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Research Article

Humic Acid and Synthesized Humic Mimic Promote the Growth of Italian Ryegrass

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ABSTRACT

Humic acid (HA) is a complex organic matter found in the environments, especially in grassland soils with a high density. The bioactivity of HA to promote plant growth depends largely on its extraction sources. The quality-control of HA and the quality improvements via an artificial synthesis are thus challenging. We recently reported that a polymeric product from fungal laccase-mediated oxidation of catechol and vanillic acid (CAVA) displays a HA-like activity to enhance seed germination and salt stress tolerance in a model plant, *Arabidopsis*. Here, we examined whether HA or CAVA enhances the growth of Italian ryegrass seedling. Height and fresh weight of the plant with foliar application of HA or CAVA were bigger than those with only water. Interestingly, enhanced root developments were also observed in spite of the foliar treatments of HA or CAVA. Finally, we proved that HA or CAVA promotes the regrowth of Italian ryegrass after cutting. Collectively, CAVA acts as a HA mimic in Italian ryegrass cultivation, and both as a biostimulant enhanced the early growth and regrowth after cutting of Italian ryegrass, which could improve the productivity of forage crops.

(**Key words** : Growth, Humic acid, Italian ryegrass, Phenol derivative, Regrowth)

Ⅰ. INTRODUCTION

Humic substances (HSs) in soils are divided into humic acid (HA), fulvic acid (FA) and humin depending on their solubility in acidic and alkaline water (Cunha-Santino and Bianchini-Júnior, 2004). Interestingly, grassland soils contain around 70% HA and 30% FA, and forest soils have *vice versa* (Stevenson, 1982). HSs are a mixture of complex soil organics that not only help plants growing fast and healthy, but also modulate soil biochemistry (Nikbakht et al., 2008; Muscolo et al., 2013). Bioactivity of HA to plants has been reported to be involved in various developmental and physiological stages such as seed germination, photosynthesis, nutrient uptakes, and root elongation (Vaughan, 1974; Cacco and Dell Agnolla, 1984; Russo and Berlyn, 1990; Trevisan et al., 2010a; 2010b). In addition, HA tolerates salt-induced growth inhibition in tomato, suggesting that HA could confer abiotic stress resistance to plants (Türkmen et al., 2004).

The annual market growth of HA for agricultural applications is remarkable, but the HA quality is inconsistent owing to dependence of its composition on the extraction sources (Stevenson, 1982; Metzger, 2010). The quality-control of HS has been thus challenged and alternative trials to employ a chemical synthesis method giving rise to humic-like materials have been issued. Analyses on HA structures showed that it contains amorphous portions and many functional groups including aromatic ring and phenols that are structurally similar with plant lignins (Duan and Gregor, 2003). Oxidative coupling of natural phenols are known to re-create a variety of natural aromatic polymers including plant lignins (Jeon et al., 2010; 2012; 2013).

The current authors recently demonstrated that an artificial

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humic mimic (CAVA) is synthesized by fungal laccase-catalyzed oxidation of catechol and vanillic acid and it boosts the ability of *Arabidopsis thaliana* (Col-0) for seed germination and salt tolerance similar to HA application (Cha et al., 2017). However, the plant stimulation activity of CAVA in respect to crop under soil systems has not been addressed yet, despite the fact that the crop cultivation in soils has a high economic value.

Italian ryegrass (*Lolium multiflorum* Lam.) native to southern Europe is a high-quality forage crop widely cultivated in temperate regions, suggesting that this plant is vulnerable to cold climates (Blount et al., 2005). To develop the cold tolerance of Italian ryegrass, NIAS (National Institute of Animal Science, RDA, Republic of Korea) have generated various hybrid ryegrasses through hybridization between Italian ryegrass and different species. Among them, new Italian ryegrass variety, "Kowinearly", displaying early-maturation showed a high productivity and a cold tolerance compared to "Florida 80" (Choi et al., 2011). Field experiments also showed that a winter survival rate of Kowinearly was an overall average of 90% in "Yunchun", northern part of South Korea, whereas that of Florida 80 was 69% during 2003-2006 (Choi et al., 2011). Italian ryegrass can be cultivated on rice paddy fields together with other forage crops including legumes (Kim et al., 2011). Due to wide supplies of excellent Italian ryegrass varieties to farmers, cultivating area during recent 10 years has been 7-fold expanded in South Korea since 2005 (http://www.rda.go.kr).

The aim of this study is to characterize the effect of HA and CAVA (i.e. HA mimic) on the biological activities of Italian ryegrass variety "Kowinearly". We found that foliar application of HA or CAVA accelerates the growth of shoots and roots of Italian ryegrass and furthermore promotes the regrowth of plants after cutting.

Ⅱ. MATERIALS AND METHODS

1. Plant materials and growth conditions

Italian ryegrass (*Lolium multiflorum* Lam. "Kowinearly") seeds were kindly provided from NIAS. Thirty seeds were directly sown in the potting soil No. 2 (Farmhannong). The germination chamber was set as 23 °C under dark condition. When the seedling leaves are protruded on the soil surface, the seedlings were transferred to the 16/8 h (light/dark) condition. To monitor the growth promotion of humic acid (HA) or a mixture of catechol and vanillic acid (CAVA), foliar application were carried twice by spray at 5 and 10 days after germination of Italian ryegrass. Plants were harvested at 17 d after germination and measured every parameter shown. To examine the regrowth activity by HA or CAVA, aerial parts of 7-day-old seedlings were cut leaving 3 cm above soil surface, and then allowed for regrowth with spraying every 5 d. Plants were harvested at 21 d after germination and measured height and fresh weight of aerial parts. Sterilized H_2O sprayed to plants as a negative control.

2. Preparation of HA and CAVA

HA was purchased from Sigma-Aldrich. A CAVA product derived from fungal laccase-catalyzed oxidative co-polymerization of catechol (CA; Sigma-Aldrich) and vanillic acid (VA; Sigma-Aldrich) was prepared via a previously reported method (Cha et al., 2017). The 100 mM sodium acetate buffer (pH 5.0)-based polymeric reactions were fully desalted with 5 kDa ultrafilter.

3. Determination of physiological parameter

Plants were harvested as mentioned above, and shoots and roots were divided to measure the fresh weight. Plant height was measured in intact plants grown in soil. Experiments were carried with three (for growth) or four (for regrowth after cutting) independent replicates.

4. Statistical Analysis

Data were analyzed the variance using SAS version 9.4 (SAS Institute) and means were separated by LSD (least significant difference) test at $P<0.05$.

Ⅲ. RESULTS AND DISCUSSION

1. HA- and CAVA-induced growth promotion of Italian ryegrass

We previously reported that natural phenols, such as CA, VA, or a mixture of CA and VA (CAVA), were oxidized by fungal laccase-catalyzed reactions, and their phenolic derivatives improved seed germination and salt stress tolerance in a model plant, *Arabidopsis* (Cha et al., 2017). While treatment of polymeric products from either CA or VA partially enhanced seed germination and salt tolerance, such bioactivity of CAVA was superior and also comparable to commercial HA. HA has been widely proven to be effective for growth and stress tolerance of various plant species, but little is known about the effects on forage crops to date (Serenella et al., 2002; Trevisan et al., 2010). First, we examined the biological effects of HA or HA mimic (i.e. CAVA) as biostimulants on early growth of Italian ryegrass seedlings. At 17 d after germination, Italian ryegrass plants were photographed after twice foliar application (at 5 and 10 d after germination) of H_2O (as a negative control), HA (86 mg L^{-1}) or CAVA (86 mg L^{-1}) by spray (Fig. 1A). In addition, HA or CAVA application significantly increased the height and fresh weight of Italian ryegrass compared to that of water (Fig. 1B and 1C). However, any significant differences were not observed between HA and CAVA, indicating that the bioactivity of CAVA to promote the early growth of Italian ryegrass is copied to that of HA. Although effects of HA in respect to molecular and biochemical levels of plants have not been revealed yet, it has been reported that HA-induced physiological responses leading to accelerated uptake of essential inorganics (e.g. nitrogen, phosphorus, potassium, and sulfur) and related plant growth promotion are feasible (Guminski, 1968; Mylonas and McCants, 1980). The similar effect of CAVA with HA strongly suggests that the artificial synthetic method (Cha et al., 2017) could be an effective way to re-create the well-known HA bioactivity.

2. Promotion of root capacity by application of HA or CAVA

Availability of nutrients in soil depends on the capacity of plant roots (Comerford et al., 1994). Previously, it has been identified that HA induces the lateral root formation via hormone-like activities in maize and tomato (Dobbss et al., 2007; Zandonadi et al., 2007; Trevisan et al., 2010). To test whether our HA and humic mimic (i.e. CAVA) promote root development in Italian ryegrass, root phenotype was monitored during 17 d-cultivation. As shown in Fig. 2A, root length and the extent of fine root formation after foliar application of HA or CAVA was largely increased compared to the water control.

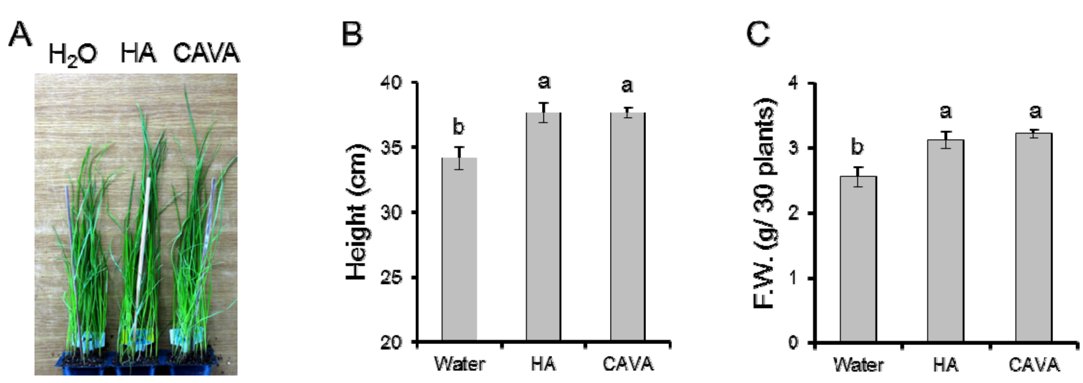

Fig. 1. Promotion of early seedling growth by foliar application of HA or CAVA in Italian ryegrass. The seedlings were grown in soil for 17 d with twice foliar application of HA or CAVA (86 mg L^{-1}). Water (H_2O) as a negative control was sprayed at the same time and method with HA or CAVA. (A) The picture taken at 17 d after germination. Plant height of canopy (B) and fresh weight (F.W.) of aerial parts (C) were measured at 17 d. Data are means ± SE (*n*=3). Means followed by the same alphabet letter are not significantly different at 5% level by the LSD test. Abbreviations: HA, commercial humic acids; CAVA, a co-polymeric product derived from fungal laccase oxidation of catechol and vanillic acid.

Moreover, the root fresh weight of HA or CAVA significantly increased (Fig. 2B). As similar with the results of growth promotion, the extent of root stimulation with CAVA was comparable to that with HA, suggesting that CAVA re-creates plant root stimulation action of HA. In plants, phytohormone auxin mainly works in root system and increases lateral root formation. Previous study suggested that HA also has an auxin-like activity with induction of mitotic sites triggering root emergence and density in maize root (Zandonadi et al., 2007). Russell et al. (2006) also demonstrated that HS application to pea leads to stomatal opening by the regulation of phospholipase A2 which is involved in auxin signaling.

3. Regrowth activity of HA or CAVA

Plant regrowth after grazing or cutting is an important characteristic for forage crops. Rapid growth and quick recovery could induce the high feeding frequency of livestock

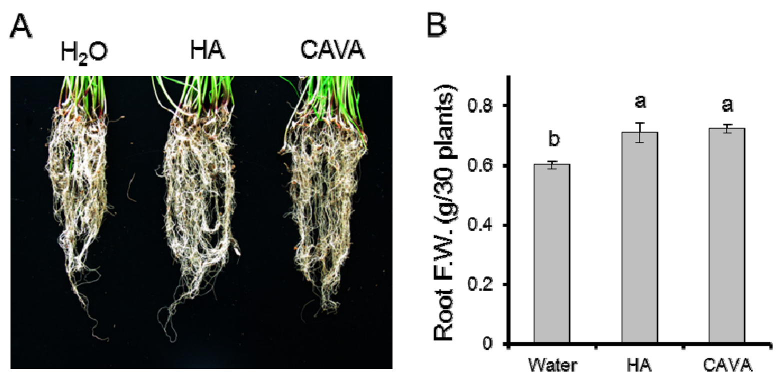

Fig. 2. Increased root capacity by foliar application of HA or CAVA. The seedlings were grown in soil for 17 d with twice foliar application of HA or CAVA (86 mg L^{-1}). Water (H_2O) as a negative control was sprayed at the same time and method with HA or CAVA. (A) The root picture taken at 17 d after germination. Fresh weight of roots (B) were measured at 17 d. Data are means ± SE (*n*=3). Means followed by the same alphabet letter are not significantly different at 5% level by the LSD test. Abbreviations: HA, commercial humic acids; CAVA, a co-polymeric product derived from fungal laccase oxidation of catechol and vanillic acid.

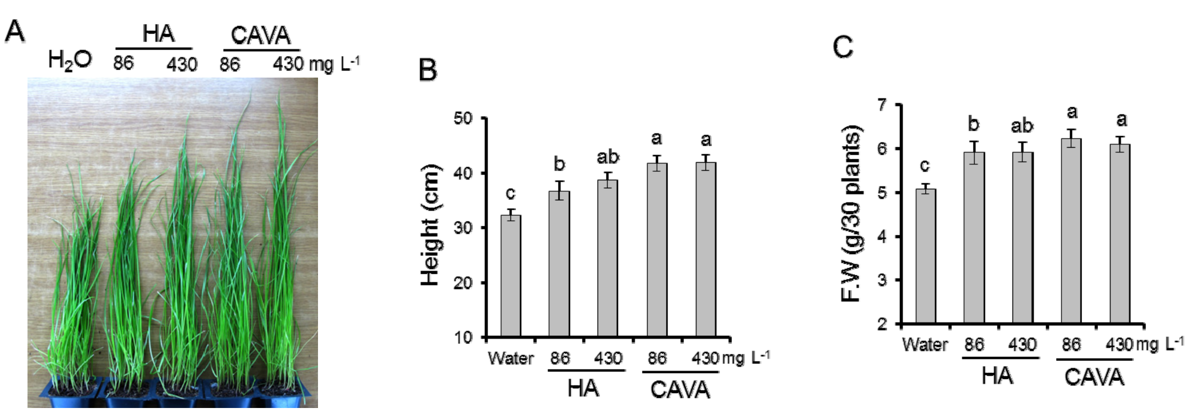

Fig. 3. Regrowth activity by foliar application of HA or CAVA after cutting. The seedlings were grown in soil for 21 d. Aerial parts of 7-day-old seedlings were cut leaving 3 cm above soil surface, and then allowed for regrowth with spraying for foliar application (86 mg L^{-1} of HA or CAVA) every 5 d. Sterilized H_2O sprayed to plants as a negative control. (A) The picture taken at 17 d after germination. Plant height of canopy (B) and fresh weight (F.W.) of aerial parts (C) were measured at 21 d after germination. Data are means ± SE (*n*=4). Means followed by the same alphabet letter are not significantly different at 5% level by the LSD test. Abbreviations: HA, commercial humic acids; CAVA, a co-polymeric product derived from fungal laccase oxidation of catechol and vanillic acid.

or the high cutting frequency, and application of nitrogen fertilizer, one of the major determinants, increase crude protein, photosynthetic pigments, and phenol contents of Italian ryegrass silage (Vincente-Chandler et al., 1958; Renlong et al., 2017). Thus, we tested whether HA or CAVA helps emergence of renewing leaves. Seven-day-old Italian ryegrass seedlings were cut and foliar application of HA or CAVA (two different concentrations; 86 and 430 mg L^{-1}) by spraying was carried out. As shown in Fig. 3A, the seedlings after treatments of HA or CAVA were re-grown faster than those treated with H_2O. Both HA and CAVA treatments increased plant height significantly (Fig. 3B). However, dose-dependent or treatment-dependent increase of plant re-growth was not observed. Fresh weight of aerial parts also increased with HA or CAVA treatment compared to H_2O treatment, but any significant differences between concentrations and treatments were not identified (Fig. 3C). This trend is consistent with the findings on the germination and salt tolerance of *Arabidopsis* (Cha et al., 2017). Overall, it can be concluded that treatment of HA or CAVA facilitates the regrowth of Italian ryegrass seedlings after cutting

Ⅳ. CONCLUSION

HA exhibiting amorphous structures and many functional groups is enriched in grassland soils. HA displays positive effects not only on plant development but also on soil fertility. Although HA is commercially available in worldwide, the quality of bioactivity to enhance crop productivity is not controlled due to the diversity of natural sources. Recently, we have manufactured an artificially synthesized HA mimic, CAVA, using catechol and vanillic acid as natural phenols. In this study, we demonstrated that foliar application of HA or CAVA promotes the growth of seedlings both in shoots and roots as well as regrowth after cutting in Italian ryegrass. Increased root capacity by HA or CAVA may trigger the nutrients uptake from soil, and consequently enhance the crop productivity. These data suggest that HA or our humic mimic, CAVA, whose quality control is readily guaranteed, could widely apply for forage crop cultivation. Furthermore, high productivity of “Kowinearly” could be achieved with the application of HA or CAVA as a powerful organic fertilizer.

Ⅴ. ACKNOWLEDGEMENTS

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