Synthesis and Application of Hydroxamic Acid: A Key Secondary Metabolite of *Piriformospora indica*

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Abbreviations

6MPTOX	6-Methoxy podophyllotoxin
DIBOA	Hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one
DIMBOA	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one
HDAC	Histone deacetylase
MeJA	Methyl jasmonate
NCED	9-cis-epoxycarotenoid dioxygenase
PTOX	Podophyllotoxin
SAHA	Suberoylanilide hydroxamic acid

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18.1   Introduction

Piriformospora indica is a root endophytic fungus which belongs to the group Basidiomycota having growth-promoting effects in several hosts (Cordoba et al. 2009). In plant species, this fungus can be seen growing both inter- and intracellularly by formation of pear shaped chlamydospores. It doesn't enter endodermis and aerial parts of the plants (Cordoba et al. 2009; McGarvey and Croteau 1995). Colonization of *P. indica* with roots of plant enhanced growth and development of host plant, disease resistance against biotic and abiotic stresses as well as phosphorus and nitrogen assimilation (Humphrey et al. 2006; Kumar et al. 2012). Fungal spore and culture filtrate of *P. indica* have beneficial effects on plant growth suggesting better nutrient uptake or hormonal signaling by the fungus. Colonized plants of *P. indica* show morphological changes in the root and physiology suggesting the induction of regulatory pathways (Yuan et al. 2007). *P. indica* can be cultured axenically and has the capability to grow on a number of complex and semisynthetic media (Zuccaro et al. 2011). These multifaceted attributes of *P. indica* led researchers to investigate its symbiotic association with a wide range of host plants and study the association on molecular basis. Association of *P. indica* with medicinal plants is reported to enhance secondary metabolites production in plants. Commercially important bioactive compounds can be enhanced by the use of plant-fungus interaction. This symbiotic association of plant-fungus can pave way for an alternative way of enhancing the accumulation of secondary metabolites. Molecular mechanisms responsible for increasing secondary metabolite content in plants associated with *P. indica* are presently unknown. The possible reason for enhanced accumulation of metabolite could be better nutrient uptake by the host and activation of defense-related pathways and associated signaling networks. This chapter reviews the most recent literature focus on plant growth promotion, defense mechanisms and accumulation of plant bioactive compounds in a diverse variety of crops associated with *P. indica.* Both nutritional and non-nutritional factors have been taken into account to suggest the biomass enhancement and accumulation of plant secondary metabolites upon association with *P. indica*.

Metabolomic analysis by using high-throughput, gas-chromatography-based mass spectrometry observed that 549 metabolites out of 1126 total compounds were produced in colonized and uncolonized Chinese cabbage roots having hyphae of *P. indica* (Hua et al. 2017). HPLC analysis of *P. indica* culture supernatant showed seven peaks in the hyphae and one main peak in the culture filtrate. Major peak was identified as benzoic acid, but the function is still not clear. The nature of the stimulatory effect of *P. indica* is yet to be known (Adya et al. 2012). Several evidences have highlighted that *P. indica* hyphae secrete many secondary metabolites such as hydroxamic acid, indoleacetic acid (IAA), chlorohydroxamic acid, etc. In this review, we focus on the role of hydroxamic acid of *P. indica* in plant growth promotion and defense mechanism.

18.2   Mechanism of Enzymatic Synthesis of Hydroxamic Acid

Amidase has broad substrate specificity which converts amides to the corresponding carboxylic acids and ammonia. Amidase exhibits "Bi-bi Ping-pong" mechanism for acyl transfer activity. First the amides react with the enzyme to give acyl-enzyme complexes (E-S complexes) which form carboxylic acids. If hydroxylamine is present instead of water (in case of acyl transfer activity) which is a strong nucleophilic agent, then its interaction with E-S complex results in the production of hydroxamic acids (Fig. 18.1). The enzyme retains its original state after the formation of the product and is ready to convert another molecule of amide and hydroxylamine to hydroxamic acid (Haron et al. 2011; Pandey et al. 2011; Sharma et al. 2012).

18.3   Levels and Effects of Hydroxamic Acid in Plants

Patanun et al. (2017) reported that histone deacetylase (HDAC) inhibitor suberoyl-anilide hydroxamic acid (SAHA), which is a derivative of hydroxamic acid, can alleviate salt stress by decreasing sodium ion concentration in stems and increase survival rates under high salinity in cassava (Table 18.1). Transcriptomic analysis reveals that SAHA upregulated the expression of allene oxide cyclase which is a catalyzing agent and catalyzes important step in biosynthesis of JA. This study demonstrated that the HDAC inhibitor is an effective small molecule for alleviating salinity stress in crops and could improve the understanding of the mechanisms by which histone acetylation regulates responses to abiotic stress in cassava. SAHA treatment can reduce Na^+ concentration in both leaves and stems. Plants are able to survive high salinity stress conditions through the maintenance of K^+ and Na^+ homeostasis using several transporters (Patanum et al. 2017).

The amount of hydroxamic acid (Hx) concentration in plant varies from species to species. There is no evidence available about level of hydroxamic acid in cereal seeds (Epstein et al. 1986), but concentration of Hx continuously increased as discussed above in wheat and maize. It reaches maximum after germination in maize
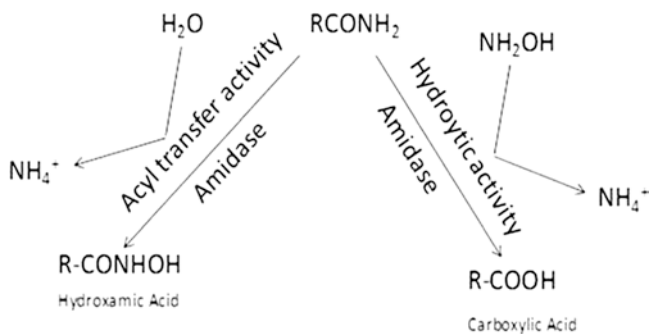
Fig. 18.1 Types of reactions catalyzed by amidase. (Modified from Bhatia et al. 2013)

Table 18.1 List of hydroxamic acid derivatives and their applications

Hydroxamic acids derivatives	Applications	References
Benzohydroxamic acid	Antitumor, antineoplastic	Bhatia et al. (2012)
Acetohydroxamic acid (Lithostat)	To treat ureaplasma, anemia, anti-HIV agent	Pandey et al. (2011)
Fatty hydroxamic acids	Anti-inflammatory to treat chronic asthma	Haron et al. (2012)
Deferoxamine B (Desferal)	Antimalarial	Giannini et al. 2015)
α-Aminohydroxamic acid	Anti-HIV agent, psoriasis inhibitor	Munster et al. (2001)
Marimastat	To treat small cell lung cancers	Muri et al. (2002)
Inhibitor of LTA4	Anti-inflammatory	
Idrapril	Render cardioprotective effects	
N-formyl hydroxylamine BB-3497	Antibacterial agent	
Cyclic hydroxamic acids	Provide resistance against pathogen and insects	Copaj et al. (2006)
Unsaturated and middle-chain hydroxamic acid	Wastewater treatment,nuclear technology	Haron et al. (2012)
Nicotinyl hydroxamic acid	Tyrosinase and melanin inhibitor	Chen et al. (2011) and Bhatia et al. (2014)
Spiropiperidine hydroxamic acid (SAHA)	Anticancerous	Bosiack et al. (2011)
Long-chain hydroxamic acids	As surfactants in detergent industry	Jahangirian et al. (2011a, b)
Poly hydroxamic acid	Used for gravimetric analysis and scavenging of heavy metal ions	Hassan et al. (2011)

and wheat (Argandona et al. 1980). Thus, the level of Hx depends upon the cultivation of crops (Klun and Robinson 1969). Hydroxamic acid is synthesized in all the plant species, but relative levels of Hx in roots and aerial part of plants are altered within species and cultivar (Argandona et al. 1981). The amount of Hx is predominantly more in stems as compared to leaf tissue. However, no significant concentration of Hx was reported in xylem exudates or guttation drops in maize and wheat (Argandona and Corcuera 1985; Guthrie et al. 1986). Subsequently, Hx level also varies within leaves. Younger leaves contain more Hx as compared to older leaves. Hx levels are more in the vascular bundles as compared to the leaves of maize (Argandona and Corcuera 1985) and wheat (Agandona et al. 1987). Furthermore, lateral veins contain higher amount of Hx as compared to the central vein of maize leaves (Argandona and Corcuera 1985). But Hx could not be detected in lower epidermal tissues of wheat leaves. Steler region contains more Hx level as compared to cortex in maize seedlings.

Broad spectrum of hydroxamic acid application has been studied in Chilean cultivars where the amount of hydroxamic acid levels was reported maximum at fourth or fifth days after seed germination. Interestingly, the level of DIBOA continuously decreased, and it became unmeasurable in some cultivars after tenth day of developmental stage, while conversion of benzoxazinoid hydroxamic acids derived from

2-hydroxy-2H-1,4-benzoxazin-3(4H) fluctuated in cereals and wheat callus culture (Zuiiiga et al. 1990). Prospective controls of hydroxamic acids in breeding programs for developing aphid-resistant cereal cultivation have been studied. Hydroxamic acid level in wheat (*Triticum aestivum* L.) reduced aphid correlation, but performance of aphid effect had considerably decreased in primitive diploid and tetraploid wheat (Thackray et al. 1990; Copaja et al. 1991).

18.4 Applications of Hydroxamic Acid

18.4.1 Histone Deacetylation by Hydroxamic Acid

Histone deacetylase (HDAC) is a class of enzymes that remove the acetyl groups from the histone proteins having an ε-N-acetyl lysine amino acid. This elimination of acetyl group allows DNA strand to wrap histone more tightly and regulates acetylation and deacetylation, thereby affecting the expression of DNA. Any change in the expression and mutations in HDACs gene leads to the development of tumor due to uncontrolled cell proliferation, cell cycle, and apoptosis (Giannini et al. 2015).

18.4.2 Effect of Hydroxamic Acid Against Antibiotic-Resistant Bacteria

Since pathogenic strains are becoming resistant to existing antibiotics, new approaches have to be explored. One such approach is the use of peptide deformylase (PDF). These are important enzymes which play a crucial role in bacteria for the synthesis of cell wall and plasma membrane. They belong to metallohydrolases family which is the most studied enzyme and an attractive target for drug design (Wei et al. 2000). These enzymes require Fe^{2+} ion for their catalytic activity. In PDF ferrous ions bond loosely and hence can easily oxidized into ferric ion, resulting in the inactivation of enzyme. Therefore, in order to develop new PDF inhibitor moieties to counteract the pathogenic bacteria, new strategies and chemical compounds must be developed. PDF can be used as antibacterial drug design because (1) it is present in all bacteria (2), the gene present with this activity is important for bacterial growth in vitro, and (3) it closely resembles with various metallohydrolases. Since PDF is a metallohydrolase, hydroxamic acid can potentially inhibit this enzyme. Actinonin is a known hydroxamate-containing inhibitor of various metallohydrolases and acts as a chelating group that binds metal ion of the enzyme and inhibits its activity (Jayasekera et al. 2000; Wei et al. 2000).

18.4.3 Antibacterial Activity of Hydroxamic Acids

Hydroxamic acids play an important role in defense mechanism of several plants and thus function as natural pesticides. The cyclic hydroxamic acids 2,

4-dihydroxy-1, 4-benzoxazin-3-one (DIBOA) and 2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA) act as a defense molecule in cereals against insects and pathogenic microorganisms. *Erwinia* spp. cause soft rot disease in maize, but maize protects itself by secreting DIMBOA. DIMBOA is also secreted for the management of *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Piriformospora aeruginosa*, *Pseudomonas indica*, *and Yersinia enterocolitica* (Varma et al. 2001; Pepeljnjak et al. 2005).

18.4.4 Insecticidal Property of Plant-Derived Hydroxamic Acid

Hydroxamic acids reduced the survival and reproduction of aphids. Different varieties of cereals like wheat, maize, and rye produce different types of hydroxamic acids that hamper the growth of aphids (*Metopolophium dirhodum*). It has been reported that aphids fed with DIMBOA have poor survival rate as compared to aphids fed with diets lacking DIMBOA. Copaj et al. (2006) reported that a high hydroxaminic acid level in maize has similar relation with the resistance to the European corn borer *Ostrinia nubilalis*. Indeed, secondary metabolites can act as shielding agents in plants against insects either causing direct toxicity or as repellent (Janzen et al. 1977). A different concentration of hydroxamic acids can have diverse effect of aphid interaction in several gramineae. Some of the derivatives of hydroxamic acids, in particular DIMBOA-l, have been demonstrated to be inhibitory against insects (Klun et al. 1967; Long et al. 1977), fungi, and bacteria (Corcuera et al. 1978; Lacy et al. 1979).

18.4.5 Hydroxamic Acid in Wastewater Treatment and Nuclear Technology

Hydroxamic acids have also been reported to have potential use in wastewater treatment and nuclear technology to evolve new methods to reduce contaminating metal ions. This serves as a promising approach to clean wastewater contaminated with heavy metal ions (Haron et al. 2012).

18.4.6 Hydroxamic Acid in Analytical Chemistry and Detergent Industry

Hydroxamic acids have important role in analytical chemistry as reagents for gravimetric and spectrophotometric analysis of metal ions (Hassan et al. 2011). Owing to their ability to form complex with metal ions, long-chain hydroxamic acids are also used as surfactants in the detergent industry (Jahangirian et al. 2011a, b).

18.4.7 Regulation of Hydroxamic Acid Derivatives in Plant Signaling

Allene oxide cyclase (AOC) plays a key rate determining step in JA biosynthesis and JA derivatives such as methyl jasmonate (MeJA) which have reduced salinity stress in soybean (Yoon et al. 2009). Similarly, accumulation constitutive transcripts of AOCs elevated plant tolerance capability against salinity stress in tobacco cell lines (Yamada et al. 2002) and wheat (Zhao et al. 2014). Interestingly, SAHA treatment sturdily induced the mRNA expression level of MeAOC4. These findings suggested that SAHA application in plant can help JA signaling pathways which improves the plants tolerance ability against salinity stress. Another plant hormone which involves abscisic acid (ABA) inhibits seed germination, and the regulation of ABA biosynthesis has a role in the maintenance of seed dormancy. 9-Cis-epoxycarotenoid dioxygenase (NCED) catalyzes the reaction and is considered as a rate-limiting enzyme during ABA biosynthesis. Previously, in vitro study has argued that two hydroxamic acids, i.e., D4 and D7, used as inhibitors of carotenoid cleavage dioxygenase (CCD) and NCED of decrease germination time of tomato (*Solanum lycopersicum* L.) seeds constitutively by greater expression of *NCED1* (Awan et al. 2017). Further, no effect on seedling growth of tomato was observed in terms of height, dry weight, and fresh weight post-seed germination. Moreover, effect of chemical on seed germination in a tetracycline-inducible LeNCE D1 transgene of tobacco was highlighted where seed germination was controlled through chemical induction of NCED gene expression and the chemical inhibition of the NCED protein. Application of tetracycline increased germination timing and delayed hypocotyl emergence as similar to exogenous application of ABA and opposite to the D4 treatment (Awan et al. 2017). Similar effect was also monitored where D4 application improved germination percentage in lettuce seeds under thermo-inhibitory temperatures.

18.5 *P. indica* Symbiosis Association with Plant Roots Modulated Phytohormone Signaling

Promotion of plant growth is most evident in *P. indica* infected plants. It is reported that phytohormones released by plants under colonization with endophytes leads to plant growth promotion (Khatabi et al. 2012). *P. indica* is reported to promote initial stage of plant vegetative growth, thus leading to an early switch to the generative stages of host development (Vahabi et al. 2013). Plant root system is a direct target of colonizing endophytes. Auxin is a key chemical signal for root development during plant-microbe interactions (Hilbert et al. 2012; Franken 2012). Promotion of root growth by beneficial microbes is widely studied (Das et al. 2012). Associated microbes change the root architecture by interfering with the plant-auxin pathways (Rajasekaran et al. 2007). The culture filtrate of *P. indica* produces substances like IAA. This helps in regulation of plant growth and lateral root development (Swanson

et al. 1992). A higher level of IAA was found in colonized roots of 3-day-old barley seedlings when compared to control. *P. indica* strains with silenced *piTam1* gene were reported to have compromised IAA production and decreased colonization of barley roots in biotrophic phase (Modi et al. 2014).

Ethylene has an important role in plant development, germination, flower and fruit ripening, leaf senescence, and programmed cell death (Vahabi et al. 2015). In *Arabidopsis*, colonization with *P. indica* interferes with ethylene signaling components resulting in increased root colonization and inhibition of growth promotion (Pal et al. 2015). It is reported that repression of ethylene-responsive genes is involved in barley when colonized by *P. indica*. Regulation of host-microbe association and root physiology is induced by phytohormones such as cytokinin, gibberellins, jasmonate, salicylic acid, and strigolactone. These are how the associated signaling networks and phytohormones work together to generate compatible fungus-host interaction. This processes lead to root growth promotion and greater biomass accumulation (Kilam et al. 2017).

The investigation of *P. indica* mycelium extracts showed that mycelium extracts (1% v/v) reduced the hairy root growth, while treatment by podophyllotoxin (PTOX) and 6-methoxy podophyllotoxin (6MPTOX) after 2 h of production significantly stimulated root dry weight (Tashachori et al. 2016). It also has the ability to synthesize hydroxamic acid a secondary metabolite, which functions like a natural pesticide (Varma et al. 2001). It has been strongly advocated that *P. indica* has significance as a biofertilizer and biocontrol agent (Waller et al. 2005; Varma et al. 2012). *P. indica* reveals several positive consequences on diverse crop plants and has become an important candidate in biotechnological and microbiological research (Barazani and Baldwin 2013). It was reported that *P. indica* induce methionine synthase activity which facilitates methionine cycle of ethylene biosynthetic pathway (Peškan Berghöfer et al. 2004) during its colonization with plant roots via immune suppression, surprisingly explains the broad host range of the fungus (Schäfer et al. 2007; Jacobs et al. 2011).

Ethylene was reported to be involved in *P. indica*-plant interaction which modulates the interaction between them via signal molecules of fungi as well as plant receptors at the root cell surface after the fungal spore reside to attain the desired compatibility. Interestingly, ethylene signal magnitude contributes to the colonization of plant roots by *P. indica* where ethylene signaling either inhibits or enhances the growth of hyphae depending on the magnitude of signaling (Camehl et al. 2013). It is now confirmed that to establish symbiotic relationship, ethylene signaling network requires definite biochemical or genetic role to establish a communication across the symbionts as well as host plants to promote physiological benefits to each partner (Ansari et al. 2013).

18.6   Symbiosis Association Elevated Nutrient Uptake

The mutual interaction with *P. indica* and host plant provides enhanced nitrate/nitrogen uptake (Sherameti et al. 2005; Yadav et al. 2010). Increase in endogenous content of N, P, and K was observed in chickpea and black lentil plants colonized with *P. indica* (Nautiyal et al. 2010). In contrast, deficiency of Fe and Cu was surpassed when inoculated with *P. indica* (Gosal et al. 2011). Kumar et al. (2011) reported that *P. indica*-treated plants were able to uptake and transport P which may be related to increased plant growth and development via their various regulatory, structural, and energy transfer processes (Fig. 18.2). Further, *Z. mays* inoculated with *P. indica* mutant where, a phosphate transporter was knocked out; there was a reduction in endogenous content of phosphate (Yadav et al. 2010; Ngwene et al. 2013).

Further, it has been highlighted that iron deficiency in the growth medium could induce Hx level in maize (Manuwoto and Scriber 1985a, b), while lower temperature reduces Hx levels in maize roots (Thompson et al. 1970). Nitrogen application has more impact on Hx level. In gramineae cultivars, nitrogen application increased Hx level, while no significant effect of nitrogen was reported in some maize cultivars (Manuwoto and Scriber 1985a, b).
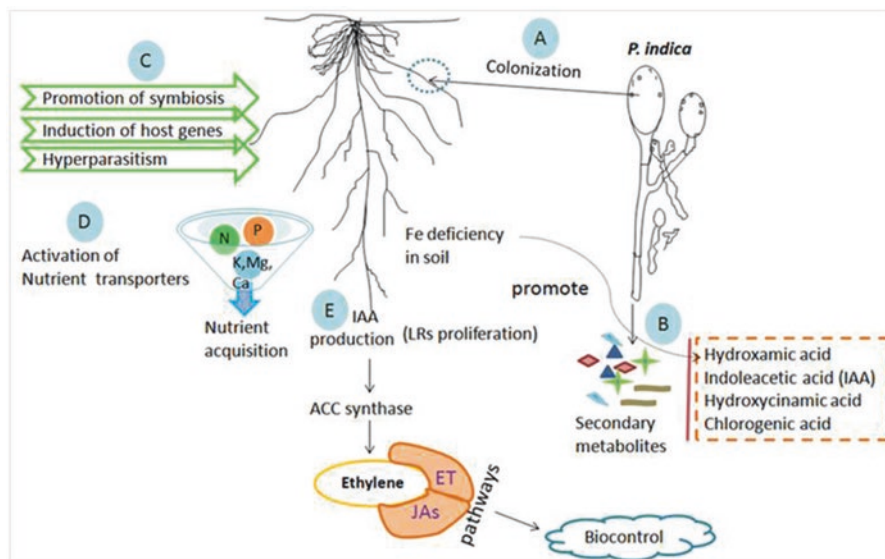
Fig. 18.2 *P. indica* association with host roots and its role in host development. The first step shows colonization of hyphae with roots (**a**). After successful colonization, several secondary metabolites are secreted by *P. indica* hypha (**b**). Secondary metabolites promote symbiosis, induction of host genes, and hyperparasitism (**c**). Subsequently, *P. indica* balances nutrients level in plants through elevated efficacy of different nutrient transporters (**d**), and activation of JAs/ET signaling pathways leads to regulation of defense response (**e**)

18.7   Conclusion and Future Prospects

Piriformospora indica synthesizes secondary metabolites including hydroxamic acid having multifunctional roles in growth, protection, stress tolerance, and plant disease management of agricultural crops. It has the potential to manipulate physio-chemical properties of the roots and might genetically reprogram root proliferation through mutualistic association. Hydroxamic acids can be synthesized naturally as well as enzymatically. Enzymatic approach can be used directly for medicinal purposes, plant growth and protection, and nutrient acquisition through hormonal regulation. Several hydroxamic acid derivatives have been chemically synthesized which can be applied in the agricultural field for improved disease control and management leading to improved crop protection. Hydroxamic acid derivatives are used as antibacterial agent, biocontrol agent, gene regulator in plant metabolism, and mineral uptake. Hydroxamic acid being an effective metal chelator, its role in iron chelation in agricultural soil needs to be investigated at molecular and cellular level in greater details, especially in those scenarios where severe iron deficiency exists in soil. Moreover, symbiotic relationship with phytohormone and *P. indica* colonized roots needs further investigation. Owing to these advantages of hydroxamic acids, research in mass production of *P. indica* in bioreactors using plant tissue culture technique can be a step closer toward commercialization of this agriculturally important compound.

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