

The Growth of Four Tree Species Seedlings on Soil and Decayed Wood of *Pinus densiflora*

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Abstract

Decayed wood provides regeneration microsites for a variety of tree seedlings, whereas the mechanisms of species-specific preferences for decayed wood, their decay types, and soil are poorly known. To evaluate the effects of chemical and biological characteristics of the substrates on seedling preference, I investigated the biotic and abiotic effects of different substrates on the above- and belowground growth of seedlings of 4 tree species commonly occurring in post-Pine Wilt Disease (PWD) forests. Seedlings were grown in microcosms including autoclaved or non-autoclaved substrates (3 types: white-rotted wood, brown-rotted wood, and soil) for 4 months. After retrieval, the above- and belowground growth of the seedlings was measured. *Pinus densiflora* seedlings experienced greater growth in nons-autoclaved rather than autoclaved substrates; however, growth was affected to a lesser extent by the differences in substrates. In contrast, *Cryptomeria japonica* seedlings experienced greater growth in soil than in woods; however, growth was not affected by autoclaving. Aboveground growth of *Clethra barbinervis* seedlings was greater in soil and autoclaved brown-rotted wood than in other wood substrates. The growth of *Eurya japonica* seedlings was greater in soil than in woods. Seedlings of the 3 species, excluding *C. barbinervis*, showed plasticity in the shoot/root ratio against substrate difference. The relative importance of biotic and abiotic factors for the above- and belowground growth of seedlings differ among tree species, which may partly explain species-dependent

preferences for different microsites on the forest floor when regenerating in post-PWD forests.

Introduction

Microsite heterogeneity plays an important role in plant population dynamics and species diversity maintenance in forest ecosystems (Harmon and Franklin 1989; Nakashizuka 2001). Different tree species favor specific microsites for seedling regeneration, reflecting their life-history traits associated with physiological and morphological responses to abiotic and biotic environmental conditions (Leck and Outred 2008). Allocation to above- and belowground growth is one of the most conspicuous traits characterizing the initial responses of seedlings to their environments (Kohyama and Grubb 1994; Doi et al. 2008). Thus, it is important to determine allocations of seedlings to above- and belowground growth for understanding their preferences pertaining to forest microsites.

Among the microsites on forest floors, coarse woody debris (CWD) provides important refuges for seedling establishment in various forest ecosystems (Fukasawa 2012). Although CWD generally contains relatively lower concentrations of nutrients compared with soil (Goodman and Trofymow 1998; Baier et al. 2006), it can contain greater concentrations of nutrients compared with soil in some cases (Takahashi et al. 2000; Fukasawa 2015a). Furthermore, the nature of microbial communities inhabiting CWD, particularly the low level of soil-borne pathogens, often contributes to good growth performances of pathogen-susceptible

tree seedlings (Cheng and Igarashi 1987; Cheng 1989; Takahashi 1991; O'Hanlon-Manners and Kotanen 2004). However, the extent to which the chemical and biotic properties of CWD contribute to seedling growth is unclear. A couple of studies have recently reported that the decay type of wood, which reflects the wood decay abilities of fungal communities that inhabit CWD, strongly affects seedling establishment on CWD (Bače et al. 2012; Fukasawa 2012). This type has been traditionally categorized into 3 types: 1) white-rot, 2) brown-rot, and 3) soft-rot determined by the chemical composition, physical structure, and outward appearance of wood (Eaton and Hale 1993). Bače et al. (2012) reported that seedlings of *Picea abies* preferentially regenerate on the logs where white-rot fungi dominate than on the logs where brown-rot fungi dominate. On the other hand, Fukasawa (2012) showed that seedlings of *Clethra barbinervis* preferentially regenerate on brown-rotted logs than on logs with other decay types. Although the reason for this preference pertaining to a specific decay type remains unclear, it is expected that wood chemical properties such as pH may have some effect because a significant relationships between wood pH and seedling density were observed (Fukasawa 2012). Furthermore, growing evidence shows that wood decay type determines wood nutrient contents (Takahashi et al. 2000; Fukasawa 2015a) as well as microbial inhabitants such as N-fixing bacteria (Aho 1974; Jurgensen et al. 1989), mycorrhizal fungi (Tedersoo et al. 2008), and myxomycetes (Fukasawa et al. 2015) of CWD. Therefore, differences in decay type may influence tree seedlings not only directly by their physicochemical properties but also indirectly by affecting symbiotic, antagonistic, and decomposer communities (Bardgett and Wardle 2010).

Forest dieback events often create huge volumes of CWD (Heilmann-Clausen 2013). In Japan, Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) is a dominant canopy tree species of temperate secondary forests, and pine wilt disease (PWD) has killed many adult pine trees over recent decades (Takemoto and Futai, 2008), generating substantial pine CWD in forests (Kato and Hayashi 2006). I recently found that decayed pine logs in the post-PWD forests present suitable regeneration sites for several tree species and that the decay type of the logs affects the seedling community (Fukasawa 2012). Because the decay type of pine logs could highly vary according to

environmental variables such as climate (Fukasawa 2015b), seedling preferences pertaining to a specific decay type may be critical for their regeneration after PWD. Thus, the aim of the present study is to compare seedling growth of tree species regenerating in post-PWD forests on different substrates such as soil and decayed wood of distinct decay types. To separate the effects of chemical and biological factors on seedling performance, sterilized substrates were used as a counterpart of non-sterile substrates for each substrate type. Seedlings of 4 tree species were used: 1) *P. densiflora*, 2) *Cryptomeria japonica* (Thunb. Ex L.f) D. Don, 3) *Clethra barbinervis* Sieb. et Zucc., 4) *Eurya japonica* Thunb. The regeneration of those 4 species has frequently been recorded in post-PWD forests (Higo et al. 1995; Fujihara et al. 2002; Kato and Hayashi 2006, 2007) and also recorded as seedlings on decayed logs of *P. densiflora* (Fukasawa 2012, 2015a; Fukasawa and Komagata unpublished). I hypothesized that 1) species with high mycorrhizal dependence (including *P. densiflora*; Sim and Eom 2006) are strongly affected by a sterile treatment rather than substrate difference, 2) species with high nutrient demands (including *C. japonica*; Nakaji et al. 2001) are strongly associated with substrate differences rather than sterile treatment, and 3) the growth of *C. barbinervis* seedlings may be better on brown-rotted wood than white-rotted wood because the seedlings of this species regenerate preferentially on brown-rotted logs (Fukasawa 2012).

Materials and methods

Tree seedlings

Properties of the 4 tree species used for the current growth experiment are given in Table 1. The used species differed with respect to their family, growth form, seed weight, nutrient demand, regeneration site, nutrient requirement, and mycorrhizal type and dependency. Seeds of *P. densiflora* and *C. japonica* were obtained from the Forest Breeding Center of Forestry and Forest Products Research Institute (FFPRI) (Ibaraki, Japan). Seeds of *C. barbinervis* and *E. japonica* were obtained from adult trees at Higashiyamato Park, Tokyo (35°45'N, 139°26'E; 114–122 m a.s.l.) and at the Yamashiro Experimental Forest of FFPRI, Kyoto (34°47'N, 135°50'E; 255 m a.s.l.), respectively. Seeds were sanded with wet paper and stored at 4°C for more than 4 months to break dormancy. Subsequently, seed surfaces were sterilized

Table 1. Taxonomy and life-history traits of the 4 tree species used in the present study

Seedling	Family	Growth form	Seed fresh weight (mg) ¹	Regeneration site ²	Nutrient requirement ³	Mycorrhizal type ⁴	Mycorrhizal dependency ⁵
<i>Pinus densiflora</i>	Pinaceae	canopy	10.0	Decayed wood, exposed mineral soil	Low	ECM	High
<i>Cryptomeria japonica</i>	Cupressaceae	canopy	4.6	Decayed wood	High	AM	unknown
<i>Clethra barbinervis</i>	Clethraceae	shrub	0.1	Decayed wood, exposed mineral soil	unknown	AM	unknown
<i>Eurya japonica</i>	Pentaphragaceae	shrub	1.0	Exposed mineral soil	unknown	AM	unknown

¹ Tateishi et al. 2001 (*P. densiflora*); Matsuda et al. 2015 (*C. japonica*); Kobayashi and Kamitani 2000 (*C. barbinervis*); Tsujino and Yumoto 2004 (*E. japonica*).

² Iwasaki et al. 1997, Obase et al. 2012 and Fukasawa 2015 (*P. densiflora*); Ota et al. 2015 (*C. japonica*); Kobayashi and Kamitani 2000 and Fukasawa 2012 (*C. barbinervis*); Manabe and Yamamoto 1997 (*E. japonica*).

³ Nakaji et al. 2001 (*P. densiflora* and *C. japonica*).

⁴ Yamato and Iwase 2005.

⁵ Sim and Eom 2006 and Dalong et al. 2011 (*P. densiflora*).

with 30% hydrogen peroxide, and the seeds were germinated on moist cotton under sterilized conditions at 20°C (12h light, 12h dark).

Experimental design

Three substrates were prepared: 1) white-rotted wood, 2) brown-rotted wood, and 3) soil. All substrates were obtained from Higashiyamato Park. The vegetation of Higashiyamato Park is pine- and oak-dominated secondary forest and has been described by Fukasawa (2012). White- and brown-rotted woods were collected from the decayed logs of *P. densiflora*. Each substrate was collected from 3 locations in the park and mixed to avoid the effects of local differences in microbe flora specific to certain vegetation. Collected substrates were milled to pass a 6 mm screen using a Retsch® SM 300 cutting mill (Verder Scientific Co. Ltd., Germany). Half of each substrate was autoclaved at 121°C for 1 h to prepare sterile substrates. In total, 6 experiments (3 substrates by 2 sterile levels) were set up. In the substrates of the 6 experiments, the pH was measured using a pH meter (Horiba, Kyoto, Japan), whereas the concentrations of cations (Na⁺, NH₄⁺, K⁺, Mg²⁺, and Ca²⁺) and anions (F⁻, Cl⁻, NO₂⁻, Br⁻, NO₃⁻, SO₄²⁻, and PO₄³⁻) were measured using an ion chromatography system (ICS-1000/2000; Dionex, CA, USA). Details of the measurements of pH and ions have been described by Fukasawa (2015a). The ion concentrations were expressed as values per 1 g of dried substrate.

A transparent polypropylene case (7.2 × 7.2 × 10 cm) (Incu Tissue; SPL Life Sciences, Korea) was first filled with approximately 173 ml vermiculite (water content, 26%), following which an approximately 43 ml substrate was filled on the upper layer.

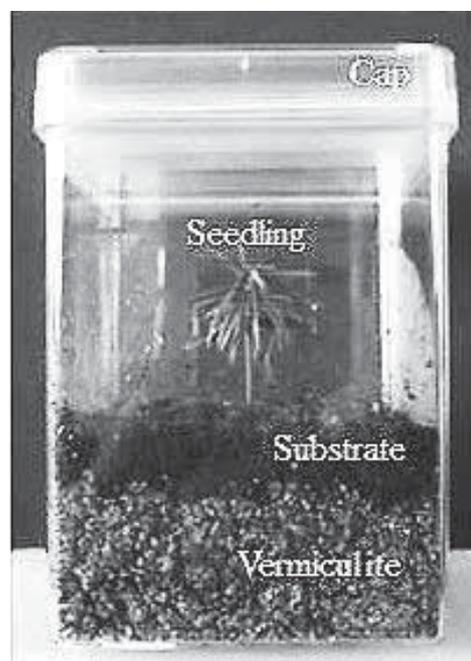


Fig. 1. Experimental microcosm system

A germinated seed was aseptically placed on the surface of the substrate in the case. All cases were capped with a transparent polypropylene cap and sealed with Parafilm® to prevent water evaporation (Fig. 1). Cases were incubated at 25°C under a 10 h dark and 14 h light cycle a day using a 11,000 lux fluorescent lamp for 4 months. Five replicated cases were prepared for each experiment and for each species. In total, 120 cases were incubated.

Measurements for retrieved seedlings

The retrieved seedlings were divided into aboveground shoots and underground roots. The heights of the shoots were measured and leaf numbers were counted. The shoots and roots were oven dried to a constant weight at 70°C and weighed. The shoot/root ratio (S/R) was calculated using the following equation:

$$S/R = \text{shoot dry weight} / \text{root dry weight}$$

Data analyses

A two-way analysis of variance (ANOVA) was applied to test the effects of substrate, autoclaving, and their interactions on pH and ion concentrations of substrates and seedling growth (shoot length, leaf number, shoot weight, root weight, and S/R). A Tukey's honest significant difference (HSD) post hoc test was performed to compare the values among the 6 experiments. A generalized liner model (GLM) was used to identify chemical properties associated with seedling growth. According to the correlations among the chemical factors (Table 3), pH, NH_4^+ , K^+ , and PO_4^{3-} were selected as variables. NH_4^+ has significant correlations with Mg^{2+} , Ca^{2+} , F^- , and Cl^- (Table 3); thus, it can act as an indicator of these elements. K^+ and PO_4^{3-} are the nutrients primarily important for plants in addition to nitrogen and have relatively less correspondence with other nutrients (Table 3). Furthermore, an alternative GLM with NO_3^- , K^+ , and PO_4^{3-} as fixed factors was tested. In that case, data of autoclaved soil were not used because the NO_3^- concentration of autoclaved soil was beyond the measurable range of ion chromatography, and accordingly, pH was removed from the factors because of the limitation of factor numbers acceptable in the GLM model. All statistical tests were conducted using R 3.1.2 (R development core team 2014).

Results

Chemical properties of the substrates

pH and ion concentrations of the substrates were different among the 6 experiments (Figs. 2, 3). Decayed wood, particularly brown-rotted wood, had a significantly lower pH than soil. Autoclaving significantly reduced pH in all substrates. Nutrient ion concentrations were affected by substrate and autoclaving, except for Na^+ , where standard errors of the data were large and significant effects were not detected (Table 2), although a trend of low Na^+ concentrations in brown-rotted wood compared with that in white-rotted wood and soil was evident (Fig. 3). Concentrations of NH_4^+ were significantly higher in autoclaved white-rotted wood and soil than brown-rotted wood and non-autoclaved white-rotted wood. K^+ was significantly and substantially higher in white-rotted wood than brown-rotted wood and soil. Mg^{2+} and Ca^{2+} were also significantly higher in white-rotted wood and soil than brown-rotted wood. F^- showed the same trend in autoclaved substrates but not in non-autoclaved substrates. A weak effect of substrates on Cl^- concentrations was detected by two-way ANOVA, whereas significant differences were not detected by Tukey's HSD post hoc test (Table 2). NO_3^- concentrations were extremely higher in non-autoclaved soil than other substrates, whereas the data for autoclaved soil were not obtained because the NO_3^- concentrations were below the limit of detection of our ion chromatography system. SO_4^{2-} concentrations were significantly higher in soil than wood. PO_4^{3-} concentrations were significantly higher in autoclaved woods than non-autoclaved woods and soil. To summarize, brown-rotted wood contained a low level of nutrients, and autoclaving increased nutrient concentrations.

Table 3 shows Pearson's correlation coefficients between the chemical properties of the substrates. Among the nutrient ions, NH_4^+ and F^- concentrations had significant correlations with 6 of the chemical properties measured. K^+ , Mg^{2+} , Ca^{2+} , and Cl^- concentrations had significant correlations with 5 of the other properties. pH showed 4 correlations, SO_4^{2-} and PO_4^{3-} concentrations showed 3 correlations, and NO_3^- concentrations showed 2 correlations with other properties. Na^+ concentrations showed no correlations with other properties.

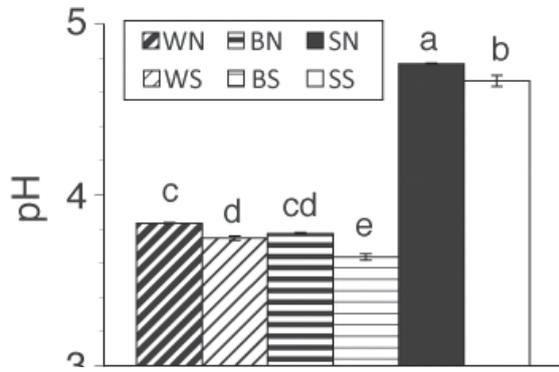


Fig. 2. pH of the substrates in the 6 experiments. WN, non-sterile white-rotted wood; WS, sterile white-rotted wood; BN, non-sterile brown-rotted wood; BS, sterile brown-rotted wood; SN, non-sterile soil; and SS, sterile soil.

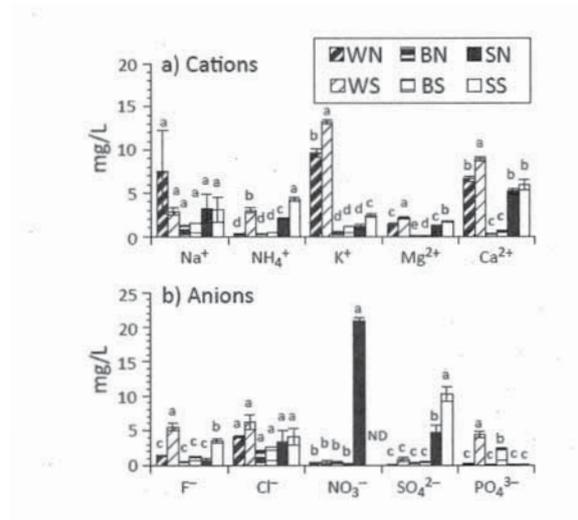


Fig. 3. Concentrations of the cations and anions in the substrates used in the 6 experiments. Abbreviations of the experiments are shown in Fig. 2.

Table 2. Results of two-way ANOVA evaluating effects of substrate, autoclaving, and their interaction on pH and nutrient ion concentrations in white- and brown-rotted wood and soil used as the substrates for seedling growth.

	Two-way ANOVA (<i>F</i> value)		
	Substrate	Autoclaving	Interaction
pH	2222.83 ***	61.50 ***	1.18
Cations			
Na ⁺	1.50	0.68	0.76
NH ₄ ⁺	130.50 ***	144.00 ***	31.70 ***
K ⁺	985.22 ***	71.02 ***	17.12 ***
Mg ²⁺	473.29 ***	78.26 ***	14.76 ***
Ca ²⁺	267.44 ***	17.75 **	5.10 *
Anions			
F ⁻	46.41 ***	137.87 ***	21.49 ***
Cl ⁻	4.72 *	2.32	0.39
NO ₃ ⁻	3545.68 ***	0.11	0.39
SO ₄ ²⁻	97.95 ***	19.46 ***	12.50 **
PO ₄ ³⁻	53.17 ***	171.23 ***	45.43 ***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3. Pearson's correlation coefficients between chemical properties of the substrates.

Variable	pH	Na ⁺	NH ₄ ⁺	K ⁺	Mg ²⁺	Ca ²⁺	F ⁻	Cl ⁻	NO ₃ ⁻	SO ₄ ²⁻	PO ₄ ³⁻
pH	1	0.02	0.62 **	-0.37	0.37	0.27	-0.03	0.004	0.98 ***	0.84 ***	-0.50 *
Na ⁺		1	-0.05	0.27	0.25	0.30	0.04	0.35	0.001	0.04	-0.15
NH ₄ ⁺			1	0.16	0.71 ***	0.60 **	0.74 ***	0.51 *	0.38	0.80 ***	0.17
K ⁺				1	0.71 **	0.79 ***	0.68 **	0.66 **	-0.38	-0.31	0.59 *
Mg ²⁺					1	0.98 ***	0.75 ***	0.66 **	0.17	0.40	0.28
Ca ²⁺						1	0.72 ***	0.72 ***	0.14	0.27	0.32
F ⁻							1	0.73 ***	-0.29	0.25	0.68 **
Cl ⁻								1	-0.06	0.20	0.42 +
NO ₃ ⁻									1	0.94 ***	0.33
SO ₄ ²⁻										1	-0.37
PO ₄ ³⁻											1

+ $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Seedling properties

Fig. 4 shows the seedling properties measured. In *P. densiflora* seedlings, the shoot length was significantly longer in non-sterile soil than in sterile white- and brown-rotted woods, whereas root weight was significantly larger in non-sterile white-rotted wood than sterile brown-rotted wood and soil. S/R was significantly larger in soil than non-sterile white- and brown-rotted woods. Leaf number and shoot weight were not significantly different among the experiments. Using two-way ANOVA, a substrate effect was detected in the shoot length and S/R, whereas an effect of autoclaving was detected in shoot length, shoot weight, and root weight (Table 4). Interactions between substrate and autoclaving were not detected in any of the seedling properties measured.

In *C. japonica* seedlings, the aboveground growth (shoot length, shoot weight, and leaf number) was generally larger in soil than decayed wood, whereas root weight was not significantly different among the experiments. S/R was significantly larger in soil than wood in non-sterile experiments. Using two-way ANOVA, a significant substrate effect was detected in all properties tested, whereas an effect of autoclaving was not detected (Table 4). Significant interactions between substrate and autoclaving were not detected.

In *C. barbinervis* seedlings, shoot weight and leaf number were larger in non-sterile soil than non-sterile decayed woods. However, in sterile experiments, shoot weight and leaf number in brown-rotted wood were significantly larger than those in other woods and reached the same growth level as that achieved in non-sterile soil. Shoot length, root weight, and S/R

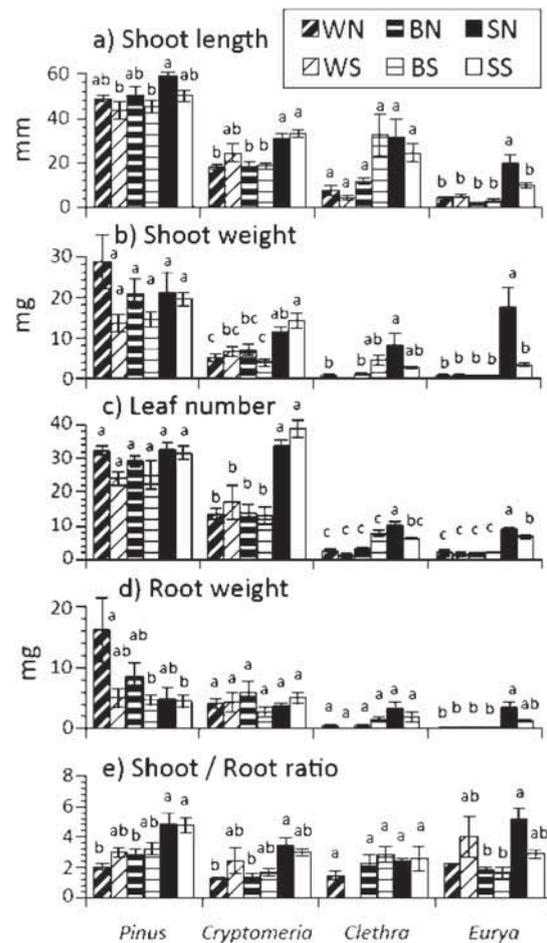


Fig. 4. Seedling properties of the 4 tree species in the 6 experiments. Abbreviations of the experiments are shown in Fig. 2.

Table 4. Results of two-way ANOVA evaluating effects of substrate, autoclaving, and their interaction on seedling growth on white- and brown-rotted wood and soil.

	Two way ANOVA (<i>F</i> value)		
	Substrate	Autoclaving	Interaction
Shoot length (mm)			
<i>Pinus densiflora</i>	4.0 *	7.3 *	0.4
<i>Cryptomeria japonica</i>	18.8 ***	2.3	0.9
<i>Clethra barbinervis</i>	3.9 *	0.3	2.1
<i>Eurya japonica</i>	25.3 ***	4.1 +	5.9 *
Shoot weight (mg)			
<i>Pinus densiflora</i>	0.6	5.5 *	1.4
<i>Cryptomeria japonica</i>	22.0 ***	0.1	2.8 +
<i>Clethra barbinervis</i>	4.4 *	0.6	4.2 *
<i>Eurya japonica</i>	14.8 ***	9.6 **	8.8 **
Leaf number			
<i>Pinus densiflora</i>	2.0	4.3 +	0.9
<i>Cryptomeria japonica</i>	39.4 ***	1.2	0.6
<i>Clethra barbinervis</i>	26.5 ***	0.7	15.1 ***
<i>Eurya japonica</i>	107.3 ***	5.7 *	3.1 +
Root weight (mg)			
<i>Pinus densiflora</i>	3.0 +	5.3 *	2.0
<i>Cryptomeria japonica</i>	0.0	0.3	2.2
<i>Clethra barbinervis</i>	5.6 *	0.3	1.7
<i>Eurya japonica</i>	19.8 ***	7.0 *	6.4 **
Shoot / Root ratio			
<i>Pinus densiflora</i>	15.81 ***	1.47	0.69
<i>Cryptomeria japonica</i>	8.59 **	0.80	1.77
<i>Clethra barbinervis</i>	1.08	0.43	0.19
<i>Eurya japonica</i>	4.93 *	1.77	2.70 +

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

were not significantly different among the experiments. Using two-way ANOVA, a substrate effect was detected in all properties, except S/R (Table 4). An effect of autoclaving was not detected, whereas significant interactions between substrate and autoclaving were detected in shoot weight and leaf number.

In *E. japonica* seedlings, above- and belowground growth was significantly larger in soil than other substrates particularly in the non-sterile experiment. S/R in non-sterile soil was larger than in brown-rotted wood. Using a two-way ANOVA, a significant substrate effect was detected in all properties (Table 4). Significant autoclaving effects were also detected on shoot weight, leaf number, and root weight. Significant interactions were detected on shoot length, shoot weight, and root weight.

Chemical factors associated with seedling properties

In *P. densiflora* seedlings, leaf number was negatively associated with PO_4^{3-} . Other properties

of *P. densiflora* were not associated with chemical factors (Table 5). In *C. japonica* seedlings, shoot length was positively associated with pH and NH_4^+ , whereas other seedling properties showed no relationships with chemical factors. Any measured properties of *C. barbinervis* seedlings were not associated with chemical factors. In *E. japonica* seedlings, leaf number was positively associated with pH and PO_4^{3-} and negatively associated with NH_4^+ , whereas other seedling properties showed no relationships with chemical factors.

Discussion

The lower pH of rotten wood compared with soil observed in the present study, particularly the lower pH of brown-rotted wood, is in line with results of previous studies (Espejo and Agosin 1991; Takahashi et al. 2000; Fukasawa 2012, 2015a; Fukasawa et al. 2015). Organic acids released from saprobic fungi during the decay process reduce the pH of decaying woody substrates (Rayner and Boddy 1988), particularly in brown rot process where brown rot fungi use

oxalic acids to generate hydroxyl radicals for non-enzymatic cell wall degradation (Espejo and Agosin 1991). Acidification after autoclaving may be partly caused by the enhanced ionization of nutrients, particularly F^- , NH_4^+ , K^+ , Mg^{2+} , Ca^{2+} , and PO_4^{3-} (Vacin and Went 1949; Matsui 1995). Sterilization of organic matter is known to cause leakage of nutrients immobilized in microbial inhabitants (Packer and Clay 2000; Troelstra et al. 2001; McCarthy-Neumann and Kobe 2010) and may result in the nutrient flush recorded in the present study. An additional cause of acidification by autoclaving may be attributed to the extraction of phenolic acids from decayed wood and plant residues in soil (Martens 2002). Hydrolysis of oligosaccharides such as sucrose could additionally contribute to a reduced pH (Druart and Wulf 1993); however, this may not have been the case in the cur-

rent study because decayed wood contains very little soluble sugar components (Eaton and Hale 1993). Observation of high concentrations of nutrient ions in white-rotted wood in the current study were additionally consistent with previous reports (Takahashi et al. 2000; Fukasawa 2015a), although the reason for this trend remains unclear.

The present study clearly showed that substrate differences and autoclaving affect seedling growth, whereas the responses were species specific. *P. densiflora* seedlings were affected by both substrate and autoclaving. Autoclaving had much more effect than substrate on pine seedling growth; autoclaving reduced the above- and belowground seedling growth, probably attributable to the lack of symbiotic microbes in autoclaved substrates. It is well-known that ectomycorrhizal symbiosis is essential for the growth

Table 5. Generalized liner model results showing parameters estimated for chemical factors associated with seedling properties of each of the 4 tree species.

	pH	NH_4^+	K^+	PO_4^{3-}	$NO_3^{-\dagger}$
Shoot length (mm)					
<i>Pinus densiflora</i>	1.46	-0.88	0.05	0.16	0.82 ⁺
<i>Cryptomeria japonica</i>	0.76 [*]	0.39 [*]	-0.06	0.19 ⁺	0.91
<i>Clethra barbinervis</i>	0.9	-0.47	-0.84	0.77	0.45
<i>Eurya japonica</i>	1.81 ⁺	-0.8	0.11	0.68	1.16 [*]
Shoot weight (mg)					
<i>Pinus densiflora</i>	0.18	-0.41	0.81 ⁺	-1.08 ⁺	-0.09
<i>Cryptomeria japonica</i>	0.30	0.67	-0.12	-0.34	0.68
<i>Clethra barbinervis</i>	1.72	-1.11	-0.48	1.06	0.86
<i>Eurya japonica</i>	1.98	-1.14	0.02	0.86	1.12 ^{**}
Leaf number					
<i>Pinus densiflora</i>	0.59 ⁺	-0.26	0.46 ⁺	-0.84 [*]	0.36
<i>Cryptomeria japonica</i>	0.65	0.41	-0.12	-0.04	0.81 ⁺
<i>Clethra barbinervis</i>	1.3	-0.76	-0.67	0.82	0.66
<i>Eurya japonica</i>	1.29 ^{**}	-0.31 [*]	-0.12 ⁺	0.29 [*]	0.97 [*]
Root weight (mg)					
<i>Pinus densiflora</i>	-0.27	-0.39	0.92 ⁺	-0.99	-0.49
<i>Cryptomeria japonica</i>	-1.43	1.27	0.03	-1.31	-0.41
<i>Clethra barbinervis</i>	1.48	-0.72	-0.43	0.69	0.87
<i>Eurya japonica</i>	1.83	-0.92	-0.05	0.74	1.11 ^{**}
Shoot / Root ratio					
<i>Pinus densiflora</i>	0.85 [*]	0.17 ⁺	-0.51 [*]	0.47 [*]	0.85 [*]
<i>Cryptomeria japonica</i>	1.29 ⁺	-0.12	-0.10	0.71	1.09 ⁺
<i>Clethra barbinervis</i> ^{††}	–	0.40 [*]	-0.94 [*]	0.98 [*]	–
<i>Eurya japonica</i>	1.82	-0.82	0.39	0.95	1.20

[†] Calculated by alternative model.

^{††} Data of autoclaved soil were not applicable because very little root growth prevented S/R calculation and, accordingly, pH and NO_3^- were excluded from analysis because of the limitation of factor number available for the model.

of *P. densiflora* seedlings (Yamada and Katsuya 1996, 2001; Guerin-Laguet et al. 2004; Sim and Eom 2006; Ma et al. 2010, 2012; Dalong et al. 2011). Because well decayed wood contains a substantial number of ectomycorrhizal propagules (Kubartová et al. 2012; Rajala et al. 2011, 2012, 2015; Fukasawa and Matsuoka 2015), seedlings colonized on decayed wood can develop ectomycorrhizal root tips to the same extent as seedlings established on the ground (Christy et al. 1982; Vogt et al. 1995; Goodman and Trofymow 1998; Tedersoo et al. 2003, 2008, 2009; Baier et al. 2006; Buée et al. 2007; Elliott et al. 2007; Iwański and Rudawska 2007; Walker and Jones 2013; Fukasawa 2015a). Autoclaving destroys the entire community of ectomycorrhizal fungi in the substrates, which may cause a significant reduction in nutrient acquisition and growth of pine seedlings. Although substrate differences had little effect on the aboveground growth of pine seedlings, significant effects on root growth and S/R were evident. Smaller S/R values in *P. densiflora* seedlings grown on decayed wood than in soil were also reported within the field survey by Fukasawa (2015). Differences in plant root allocation between substrates are generally attributable to water and nutrient (particularly nitrogen) availability of the substrates (Lambers et al. 1998). Because in the present study, water content was regulated to be similar among experiments, the differences in plant root allocation were likely attributable to nutritional differences between substrates. Chiwa et al. (2012) showed that *P. densiflora* seedlings with good nitrogen uptake allocate more carbon to shoots than roots. In the present study, although NH_4^+ concentrations showed no significant associations with root growth and S/R in the GLM model, an alternative model with NO_3^- instead of NH_4^+ , detected a significant positive effect of NO_3^- on S/R of *P. densiflora* seedlings. These results suggested that *P. densiflora* seedlings have high potential for morphological plasticity and can colonize both soil and decayed woods regardless of the decay type as long as the symbiotic fungal communities exist. Previous field observations additionally support the assertion of allocation plasticity and colonizability on a variety of microsites (Fukasawa 2015a; Han et al. 2015).

Growth, particularly aboveground growth, of *C. japonica* seedlings differed among substrates and was clearly explained by pH and NH_4^+ , whereas growth was not affected by autoclaving. These results

suggested that *C. japonica* seedlings prefer relatively high pH and nitrogen rich substrates rather than viability of soil symbiotic organisms. The sensitivity of *C. japonica* seedlings to NH_4^+ was also reported by Yamaguchi et al. (2014), where NH_4^+ substantially promoted the net photosynthetic rate in current-year needles of *C. japonica* seedlings, probably because of increased stomatal conductance and concentrations of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and chlorophyll. The relationships between pH and seedling growth have additionally been extensively researched. Matsui (1995) reported that the acidic condition near pH 3.0 causes extensive damage to biological activity of *C. japonica* seedlings and found pH 3.2 to be the lower limit threshold of seedling tolerance in a water culture experiment, although the effects were reduced when humus-containing forest soil was used as a growth substrate (Hirano and Hijii 2000). Soil acidic conditions are known to reduce plant growth not only by the root-growth inhibition effect (Mallik 2003) but also by accelerating the release of toxic aluminium ions from soil particles (Kramer and Kozłowski 1979). Although the concentration of aluminium ions was not measured in the present study, Ostrofsky et al. (1997) reported that aluminium concentration in decayed wood is $<8 \mu\text{M}$ even when the wood is decayed by certain brown rot fungi that accumulate relatively high concentrations of aluminium. This concentration ($8 \mu\text{M}$) is lower than the previously-tested minimum effect level on *C. japonica* growth ($20 \mu\text{M}$) (Hirano et al. 2007). *C. japonica* is an arbuscular mycorrhizal plant (Fujimaki et al. 2001). However, the importance of mycorrhizal colonization on the performance of their seedlings is rarely studied. In the present study, autoclaving of the substrates had no significant effects on *C. japonica* seedling growth, which was opposite to the response of *P. densiflora* seedlings. These results suggested that *C. japonica* seedlings are highly sensitive to the nutrient level of the substrates; thus, they are likely to preferentially colonize on soil rather than decayed wood.

The aboveground growth of *C. barbinervis* in brown-rotted wood was, though limited to the sterile experiment, recorded as the same level as that in soil, whereas less growth was recorded on white-rotted wood. This result is consistent with observations of previous field studies where the seedling density of *C. barbinervis* was high on brown-rotted wood

(Fukasawa 2012) as well as soil without a litter layer (Nakashizuka 1989; Kobayashi and Kamitani 2000). Because none of the chemical factors showed significant associations with seedling growth, the reason for the increased growth of *C. barbinervis* seedlings on brown-rotted wood than white-rotted wood remains unclear. *C. barbinervis* is an arbuscular mycorrhizal tree species, and it was suggested that mycorrhizal symbiosis is important for the growth of *C. barbinervis* seedlings on decayed logs (Fukasawa 2012). However, any evidence showing positive effects of fungal symbionts on *Clethra* seedling growth (i.e., greater growth in non-sterile experiments than sterile experiments) was not detected.

The above- and belowground growth of *E. japonica* was larger in non-sterile soil than wood and was positively associated with NO_3^- concentration. *E. japonica* is a small-seeded shrub species regenerating on exposed mineral soil on ridges or upper slopes in the forest understory (Manabe and Yamamoto 1997). Fukasawa (2012) reported that seedling density of *E. japonica* is larger on soil than decayed logs. The observation of good growth in soil in the present study is in line with these previous field observations and suggests that *E. japonica* seedlings have high nitrogen (notably nitrate) demands, although high nitrate demands are not consistent with their preference for ridges or upper slopes where nitrate-nitrogen is typically very low (Koyama et al. 2013).

In summary, the hypothesis that “species with high mycorrhizal dependence (*P. densiflora*) are strongly associated with a sterile treatment rather than substrate difference and species with high nutrient demands (*C. japonica*) are strongly associated with substrate difference rather than sterile treatment” was supported by the results of the present study. Similarly, the improved growth of *C. barbinervis* on brown-rotted wood than white-rotted wood supports my previous field observations (Fukasawa 2012) and confirms the preference for brown-rotted wood during seedling colonization. From the growth data of *P. densiflora*, *C. japonica*, and *E. japonica* on the 3 different substrates, it is expected that *P. densiflora* seedlings may be able to regenerate within the 3 substrates as long as mycorrhizal symbionts exist, whereas *C. japonica* and *E. japonica* seedlings may prefer soil rather than decayed wood because of the nutrient (especially nitrogen) conditions of soil. However,

a recent report regarding regeneration microsites of *C. japonica* indicates that they prefer decayed stumps rather than soil (Ota et al. 2015). Such disagreement between laboratory and field observations provides motivation for further research and may result in the discovery of new and unexpected mechanisms of regeneration site preferences in forest tree species.

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