Research Article

Structure and action mechanism of humic substances for plant stimulations

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ABSTRACT

Humic substances that can be obtained from coal resources such as leonardite in a bulk scale have been employed as crop stimulators and soil conditioners. The polymeric organics containing a variety of aromatic and aliphatic structures are known to activate plants in a multifunctional way, thus resulting in enhanced germination rate and abiotic stress resistance concomitant with induction of numerous genes and proteins. Although detailed structural-functional relationship of humic substances for plant stimulations has not been deciphered yet, cutting-edge analytical tools have unraveled critical features of humic architectures that could be linked to the action mechanisms of their plant stimulations. In this review article, we introduce key findings of humic structures and related biological functions that boost plant growth and abiotic stress resistance. Oxygen-based functional groups and plant hormone-like structures combined with labile and recalcitrant carbon backbones are believed to be critical moieties to induce plant stimulations. Some proteins such as HIGH-AFFINITY K^+ TRANSPORTER 1, phospholipase A2 and H^+-ATPase have been also recognized as key players that could be critically involved in humic substance-driven changes in plant physiology.

(**Key words** : Humic substances, Molecular mechanism, Plant stimulation, Structure-function relationship)

Ⅰ. Humic substances in Agricultures

Humic substances (HS) belong to colorful organic polymers whose molecular formulas are irregular, unlike other distinct organics such as carbohydrates, proteins and alkanes. Depending on alkali solubility, HS are classified into three subtypes (i.e., humic acid, fulvic acid and humin) (Muscolo et al., 2013). HS are ubiquitous in several environments including rivers and soils, but the relative distribution of HS is variable depends on the environment types (Weber and Wilson, 1975; Lobartini et al., 2008). For instance, grassland soils are known to possess relatively large amounts of humic acid (~70%) compared with fulvic acid (~30%), whereas forest soils have vice versa (Stevenson, 1982). The contrasting distribution pattern of HS may be attributable to use of grassland for animal feeding producing the remnants of pastures and manures, thus triggering different decomposition ways of plant materials.

Versatile interaction of HS with clays and minerals in the environments including soil induces the microbial recalcitrance of HS, allowing for their extremely long-term persistence (Marschner et al., 2008). The rare contents of NPK (i.e. nitrogen, phosphorus and potassium) elements in HS represent little likelihood of the mixtures acting as plant nutrients, but their detailed organic structures directly stimulate plants to express numerous proteins associated with germination, secondary metabolites and abiotic stress resistance (Garcia et al., 2016). Indeed, HS-induced plant stimulations to affect developmental and physiological processes (e.g., germination, root development, abiotic stress resistance and nutrient uptake) have been demonstrated in various crops (Vaughan, 1974; Cacco and Dell Agnolla, 1984; Russo and Berlyn, 1990; Trevisan et al., 2010a; 2010b). More recently, the current authors also reported that humic acids and artificially synthesized mimics enhance the productivity of alfalfa and Italian ryegrass, wherein their root densities are dramatically increased (Khaleda et al., 2017a; 2017b).

Beyond the direct stimulation of plants, polymeric features

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of HS with widespread negatively charged functional groups are capable of aggregating soil particles and adsorbing plant metallic nutrients, thus making healthy soils for crop cultivation (Piccolo et al., 1997). Commercialization and market of HS have been successfully established due to the beneficial effectiveness of HS on crops and annual market of HS grows rapidly owing to a need for eco-friendly plant stimulants and a drawback of currently used NPK-based chemical fertilizers (P&S Market Research, 2015). Overall, it seems to be evident that HS are beneficial to crops, but their action mechanisms are still controversial because structure analyses and activation targets of HS are hardly characterized.

Ⅱ. Structure-function relationship of humic substances

HS are regarded as an assembly that is hardly separated, thus making them not to be fully analyzed. However, the polymeric features of HS are readily characterized through gel permeation chromatography. Many researchers have tried to fractionize HS into low-molecular and high-molecular sizes to decipher core components responsible for plant stimulations (Nardi et al., 2002). In general, the low-molecular-size fractions exhibit a relatively strong activity to stimulate plants. The fractions are more likely to penetrate into plants due to the small sizes. Such translocation may facilitate induction of genes and proteins responsible for metabolic and physiological changes in plants. The small molecule assembly is also proven to have oxygen-based functional groups such as phenols, alcohols and carboxylic acids (Canellas et al., 2002). Such groups that are prone to lose proton can bind to several organic and inorganic structures, based on dipole and negatively charged groups. They may interact with several gene regulators through relatively non-specific bindings. In spite of the lack of direct evidence on HS-induced regulator activation, physical interactions between HS and proteins have been reported (Tomaszewski et al., 2011). In addition, plant hormones originated from microbes can be liberated more easily from the humic assembly with low-molecular-weight compounds, thus activating plants through hormone-based pathways (Nardi et al., 2002; Canellas et al., 2002).

High-molecular-weight compounds in HS that are hardly translocated into plant cell interiors can activate plant growth. They are known to be adsorbed on the surfaces of plant roots and this behavior may derive from oxygen-based functional groups widely distributed in the HS (Maggioni et al., 1987). In fact, oxygen-based functional groups with aromaticity allow for versatile binding with several surfaces (Jeon et al., 2013). Through the attachments, the high-molecular-weight compounds accelerate the transport of some ionic molecules including NO_3^-, SO_4^{2-} and K^+ into plant cell interiors. To date, the detailed mechanism for this transportation has not been suggested, but increased expression of related transporters including nitrate carrier proteins have been reported (Dell'Agnola et al., 1981).

Together with this kind of gene induction on the plant root surfaces, the high-molecular-weight part of HS acts as polyelectrolytes that can efficiently donate proton ions from oxygen-based functional groups, resulting in partial acidity of the attached regions (Nardi et al., 1991). This action makes the plant surfaces active, thus affecting metabolic and translocation pathways occurring in plasma membranes of the surface cells. For instance, alkalinization induced by utilization of NO_3^- as an N source could be neutralized in the presence of HS on the root surfaces, wherein transport of plant inorganic nutrition actively happens (Nardi et al., 2002).

Another interesting view of HS structure is a supramolecular interaction of small humic components leading to formation of micellar architecture. The distribution of amphiphilic groups and the complexation with metallic ions playing an important role as a bridge allow for polymeric features of HS that are in fact an assembly of relatively small humic components (Sutton and Sposito, 2005). This model has been evidenced by some experimental observations. First, the addition of small organic acids that may disrupt supramolecular interactions of humic components caused severe changes in the polymeric lengths of HS (Piccolo, 2001). Second, ubiquitous distribution of amide forms of nitrogen within humic structures is hardly matchable with key polymerization processes bringing a huge size of HS (Sutton and Sposito, 2005). Third, membrane-like coating phenomena of HS on alumina with very limited rotations are consistent with the action of simple surfactants. Fourth, direct observation of 400 – 800 nm micelle-like shapes through transmission electron microscopy was achieved with

dissolved organic matters from aquatic sources that exhibit similar structural characteristics of HS (Kerner et al., 2003). The non-covalent bonding-based supramolecular association supports that recognizable and biodegradable biomolecules such as lipids, lignin-related aromatics and proteins could be a member of small humic components, thus affecting action mechanisms of HS for plant stimulations.

Beyond the oxygen-based functional groups, the presences of plant hormones or hormone-like structures in HS could be involved in plant stimulations through regular plant hormone pathways. In fact, their existences have not been demonstrated clearly due to the structural complexity of HS, but there are some experimental clues to support this hypothesis. First, HS-based plant activation is very similar with plant response to auxin, one of the well-defined plant hormones (Nardi et al., 2002). Toward this end, increased growth and surface area of roots, one of the main morphological characteristics through the action of auxin hormones, were observed with the treatments of HS derived from various sources. The experiments with auxin inhibitors also suggest that auxin-like activities of HS are inducible via different signal cascades with auxin compounds. Second, some researchers have shown that auxin groups are identifiable in architecture of HS by employing a mass spectrometry (Canellas et al., 2002).

Overall, polymeric shapes derived from either random-coiling of long strands or micellar assembly of small humic components are the final form that interacts with plants. Oxygen-based functional groups associated with relatively non-specific adsorption and acidity and auxin-like moieties capable of activating plant hormone-like pathways may be core structural features responsible for plant stimulations.

III. Molecular evidences on humic substance-induced plant stimulations

In general, HS are able to promote the mitotic sites in roots triggering lateral root developments concomitant with enhanced root density. Such root development may coincide with strengthened uptake of plant nutrients from soils (Zandonadi et al., 2007). It is noticeable that this kind of stimulation is comparable to an auxin-like activity. Purified humic acids from

leonardites activate FRO1 and IRT1 of cucumber encoding a Fe(III) chelate-reductase and a Fe(II) root transporter, respectively. The genes that are closely associated with Fe uptake contribute to increased Fe availability of plants (Elena et al., 2008). Nitrate uptake in maize is also accelerated by treatments of HS, which is coupled with action of H^+-ATPase (Mha2) capable of driving electrochemical gradients of H^+ with the enhancement of nitrate influx across the plasma membranes of root cells (Pinton et al., 1999; Quaggiotti et al., 2004). Another example for induction of Mha2 is revealed by earthworm-derived low-molecular-weight HS treated in maize roots (Quaggiotti et al., 2004). Phospholipase A2-involved stomatal openings renowned for a result of auxin-based signaling pathways are proven to be inducible with both the low- and the high-molecular-weight factions of HS (Scherer, 2002; Russell et al., 2006).

Beyond the auxin-like activation of HS, gibberellin (GA)-like signaling to break seed dormancy can be involved. Inhibition of seed germination caused by abscisic acid (ABA) and a GA biosynthetic inhibitor (i.e., paclobutrazol) was escaped with HS treatments, suggesting that HS also contain GA analogues capable of activating GA signaling (Cha et al., 2017). HS are able to allow plants such as Arabidopsis, bean and corn to resist salt-induced abiotic stresses (Khaled and Fawy, 2011; Aydin et al., 2012; Cha et al., 2017). In more detail, HS delay the salt-mediated degradation of a sodium influx transporter HIGH-AFFINITY K^+ TRANSPORTER 1 (HKT1), thus resulting in the facilitation of sodium flux (Khaleda et al., 2017c). There is a still need to investigate the systemic mechanisms of HS at a molecular level by employing transcriptomic, proteomic and phenomic analysis, which finally links to a full understanding of the roles of HS as a plant stimulator. Specific structure-function relationship of HS for plant stimulations is summarized in Fig. 1.

IV. ACKNOWLEDGEMENTS
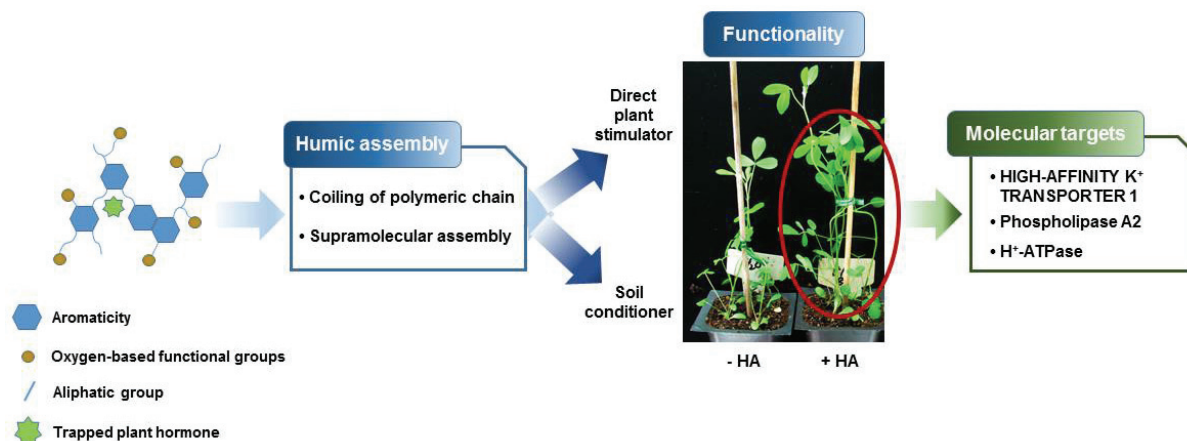
Fig. 1. A scheme for structure-function relationship of humic substances.

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