in September 2017. The measurement was

conducted after an overnight dark adaptation to evaluate the chronic photoinhibition (Krause et al., 1995; Kitao et al., 2003).  $F_v/F_m$  was calculated with the following parameters and formula:  $F_0$  for the steady state yields,  $F_m$  for the maximum yields measured under irradiance of approximately 5,000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux density for 1 s, and  $F_v/F_m = (F_m - F_0)/F_m$  as defined by Schreiber et al. (1986).

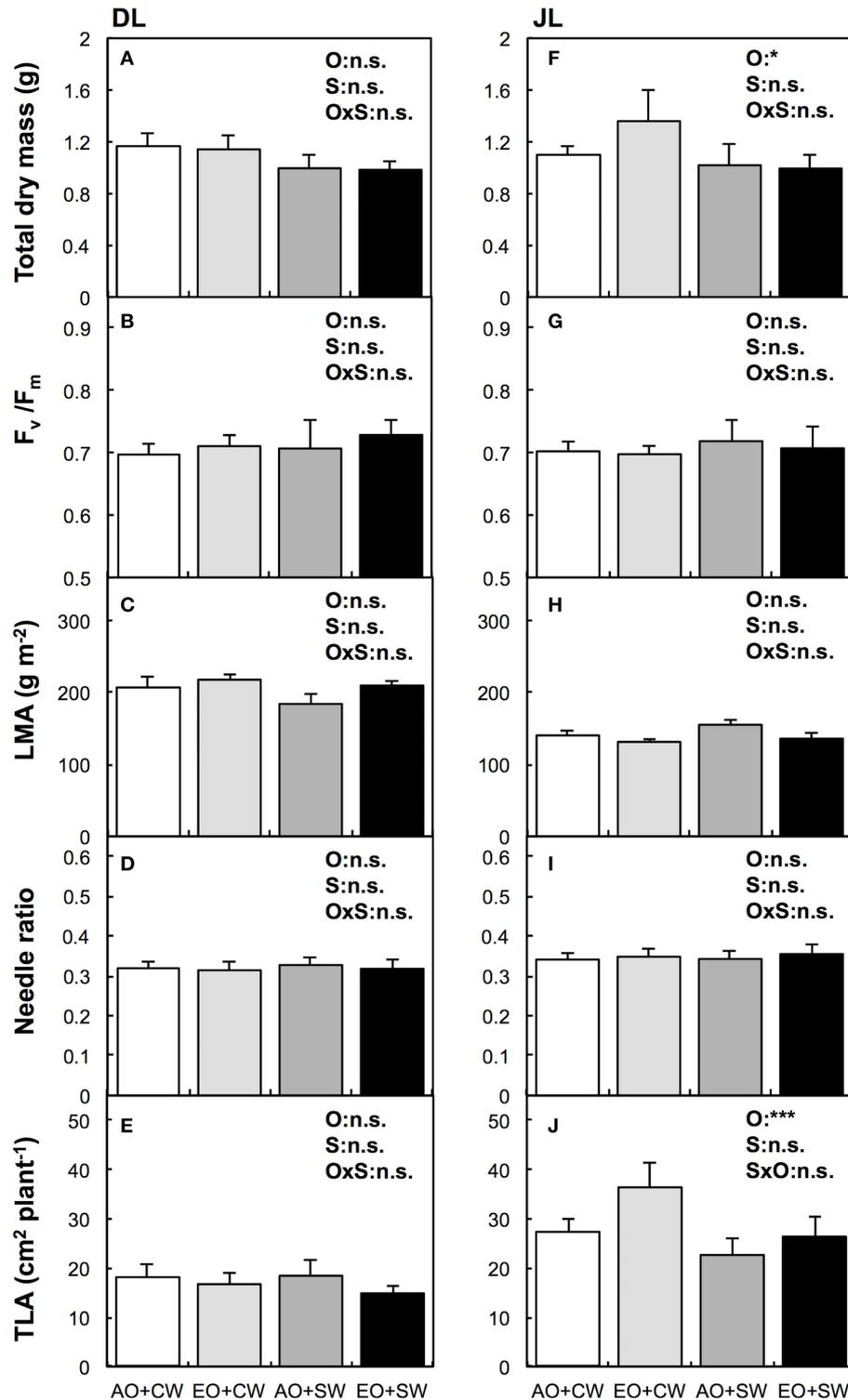
## Nutrition and Chlorophyll Contents in Needles

Approximately 15 mg powder samples obtained from mature needles sampled at the end of September 2017 were weighed and used to measure nitrogen (N) content with a NC analyzer (Elementar, Vario EL III, Japan). Approximately 50 mg of powder samples were weighed and digested with 2 ml of 61% nitric acid (Kanto Chemical, Tokyo, Japan) at 110°C for ~2 h. A 0.5 ml solution of hydrogen peroxide was added and the sample was heated continuously at 110°C for 20 min. After cooling to room temperature, 10 ml of 2% nitric acid were added. The concentration of K, Ca, Mg, and Na in these samples was measured by ICP-MS (ELan, DRC-e; PerkinElmer, Waltham, MA, U.S.A.). Element contents were calculated as the dry weight unit and converted as the leaf area unit by LMA.

Samples were stored at -75°C after sampling and weighing. Needles were immersed in 2 ml dimethyl sulfoxide and incubated at 65°C in the dark to extract chlorophyll (Chl). Then, the extracts were measured with a spectrophotometer (Gen spec III, Hitachi, Tokyo, Japan) with two waves; 665 nm ( $A_{665}$ ) and 648 nm ( $A_{648}$ ). Chl-a and Chl-b contents were calculated according to Barnes et al. (1992) and Shinano et al. (1996). The total Chl (i.e., Chl a+b) and Chl a/b were also calculated. We also obtained ratio of total Chl/needle N content (Chl/N) as an index of allocation in foliar nitrogen (Kitaoka and Koike, 2004).

## Statistical Analyses

Statistical analyses were conducted using R (R Core Team, 2017). All the data of response variables were set as the four mean values per treatment, which were obtained from four seedlings per species in each chamber ( $n = 4$ ). The effects of eO<sub>3</sub> and salt stress were determined by analysis of variance in each species (Two-way ANOVA). The relationships between needle Na and mineral element contents at all treatments in both species were analyzed using a generalized linear mix model (GLMM,  $n = 32$ ). The random effect was set as the experimental unit. When species differences and their interactions were significant, correlation analysis was performed in each species ( $n = 16$ ). Relationships between TLA and content of each element in needles were analyzed by the same GLMM. In each model, explanatory variables (= fixed effects) were set with each mineral element, species, and its interaction, whereas the response variables were the mean values at all treatments in both species. In all the linear analyses, the coefficient of determination ( $R^2$ ),  $P$ -value, and the Akaike's information criterion were calculated (AIC, Akaike, 1974). AIC is one of the most popular criterion for linear model identification and the optimal model is selected when AIC is minimized.



**FIGURE 1** | Biometric responses to salt treatment and elevated ozone (O<sub>3</sub>) in Dahurian larch [DL, *Larix gmelinii*, (A–E)] and Japanese larch [JL, *Larix kaempferi*, (F–J)]. White bar means ambient O<sub>3</sub> and control tap water (aO<sub>3</sub>+CW), light gray bar means elevated O<sub>3</sub> and control tap water (eO<sub>3</sub>+CW), dark gray bar means ambient O<sub>3</sub> and salt water (aO<sub>3</sub>+SW), and black bar means elevated O<sub>3</sub> and salt water (eO<sub>3</sub>+SW). The explanatory variables were set as elevated O<sub>3</sub> (O), salt treatment (S), and its interaction (OxS). Results of Two-way ANOVA ( $n = 4$ ) with statistical significant difference are shown as: \*\*\* $P < 0.001$ , \*\* $P < 0.05$ , and n.s.  $P \geq 0.05$ .  $F_v/F_m$ , the maximal photochemical efficiency of photosystem II; LMA, leaf mass area; Needle ratio, needle dry mass to total dry mass; TLA, total leaf area index.

## RESULTS

### Response to Elevated O<sub>3</sub> and Salt Stress

No significant effects of elevated O<sub>3</sub> (eO<sub>3</sub>) were observed on the total dry mass of DL. We did not observe significant inhibition of O<sub>3</sub> on F<sub>v</sub>/F<sub>m</sub> between both larch species. However, O<sub>3</sub> significantly increased total dry mass and TLA of only JL ( $P < 0.05$ , **Figure 1**). The mean value of total dry mass and TLA at eO<sub>3</sub> were ~23 and 34% higher than at the ambient O<sub>3</sub> condition, respectively. No significant effects of O<sub>3</sub> were observed in other biometric (i.e., needle ratio and LMA, **Figure 1**) and biochemical parameters (i.e., element contents and chlorophyll in needles, **Table 2**).

While salt stress had also no significant effects on all biometric and physiological parameters of the two species, several element contents in needles were significantly changed (**Table 2**). Needle Na contents were significantly increased under salt stresses in both larches. In the dry mass unit, needle Na content of DL with salt treatment was approximately eight times greater than in the salt-free condition ( $P < 0.05$ ) whereas Na of JL with salt treatment was at least 13 times greater ( $P < 0.001$ ). Salt stress increased Chl ratio only in DL, which showed an ~14% increase in average values ( $P < 0.01$ ). In contrast, salt stress significantly reduced K, Ca, and Mg contents of JL. In the dry mass unit, the K, Ca, and Mg contents of DL decreased ~17% under salt treatment ( $P < 0.05$ ), 36% ( $P < 0.001$ ), and 34% ( $P < 0.001$ ), respectively. In the needle area unit, however, the increment of N content was observed in only JL ( $P < 0.05$ ).

### Relationship Between Needle Na and Other Elements

The overall relationship between Na and K contents was not significant in the larch seedlings (**Figure 2**). However, a significant relationship between Na and K contents was observed in JL ( $R^2 = 0.2$ ,  $P < 0.05$ , AIC = 48.6) but not in DL ( $R^2 = 0.02$ ,  $P = 0.33$ , AIC = 47.1). A significant relationship between Ca and Mg were observed in both larch species, as was a significant relationship between Na and Mg content (DL;  $R^2 = 0.35$ ,  $P < 0.01$ , AIC = -10.22, JL;  $R^2 = 0.23$ ,  $P < 0.05$ , AIC = -4.63). On the contrary, there were species differences in the relationship between Na and Ca content ( $P < 0.05$ , **Figure 3**). The coefficient value of Na on Ca content was ~1.8 times higher in JL than DL (**Table 3**). All the relationships between Na and N contents in both and each larch species were not significant (**Figure 2**, **Table 3**).

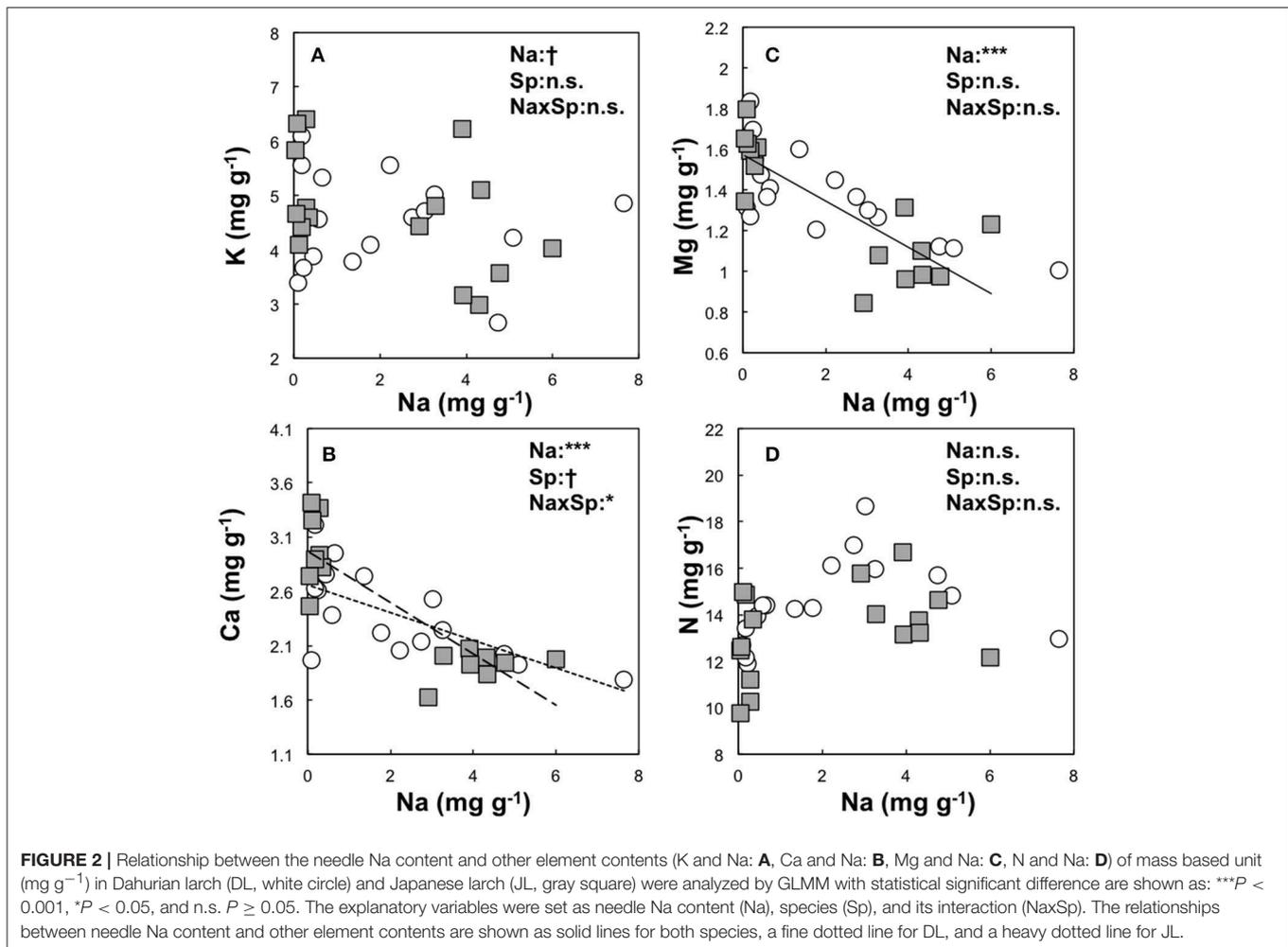
### Relationship Between TLA and Needle Mineral Elements

**Table 3** shows the summary of model formulas to explain TLA by each element in needles. The best model to explain TLA of both larches determined by AIC was constructed with N ( $R^2 = 0.54$ ,  $P < 0.001$ , AIC = 211). This significant relationship was also observed within each species (DL;  $R^2 = 0.35$ ,  $P < 0.01$ , AIC = 94, JL;  $R^2 = 0.23$ ,  $P < 0.05$ , AIC = 116) whereas the highest  $R^2$  in model of DL was performed by K ( $R^2 = 0.36$ ,  $P < 0.01$ , AIC = 94) although AIC was the same value with N (**Figure 3**). The

**TABLE 2 |** Summary of the biochemical needle traits; mineral element contents, total chlorophyll (Chl) contents, Chl a, Chl b, the ratio of Chl a/b, and the ratio of total Chl/needle N contents in Dahurian larch (DL) and Japanese larch (JL) seedlings at all the treatments.

Species	Factor	Treatments				Two-way ANOVA		
		aO <sub>3</sub> +CW	eO <sub>3</sub> +CW	aO <sub>3</sub> +SW	eO <sub>3</sub> +SW	O <sub>3</sub>	Salt	O <sub>3</sub> × Salt
DL	Na (mg g <sup>-1</sup> )	0.64 ± 0.27	0.29 ± 0.10	3.13 ± 0.62	4.50 ± 1.21	n.s.	*	n.s.
	K (mg g <sup>-1</sup> )	4.10 ± 0.42	4.97 ± 0.54	4.09 ± 0.51	4.84 ± 0.28	n.s.	n.s.	n.s.
	Ca (mg g <sup>-1</sup> )	2.61 ± 0.22	2.71 ± 0.18	2.16 ± 0.05	2.07 ± 0.16	n.s.	n.s.	n.s.
	Mg (mg g <sup>-1</sup> )	1.45 ± 0.06	1.54 ± 0.13	1.24 ± 0.05	1.22 ± 0.10	n.s.	n.s.	n.s.
	N (mg g <sup>-1</sup> )	13.8 ± 0.38	13.0 ± 0.58	15.7 ± 0.55	15.6 ± 1.19	n.s.	n.s.	n.s.
	Total Chl (mg g FW <sup>-1</sup> )	8.75 ± 0.39	10.09 ± 1.10	9.24 ± 1.49	7.78 ± 0.97	n.s.	n.s.	n.s.
	Chl-a (mg g FW <sup>-1</sup> )	6.87 ± 0.29	7.83 ± 0.76	7.58 ± 1.25	6.18 ± 0.82	n.s.	n.s.	n.s.
	Chl-b (mg g FW <sup>-1</sup> )	1.88 ± 0.11	2.26 ± 0.36	1.67 ± 0.24	1.60 ± 0.15	n.s.	n.s.	n.s.
	Chl a/b	3.62 ± 0.16	3.60 ± 0.25	4.46 ± 0.18	3.78 ± 0.14	n.s.	**	n.s.
	Chl/N (mg mg <sup>-1</sup> )	0.63 ± 0.02	0.79 ± 0.11	0.59 ± 0.11	0.50 ± 0.03	n.s.	n.s.	n.s.
JL	Na (mg g <sup>-1</sup> )	0.28 ± 0.04	0.08 ± 0.02	3.82 ± 0.43	4.55 ± 0.49	n.s.	***	n.s.
	K (mg g <sup>-1</sup> )	5.05 ± 0.46	5.23 ± 0.51	3.95 ± 0.41	4.63 ± 0.66	n.s.	*	n.s.
	Ca (mg g <sup>-1</sup> )	3.01 ± 0.12	2.97 ± 0.22	1.89 ± 0.09	1.95 ± 0.05	n.s.	***	n.s.
	Mg (mg g <sup>-1</sup> )	1.58 ± 0.02	1.60 ± 0.09	1.00 ± 0.06	1.12 ± 0.09	n.s.	***	n.s.
	N (mg g <sup>-1</sup> )	12.5 ± 1.08	12.5 ± 1.06	14.5 ± 0.45	13.8 ± 0.98	n.s.	n.s.	n.s.
	Total Chl (mg g FW <sup>-1</sup> )	8.41 ± 0.68	9.21 ± 0.99	7.64 ± 0.99	8.04 ± 1.49	n.s.	n.s.	n.s.
	Chl-a (mg g FW <sup>-1</sup> )	6.44 ± 0.53	7.11 ± 0.75	5.95 ± 0.77	6.32 ± 1.30	n.s.	n.s.	n.s.
	Chl-b (mg g FW <sup>-1</sup> )	1.97 ± 0.16	2.10 ± 0.24	1.69 ± 0.25	1.72 ± 0.20	n.s.	n.s.	n.s.
	Chl a/b	3.28 ± 0.16	3.38 ± 0.05	3.61 ± 0.25	3.55 ± 0.32	n.s.	n.s.	n.s.
	Chl/N (mg mg <sup>-1</sup> )	0.68 ± 0.07	0.74 ± 0.02	0.52 ± 0.06	0.57 ± 0.06	n.s.	n.s.	n.s.

All the values are shown as the mean ± SE with the results of two-way ANOVA ( $n = 4$ ). Statistical significant differences are shown as: \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ , and n.s.,  $P \geq 0.05$ .



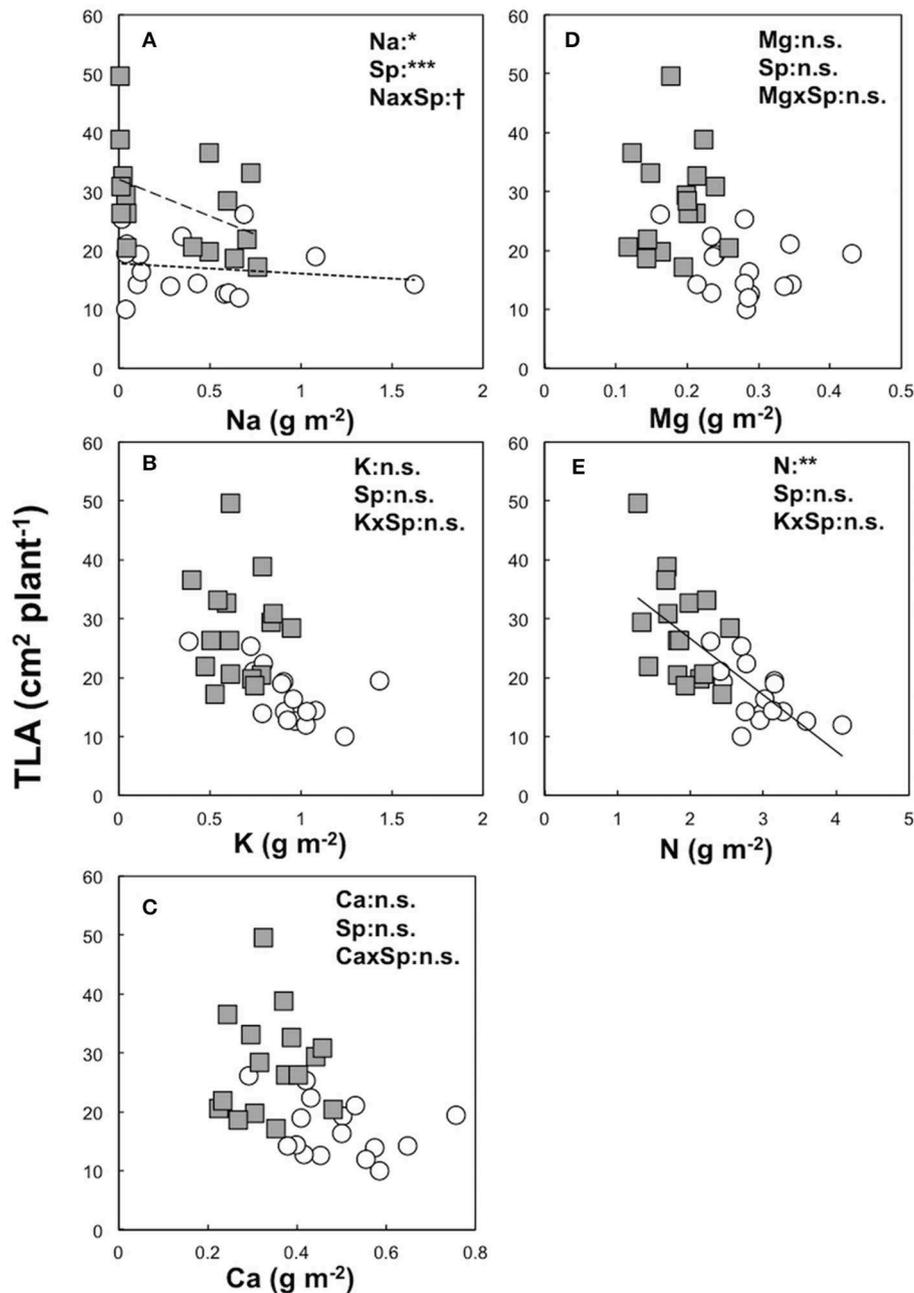
second best model of both larches was constructed with K ( $R^2 = 0.28$ ,  $P < 0.01$ ,  $\text{AIC} = 225$ ) while the third models contained almost equal  $R^2$  and AIC values (Ca;  $R^2 = 0.20$ ,  $P < 0.01$ ,  $\text{AIC} = 229$ , Mg;  $R^2 = 0.20$ ,  $P < 0.01$ ,  $\text{AIC} = 229$ ). However, no significant relationships between TLA and K, Ca, and Mg content were observed in JL (Table 4). Although needle Na content was significantly correlated with TLA in both larch species ( $P < 0.05$ ), the result of GLMM showed a species difference in TLA ( $P < 0.001$ ). When the effects of Na contents on TLA were analyzed in each species, the significant relationship was observed only in JL ( $R^2 = 0.20$ ,  $P < 0.05$ ,  $\text{AIC} = 116$ ) but not in DL ( $R^2 = 0.02$ ,  $P = 0.28$ ,  $\text{AIC} = 101$ ).

## DISCUSSION

We evaluated initial growth of Dahurian larch (DL) and Japanese larch (JL) seedlings treated with the simulated eO<sub>3</sub> and alkaline salt stress. In contrast to our expectations, eO<sub>3</sub> did not suppress the total dry mass of both larch species. Moreover, eO<sub>3</sub> did not induce significant nutritional imbalance in needles even with salt stress (Table 2). Elevated O<sub>3</sub> significantly increased the dry mass of JL while we did not observe any significant inhibitions of

biometric and physiological parameters, except for TLA. In fact, the similar results indicated that JL can show hormetic responses of photosynthesis rates and dry mass to mild O<sub>3</sub> stress (Dong-Gyu et al., 2015; Sugai et al., 2018). Since leaf quantity as well as photosynthetic capacity are related to plant growth (Poorter, 1989), the dry mass increment might be related to the TLA response (Figure 1).

The best factor explaining variables of TLA was needle N contents. The results of negative correlation between TLA and N contents suggests that DL originally may not produce TLA regardless of any growth conditions as relatively higher N contents per needle area, while JL may be able to adjust producing TLA as relatively lower N contents. This result suggests that the N use efficiency in forming needles may vary between the two larches, and DL may have adaptive abilities for harsh conditions, such as low temperature and limited soil nutrient availability more than JL (Gower and Richards, 1990). Koike et al. (2012) reported that the photosynthetic N use efficiency of DL was higher (over 20%) compared to JL. Instead of these higher abilities in a needle scale, DL may be inferior to adjust TLA along with abiotic environments (Abaimov, 2010; Polezhaeva et al., 2010; Mamet et al., 2019).



**FIGURE 3 |** Relationship between the total leaf area index (TLA, cm<sup>2</sup> plant<sup>-1</sup>) and needle element contents (TLA and Na: **A**, TLA and K: **B**, TLA and Ca: **C**, TLA and Mg: **D**, TLA and N: **E**) of leaf area-based unit (g m<sup>-2</sup>) in Dahurian larch (DL, white circle) and Japanese larch (JL, gray square) were analyzed by GLMM with statistical significant difference are shown as: \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ , and n.s.  $P \geq 0.05$ . The explanatory variables were set as each needle element content, species (Sp), and its interaction. The relationships between TLA and each element content are shown as solid lines for both species, a fine dotted line for DL, and a heavy dotted line for JL.

In a previous study, 1-month-old saplings of European larch (*Larix decidua* Mill) showed a 16% lower dry mass at 150 mM NaCl compared to controls (Plesa et al., 2018). While salt treatment showed no significant suppression in any initial growth, we observed significant biochemical responses in needles (i.e., several element contents, **Table 2**). As expected, DL showed

a relatively lower degree of needle Na content under salt stress compared to JL. The increased degree based on mean values was up to 3.5 times, indicating that the capacity to accumulate Na into needles may be higher in JL. Furthermore, DL showed no change in K contents under salt stress as compared with JL. High Na concentration can have negative effects on

**TABLE 3** | Results of the generalized liner mixed models (GLMM) to describe the relationship between needle Na and other element contents in Dahurian larch (DL) and Japanese larch (JL).

Model formula	R <sup>2</sup>	P-value	AIC
<b>Both larch species</b>			
K = -0.13 Na + 4.90	0.087	0.057	91.43
Ca = -0.18 Na + 2.81	0.586	<0.001	24.14
Mg = -0.09 Na + 1.54	0.577	<0.001	-17.68
N = 0.28 Na + 13.3	0.099	0.954	134.36
<b>DL</b>			
K = -0.05 Na + 4.61	0.015	0.326	47.05
Ca = -0.13 Na + 2.66	0.467	<0.01	11.61
Mg = -0.07 Na + 1.52	0.521	<0.001	-10.22
N = 0.27 Na + 14.0	0.107	0.891	67.51
<b>JL</b>			
K = -0.22 Na + 5.19	0.202	<0.05	48.63
Ca = -0.24 Na + 2.97	0.723	<0.001	13.59
Mg = -0.11 Na + 1.57	0.654	<0.001	-4.63
N = 0.30 Na + 12.7	0.118	0.904	68.89

All the values of element content were mass based unit (mg g<sup>-1</sup>). R<sup>2</sup>, the coefficient of determination; AIC, Akaike's information criterion.

physiological functions due to the inhibition of enzyme activities and K uptakes (Marschner, 2012; Polle and Chen, 2015). K has an outstanding role in plant-water relations, contributing to the osmotic potential of cells and tissues (Marschner, 2012). Reductions of K contents under salt stress in JL, therefore, supported this competitive absorption with Na. However, the salt effect on needle K content was not significant in DL. Previous studies have also reported stable values of K of other *Larix* species (e.g., *Larix laricina* Du Roi; Renault, 2005, *L. decidua* Mill; Plesa et al., 2018). The K homeostasis can be accomplished by cation selectivity in uptake systems from the cytoplasm into vacuoles. Plesa et al. (2018) have pointed out that needle K content of *L. decidua* increased under salt treatment. Since we did not observe any increments of K contents across all treatments, future investigations should compare the specific mechanism differences of K homeostasis within the genus *Larix*.

We observed homeostasis of other mineral contents. Regression analysis showed negative correlations between needle Na and Ca, and between Na and Mg contents in both larches and each larch seedlings (Figure 2), while significant salt effects were only observed in JL (Table 2). In particular, we observed significant species differences regarding the relationship between needle Na and Ca contents, suggesting that the Na sensitivity to needle Ca contents was higher in JL than DL. Ca plays an important role in the cell membrane, cell wall stabilization, signaling functions, and the mitigation of salinity (Köster et al., 2019). These results supported our hypothesis that salt tolerance would be greater in DL, and that mineral homeostasis was related to other mineral elements apart from K. DL may obtain the adaptive capacity to salt stress as the original distribution of the species where the primary salinization is likely to occur cause to drought condition (Polezhaeva et al., 2010; Mamet et al., 2019). In contrast, JL is naturally distributed in central Japan. Species

**TABLE 4** | Results of the generalized liner mixed models (GLMM) to describe the relationship between total leaf area (TLA, cm<sup>2</sup>) and needle mineral element contents in Dahurian larch (DL) and Japanese larch (JL).

Model formula	R <sup>2</sup>	P-value	AIC
<b>Both larch species</b>			
TLA = -7.31 Na + 25.3	0.097	<0.05	232
TLA = -19.9K + 38.5	0.278	<0.01	225
TLA = -32.2 Ca + 36.0	0.196	<0.01	229
TLA = -56.5Mg + 35.8	0.204	<0.01	229
TLA = -9.57N + 45.8	0.535	<0.001	211
<b>DL</b>			
TLA = -1.71 Na + 17.8	0.024	0.282	101
TLA = -12.5K + 28.7	0.355	<0.01	94
TLA = -13.5 Ca + 23.7	0.101	0.115	99
TLA = -15.5Mg + 21.4	0.041	0.227	100
TLA = -6.30N + 35.7	0.346	<0.01	94
<b>JL</b>			
TLA = -12.5 Na + 32.1	0.198	<0.05	116
TLA = -2.50K + 29.8	0.002	0.435	120
TLA = 7.55 Ca + 25.6	0.005	0.600	120
TLA = 15.5Mg + 25.3	0.005	0.607	120
TLA = -11.5N + 49.8	0.232	<0.05	116

All the values of element content were area-based unit (g m<sup>-2</sup>).

differences in adaptive traits, including salt tolerance, may be induced by the geographical isolation from other continental *Larix* species in the interglacial period (Polezhaeva et al., 2010)

While we hypothesized that the increment of N content via the synergy with K<sup>+</sup> uptake would be either a species specific responses or related to osmotic regulations, we did not observe significant changes in needle N content, except for the area-based value of JL (Table 2). However, we observed higher Chl a/b under salt stress in DL, but not in JL. In tamarack saplings (*L. laricina* Du Roi), 60 mM NaCl reduced the total Chl and the Chl a/b (Renault, 2005). However, in *L. decidua*, 150 mM NaCl reduced the total Chl, while the Chl a/b did not change (Plesa et al., 2018). A high value of Chl a/b under salt treatment was previously reported in herbaceous plants, including sunflower (*Helianthus annuus* L., Liu and Shi, 2010) and wheat (*Triticum aestivum*, Yang et al., 2008), while a lower Chl a/b was reported in poplar (*Populus alba* x *P. berolinensis*, Wang et al., 2013). Given the preferential reduction in Chl-b than Chl-a under salt stresses, it may act to protect the reaction center core more than the light harvesting capacity related to Chl-b. Future studies should continue to investigate N metabolism, with the consideration that this process may be related closely to Chl metabolic regulation (e.g., Yang et al., 2008).

Irrespective of our hypothesis, we observed no clear combined effect of eO<sub>3</sub> and salt stress on physiological parameters in larch seedlings (Table 2). Therefore, even if we employ these larch species to manage soil salinization under eO<sub>3</sub>, their initial growth may not be suppressed by eO<sub>3</sub> and salinity stress. Non-significant changes of needle element contents indicated that the element variables were maintained even under the combined effect. These results suggested that eO<sub>3</sub> may not inhibit the

element homeostasis of larch species, especially in DL. This may be attributed to non-significant changes of the total dry mass in DL treated with single O<sub>3</sub> and the combined effect. It was reported that the stable P and iron (Fe) contents in leaves implied homeostatic readjustment, contributing to preventing physiological inhibition of eO<sub>3</sub> (Agathokleous et al., 2018). It is possible that the combined effect, and even single O<sub>3</sub> effect, were not detected due to the physiological status of seedlings, which were shown in F<sub>v</sub>/F<sub>m</sub> (Figure 1). In all treatments, F<sub>v</sub>/F<sub>m</sub> showed almost the same value from 0.70 to 0.72, which were significantly lower than the standard normal value of health condition ranged between 0.81 and 0.83 (Schreiber et al., 1986).

We conclude that the effects of eO<sub>3</sub> and alkaline salt stress did not suppress the total dry mass of both larch species. Although we did not find any specific factors explaining species difference in O<sub>3</sub> sensitivities between species, TLA may be involved. Stress adaptive capacity to salt was higher in DL than JL, suggesting that salt treatment did not reduce needle K content in DL, and DL also maintained Ca and Mg contents in needles. Specifically, DL may show the greater salt tolerance to the mineral homeostatic capacity even under eO<sub>3</sub>. Further study should evaluate the relationship between the stomatal responses and K homeostasis under eO<sub>3</sub> and salt stress, which may reveal how larch achieves the optimal nutrient balance for these stress resistances. Our results suggest that *L. gmelinii* seedlings may be future candidates for afforestation, even under eO<sub>3</sub> condition at saline soils.

## DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/Supplementary Files.

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## AUTHOR CONTRIBUTIONS

TS and WY were the same contribution to the planning and working. TS primarily worked on drafting and editing the manuscript with figures and tables. WY performed cultivation and measurements at the field. FS had responsible for managing the experimental fields. All other co-authors (TW, LQ, and TK) equally contributed to funding, manuscript writing, and revision.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ffgc.2019.00053/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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