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PHYTOTOXIC EFFECTS AND CHEMICAL ANALYSIS OF LEAF EXTRACTS FROM THREE *Phytolaccaceae* SPECIES IN SOUTH KOREA

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Abstract—We analyzed phenolic compounds and other elements in leaf extracts and compared morphology of three species of the Phytolaccaceae family found in South Korea. To test allelochemical effects of the three *Phytolacca* species, we also examined seed germination and dry weight of seedlings of *Lactuca indica* and *Sonchus oleraceus* treated with leaf extracts. The concentrations of total phenolic compounds were exotic *Phytolacca esculenta* (3.9 mg/l), native *Phytolacca insularis* (4.4 mg/l), and exotic *Phytolacca americana* (10.2 mg/l). There was no significant difference in concentrations between *P. esculenta* and *P. insularis*, but the concentration of total phenolics in *P. americana* was two times higher than either *P. esculenta* or *P. insularis*. Analysis of aqueous extracts by HPLC showed seven phenolic compounds (gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, *m*-hydroxybenzoic acid, ρ-coumaric acid, and cinnamic acid). Total phenolics in *P. americana* were eight to 16 times higher than either *P. esculenta* or *P. insularis*, respectively. *P. americana* inhibited seed germination and dry weight of the two assay species. The phytotoxic effects of the two *Phytolacca* species were different, despite the fact that *P. esculenta* and *P. insularis* had similar levels of total phenolic compounds. We also found that *P. americana* had invaded Ullung Island, which suggested that *P. americana* had excellent adaptability to the environment. The three species of Phytolaccaceae in South Korea can be distinguished by their different allelopathic potentials and morphologies.

Key Words—Allelochemical effects, Phytolaccaceae, total phenolic compounds, seed germination, morphology.

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INTRODUCTION

Many plants in the Phytolaccaceae are distributed throughout tropical and subtropical zones. Three species of Phytolaccaceae are found in South Korea: *Phytolacca esculenta*, *Phytolacca insularis*, and *Phytolacca americana* (Lee, 1985). Among these, *P. americana* is native to North America, but is distributed widely in Europe and Asia. It is a perennial weed in the central and southern provinces and Cheju Island. Since 1993, it has become an invasive exotic in South Korea and is threatening native species (Lee et al., 1997; Han et al., 1998; Kim et al., 2000), especially in polluted areas (Park, 1995a; Park et al., 1999). *P. insularis* is native to Ullung Island, and *P. esculenta* is native to China, but occurs rarely in the central province including Cheju Island in South Korea (Lee, 1985; Park, 1995b). There have been several reports of the role of allelopathy in exotic plant invasions and its defense against the spread of invaders to maintain biodiversity of natural systems, community structure, and ecosystem function (Callaway and Aschehoug, 2000; Keane and Crawley, 2002; Kennedy et al., 2002; Bais et al., 2003; Vila and Weiner, 2004). Little is known about the Phytolaccaceae except for a few papers dealing with the invasive threat of *P. americana* in South Korea (Han et al., 1998; Kim et al., 2000). Researchers in South Korea have claimed that *P. esculenta* and *P. insularis* are not morphology distinct, although their distributions are limited to different areas. The opinion of researchers is that *P. insularis* is the same species as *P. esculenta* and that there are no native species of Phytolaccaceae in South Korea. To address this issue, morphological and chemical analyses of these three species were conducted.

Allelopathy is an ecological mechanism operating in natural and managed ecosystems. In competition between exotic and native species, exotic species that produce large amounts of allelochemicals effectively repel other species, and their ability to invade plant communities increases (Pellissier, 1993; Inderjit and Dakshini, 1998; Kim et al., unpublished). There are many secondary metabolites that act as plant allelochemicals including phenolic, terpenoid, flavonoid, and alkaloids. Among these, phenolic compounds are the most abundant under field conditions and are known to affect seed germination, seedling growth, cell division, and fungal activity (Lodhi, 1976; Bhowmik and Doll, 1984; Rice, 1984, 1995; Inderjit, 2003; Kim et al., 2000).

Recently, phenolic-induced oxidative damage was found to be mediated by aluminum (Sakihama and Yamasaki, 2002) and that accumulation of such metals occurs by conjugation with phenolic compounds in various plant organs (Santiago et al., 2000; Lavid et al., 2001). Under field conditions, phenolics interfere with the growth and establishment of competing plant species by releasing water-soluble compounds into the soil. Furthermore, extracts of *P. americana* inhibit seed germination, seedling growth, and exhibit antifungal activity at low concentrations (Lee et al., 1997; Kim et al., 2000).

Phenolic compounds were not studied in the context of physiological responses associated with this family. Therefore the goal of this study was to characterize the three species located in different environments by comparing physioecology of allelochemical effects, heavy-metal accumulation, and morphology in South Korea. The objectives of the present study were to (1) determine the effect of the leaf extracts of Phytolaccaceae (*P. esculenta*, *P. insularis*, and *P. americana*) on seed germination and seedling dry weight of *Sonchus oleraceus* and *Lactuca indica* and (2) differentiate species of Phytolaccaceae according to morphology, phenolic compounds, and allelochemical effect.

METHODS AND MATERIALS

Study Areas. We examined the bioassay of seed germination and seedling growth with the extract of roots, leaves, fruits, and stems. Leaf extract had the strongest effect among these, so we used leaf extracts in this experiment. Plant extracts were made from fresh leaves of 10 individual plants per species (*P. esculenta* V. Houtte, *P. insularis* Nakai, and *P. americana* L.). Leaves were collected from Youngdong (36°10′N, 127°46′E) in Chungcheong Province (*P. esculenta*), Dodong, Ullung Island (37°28′N, 130°54′E) in Kyungpook Province (*P. insularis*), and Suwon (37°16′N, 127°00′E) in Gyeonggi Province (*P. americana*), South Korea, in August 2001. Seeds of *S. oleraceus* L. and *L. indica* Hara were collected in October and November 2000 from old fields in Seoul, South Korea (37°30′N, 127°12′E), and were used for the seed germination bioassay. Youngdong (*P. esculenta*) and Dondong (*P. insularis*) sites are located on rural hillsides with less anthropogenic pollutants. On the other hand, Suwon (*P. americana*) site is at a recently developed suburban hillside, influenced by heavy vehicle traffic. The climate is temperate, with four distinct seasons. June, July, and August, corresponding to the summer season, account for more than half of the total annual rainfall.

Analysis of Phenolic Compounds and Heavy Metals. Chemical standards used for analysis of phenolic compounds were purchased from Sigma Chemical Co. Two hundred grams of fresh leaves of Phytolaccaceae (*P. esculenta*, *P. insularis*, and *P. americana*) were extracted with 1-l distilled water at room temperature for 48 hr and then centrifuged at 15,000 rpm for 30 min (Centrikon T-1045, Kontron Co.). The supernatant was collected and stored at 4°C until used for bioassay and analysis. Phenolic compounds were purified from water extracts by adding 10 ml of saturated NaCl to 40 ml of aqueous extract in a funnel. The pH was adjusted to pH 2 with 1 N HCl. After adding 20 ml of 5% $NaHCO_3$ and mixing, we collected the $NaHCO_3$ solution. This was adjusted to pH 2 with HCl and mixed with 20 ml of ether. After mixing, the ether layer was evaporated under reduced pressure with a rotary evaporator. The residue was dissolved into 5 ml of acetonitrile. Individual phenolics were analyzed by HPLC (Hewlett Packard Series 1050, USA), with diode-array detection (250, 254, 284 nm). Twenty microliters of extract was injected onto a μBonda-Pak C18 Radial Pak (0.8 × 10 m) column, with a mobile phase of acetonitrile and 0.02 M sodium acetate (pH 4.3) and a flow rate of 1.3 ml/min. Total phenolics were measured using the Folin-Ciocalteu phenol reagent (Swain and Hillis, 1959). Leaf extracts were analyzed for elements B, Al, Co, Cd, Cu, Fe, Mn, Ni, Pb, and As (Agricultural Improving Institute, 1988).

Morphological Comparison of Phytolaccaceae. The morphology of Phytolaccaceae was observed by anatomy microscope (36, EPT, 160T). The anther of *P. esculenta* was observed at 2.5 × 50 and the flower and the fruit at 2.5 × 8.5. The anther of *P. insularis* was observed at 2.5 × 25 and the flower and the fruit at 2.5 × 10, and the anther of *P. americana* at 2.5 × 30 and the flower and the fruit at 2.5 × 10. Those were compared between Phytolaccaceae. Photographs were taken using a Nikon F801 camera, using T-MAX 100 Kodak film (Phillips et al., 2000).

Seed Germination and Seedling Bioassay. Leaf extracts were filtered through Whatman No. 1 filter paper and then diluted to concentrations of 0, 12.5, 25, 50, 75, and 100% for bioassay. After being selected for uniform size, seeds from *S. oleraceus* and *L. indica* were sterilized for 3 min in a solution of 5% sodium hypochlorite and then rinsed with distilled water. Thirty seeds were sown in a Petri dish (diam 90 mm) on filter paper treated with leaf extract solution. The bioassay was repeated four times in a growth chamber set at a constant temperature of 26°C and 80% relative humidity, with an alternating photoperiod of 12 hr light/12 hr dark for 10 days. The germination percentage

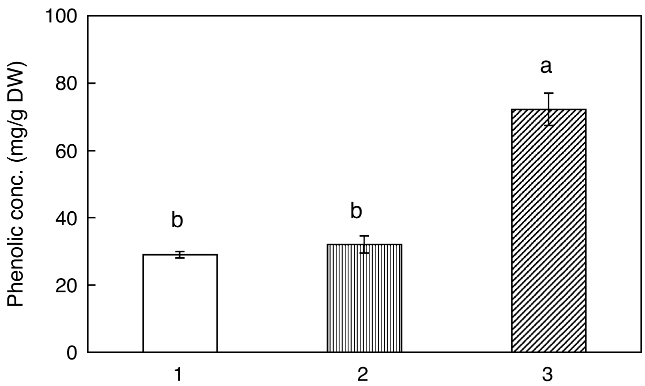

FIG. 1. Comparison of total phenolic compounds from leaf extracts of (1) *P. esculenta*, (2) *P. insularis*, (3) *P. americana*. Means (±SE, $N = 5$) with the same letter are not significantly different (Duncan's multiple range test, $P < 0.001$).

and dry weight for each seedling were measured at each concentration of extract. Seed germination was assessed daily, whereas the dry weight of seedlings was measured after 1 wk. This bioassay was modified from Lodhi (1976).

Statistical Analysis. The data were normally distributed, and significant differences at $P < 0.001$ between the total phenolic compound, treatments, and controls of the bioassay were calculated using Duncan's multiple-range test (SAS Institute 2000, Figures 1 and 4).

RESULTS

Among the species examined, *P. americana* had the highest total phenolic concentrations at 72.2 mg/l, whereas *P. esculenta* and *P. insularis* had concentrations of 29.0 and 32.1 mg/l, respectively (Figure 1, $P < 0.001$). Seven phenolic compounds were identified by HPLC analysis of Phytolaccaceae extracts: gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, *m*-hydroxybenzoic acid, *ρ*-coumaric acid, and cinnamic acid. Chlorogenic acid in *P. esculenta* and *P. americana* exhibited the highest concentration, whereas *P. insularis* appeared to have none. A small concentration of *m*-hydroxybenzoic acid appeared in *P. esculenta*, but not in *P. americana* and *P. insularis*. An unknown peak was present at Rt = 5.5 min in *P. esculenta* and *P. insularis* that was not observed in *P. americana*. Another unknown peak was observed at 14.5 min in *P. esculenta* that was not present in either *P. insularis* or *P. americana* (Figure 2).

Most of the elements (B, Al, Mn, Ni, Cu, Zn, As, Cd, Pb, except Fe) were found about 3–16 times more concentrated in the leaves of *P. americana* than those of *P. esculenta* and *P. insularis*, respectively (Table 1).

Fruit from the three Phytolacca species had different characteristics. The fruit of *P. esculenta* was berry-like with the ovary separated into eight cells. The number of stamens was eight, the anther was pink, and the stem was pale green (Figure 3A). The fruit of *P. insularis* was also berry-like, the number of stamens was eight, the anther was white, and the stem was green (Figure 3B). The fruit of *P. americana* was spherical, the number of stamens was 10, the anther was white, and the stem was green and reddish (Figure 3C).

Seed germination and dry weight of *S. oleraceus* and *L. indica* were inhibited with increasing concentrations of Phytolaccaceae extract (Figure 4). Germination rates of the two species were greatly reduced in the leaf extracts of *P. americana* in comparison to *P. insularis* and *P. esculenta*, and were slightly stimulated by 12.5 and 25% concentration of *P. insularis* and *P. esculenta* (Figure 4A and C), respectively. The dry weights of *S. oleraceus* and *L. indica* were similar to the germination result, but the dry weights of *S. oleraceus* were slightly stimulated by 12.5–75% leaf extracts of *P. insularis* and *P. esculenta* (Figure 4B). *L. indica* seedling dry weight was stimulated by 12.5–75% of

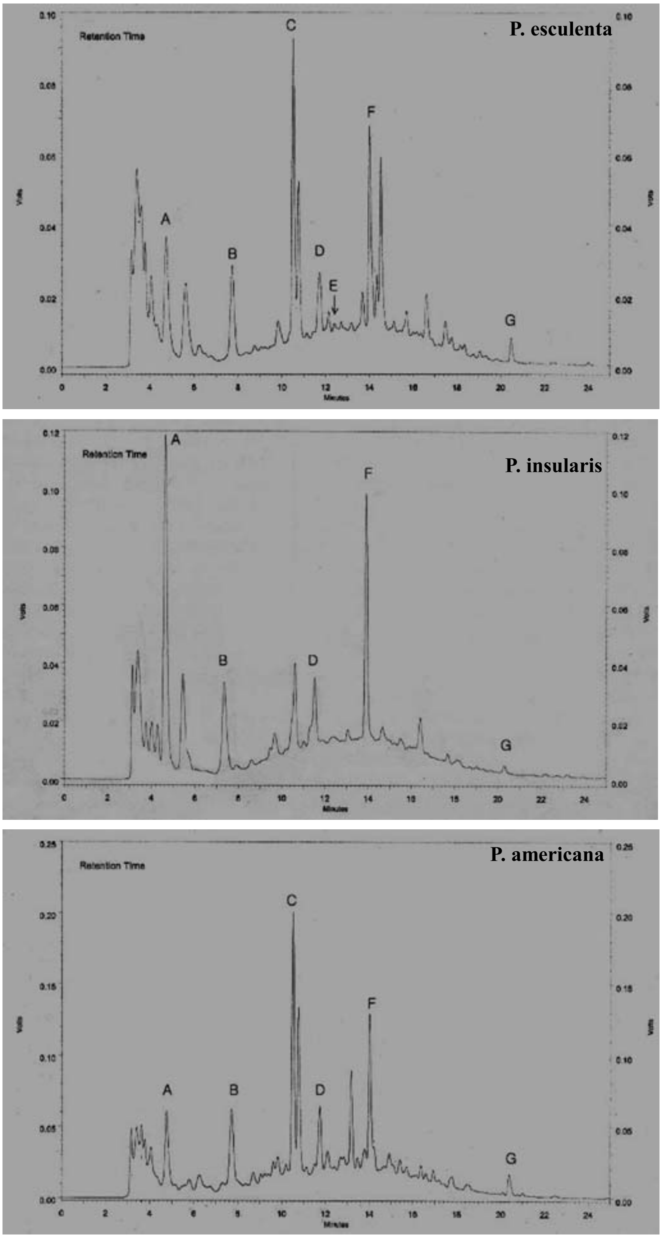

FIG. 2. Analysis of phenolic compounds in leaf extracts of *P. esculenta* (1), *P. insularis* (2), and *P. americana* (3) by HPLC. Identified phenolics are (A) gallic acid, (B) protocatechic acid, (C) chlorogenic acid, (D) caffeic acid, (E) *m*-hydroxybenzoic acid, (F) *ρ*-coumaric acid, and (G) cinnamic acid.

TABLE 1. HEAVY METAL AND NUTRIENT CONCENTRATIONS OF *Phytolacca* LEAF EXTRACT

	P. esculenta (mg/l)	*P. insularis* (mg/l)	*P. americana* (mg/l)
B	0.39	0.31	**0.81**
Al	1.49	1.32	**35.99**
Mn	8.11	0.42	**66.33**
Fe	2.80	**4.00**	2.32
Ni	0.0	0.0	**0.04**
Cu	0.04	0.03	**0.25**
Zn	0.72	0.05	**3.49**
As	0.04	0.04	**0.20**
Cd	0.0	0.0	**0.01**
Pb	0.0	0.0	**0.02**
Total	**13.59**	**6.17**	**109.46**

Highest concentrations of each element are in bold.

P. insularis (Figure 4D). Leaf extracts of the three species were significantly different (Figure 4, $P < 0.001$). Seed germination of *P. americana* was little affected by extracts of *P. americana* (data not shown).

DISCUSSION

Phenolic compounds have been studied as candidate allelochemicals because they are water-soluble and can complex with ions in the soil (Rice, 1984; Appel, 1993). *P. esculenta* and *P. americana* are exotic species, whereas *P. insularis* is the only native species of the Phytolaccaceae distributed in South Korea. *P. americana* is widely distributed not only in polluted areas, but also occurs in fields throughout South Korea, whereas the distribution of *P. esculenta* and *P. insularis* is limited and is decreasing in South Korea (Lee, 1985; Park, 1995a; Park et al., 1999). This phenomenon may be explained by total leaf phenolic concentration shown in the present study. *P. americana* had a much higher total phenolic concentration (72.18 mg/l) than those of either *P. esculenta* (29.04 mg/l) or *P. insularis* (32.08 mg/l) (Figure 1). The phenolic compounds in leaf extracts of *P. americana* were found to inhibit growth of 35 plant species and nine fungal species (Kim et al., 2000). The quantity and chemical nature of phenolics have important effects on the distribution of plant species and a plastic response that contributes to survival in adverse environmental conditions (Del Moral, 1972; Inderjit and Dakshini, 1998). Wu et al. (1998) isolated 14 phenolic compounds from *Buchloe dactyloides* with the most abundant phenolic compounds being salicylic and cinnamic acids. Among seven phenolic compounds analyzed, chlorogenic acid was the most abundant compound in the exotic species, *P. americana* and *P. esculenta*, whereas the

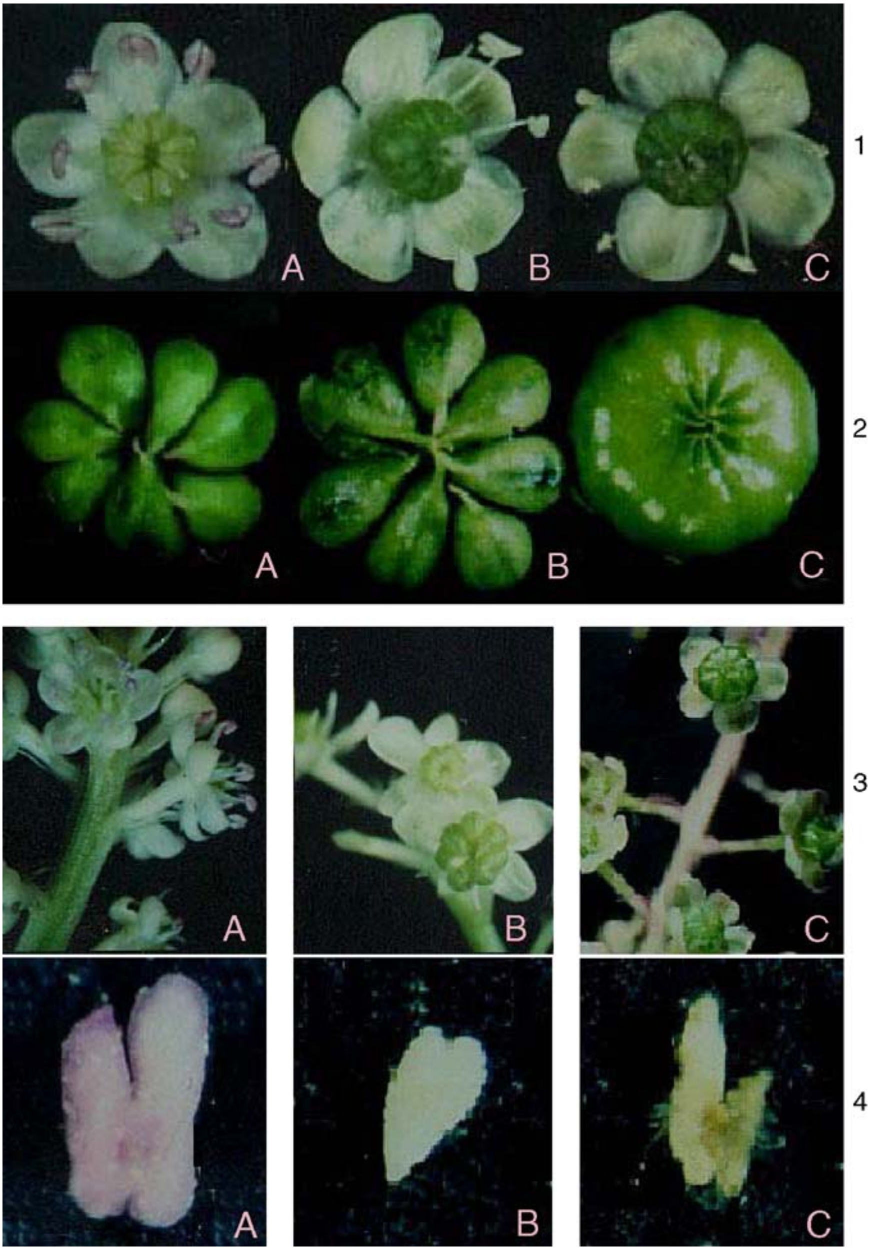

FIG. 3. Morphological comparison of flowers (1), fruits (2), stems and flowers (3), and anthers (4) on *P. esculenta* (A), *P. insularis* (B), and *P. americana* (C).

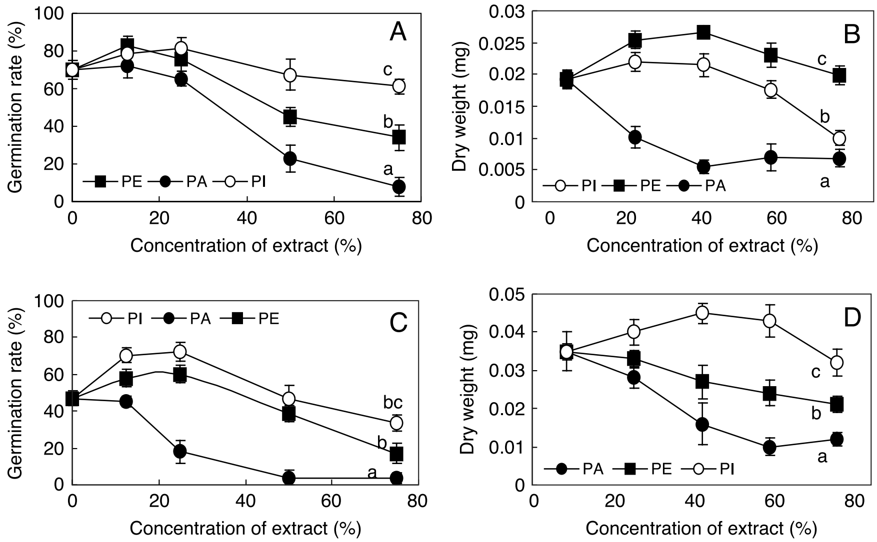

FIG. 4. Effect of the leaf extracts of *Phytolaccaceae* (PA: *P. americana*, PE: *P. esculenta*, PI: *P. insularis*) on seed germination and dry weight of *S. oleraceus* (A and B) and *L. indica* (C and D). Means (±SE, $N = 3$) with the same letter are not significantly different (Duncan's multiple range test, $P < 0.001$).

native species, *P. insularis*, had none (Figure 2). Therefore chlorogenic acid appears to be a candidate phenolic compound that could be used to differentiate between *P. esculenta* and *P. insularis*. Chlorogenic acid is derived from phenylalanine or tyrosine through the shikimic acid pathway and is more widespread in higher plants (Neish, 1964; Rice, 1984). In analysis of phenolic compounds from several plants, the unknown compounds in Figure 2 with Rt = 3.8, 5.5, and 14.5 min were identified as ρ-hydroquinone, gentistic acid, and scopoletin, respectively (Kim et al., 2000; Lee et al., 1997).

P. americana accumulates high concentrations of elements primarily in the leaves, whereas *P. esculenta* and *P. insularis* had lower concentrations (Table 1). Sakihama and Yamasaki (2002) reported that Al, Zn, Mn, and Cd stimulated phenoxyl radical-induced lipid peroxidation. The element concentrations in *P. americana*, especially Al and Mn, were higher than in *P. esculenta* or *P. insularis* (Table 1). *Ruta graveolens* treated with a mixture of Ni, Cu, Co, Cr, Cd, and Pb exhibited both qualitative and quantitative differences in phenolic concentration (Zobel et al., 1999), and phytoremediation plants in field accumulated high concentrations of metals in their foliage (Baker and Brooks, 1989; Raskin et al., 1997). Therefore, the accumulation of elements in the leaves of *P. americana* may be related to the high total phenolic concentration that was observed in the leaves and may account for the species distribution. *P. americana* is widely distributed in polluted areas throughout South Korea, and its distribution has been correlated with absorption of heavy metals from polluted soil (Park, 1995a; Park et al., 1999).

Lee (1985) and Park (1995b) reported that the only difference between *P. esculenta* and *P. insularis* was the color of the anther. We found that the anther color of *P. esculenta* was pink and *P. insularis* was white and yellow (Figure 3A and B). *P. americana* showed different fruit, flower, stem, and anther color (Figure 3C). Recently *P. americana* has been found around Dodong on Ullung Island, which is visited by many people. This suggests that *P. americana* has invaded Ullung Island, possibly as a result of its high total leaf phenolic concentration and its ability to adapt to this environment. This invasion may threaten the distribution of *P. insularis*, the native species present on the island.

Allelopathic effects typically are the result of an interaction of a mixture of compounds rather than due to a single compound (Veronneau et al., 1997; Inderjit, 2003). Under field conditions, additive or synergistic effects among allelochemicals become more influential, especially at low concentrations (Dalton, 1989; Einhellig, 1995). Thus, we investigated the seed germination and dry weight of *S. oleraceus* and *L. indica* to test physiological effects of leaf extracts. The seed germination and dry weights of *S. oleraceus* and *L. indica* were dramatically inhibited by increasing concentration of *P. americana* extracts. The phytotoxic effects of *P. esculenta* and *P. insularis* were significantly different, although they had similar levels of total phenolic compounds (Figures 1, 2, and 4). We also investigated self-limiting phytotoxic compounds in *P. americana* to confirm if *P. americana* had self-constraining effects on its own seed germination and seedling growth. Seed germination of *P. americana* was little affected by extracts of *P. americana* (data not shown). Consequently, the extract of *P. americana* may decrease the invasiveness of neighbor species more than those of *P. insularis* and *P. esculenta* ensuring survival of their offspring. The phenolic compounds found in leaf extracts have been reported to reduce seed germination, seedling growth, and dry weight (Einhellig, 1995; Inderjit, 2003) as shown in the present study (Figure 4). Since 1993, *P. americana* has become a threat to native species in South Korea (Han et al., 1998; Park et al., 1999; Kim et al., 2000). The overall pattern of seed germination inhibition by leaf extracts containing phenolic compounds corresponds with the findings of previous studies (Pellissier, 1993; Lydon et al., 1997). Species containing abundant phenolic compounds strongly suppressed seed germination of other species. In our study, seedling growth and dry weight was slightly stimulated by 12.5–25% concentration of *P. insularis* and *P. esculenta* extract, but not with *P. americana* extract. Similarly, seed germination is stimulated by low concentrations of phenolic compounds (Heisey, 1990; Inderjit and Dakshini, 1998). Our results showed that *P. americana* also has abundant phenolic compounds that inhibit seed germination and seedling biomass. We suggest that *P. americana* is a successful invader of existing plant communities and may be able to expand its habitats by employing foliar allelochemicals. We also predict that *P. americana* is more adaptable than *P. insularis* and *P. esculenta* to environmental changes through the production of self-defense allelochemicals. Therefore, we need to protect *P. insularis* and *P. esculenta* from *P. americana* to maintain species diversity in South Korea.

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