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Sugar, amino acids and nitrate as nitrogenous source of fertilization influence growth of *Xanthomonas oryzae* pv. *oryzae* and *Aspergillus* species in hypersensitive response development of tobacco crop

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ABSTRACT

The relationship between nutrient supplement and pathogen colonization on the leaf surface of plant was studied by measuring the colony forming unit. Data obtained indicate that the population of *Xanthomonas oryzae* pv. *oryzae* and *Aspergillus* species in plant was affected by exogenous supply of sugar and amino acids as feed, and further nitrate as nitrogenous source of mineral nutrition favoured hypersensitive response development. Carbon (20 mM sucrose) and amino acids (10 µM amino acids such as gluatamic acid, aspartic acid, alanine, cystein and methionine) were used as exogenous supplements as feed along with pathogens. The abundance of colonization on the leaf surface of ammonium nutrient growing tobacco plants was high as compared to nitrate. Carbon and nitrogen sources were directly linked with maximum population sizes of bacteria and fungus on the leaf surface. Population sizes of bacteria and fungal pathogens in ammonium nutrient media capable to support were high and indirectly linked to disease resistance. However, nitrate growing plants had low population size with bigger role in hypersensitive response development, thus helping the plants against disease. It is hypothesized that sugars and amino acids are able to increase the severity of pathogens and nitrate nutrient was able to overcome the same through hypersensitive response development.

Keywords : Amino acid, *Aspergillus* species, Colony forming unit, Hypersensitive response, Sucrose, *Xanthomonas oryzae* pv. *oryzae.*

A number of microorganisms are colonized on the aerial surfaces of plant. Among these only few do not multiply on leaf surface (Lindow & Andersen1996). Most of the pathogenic and saprophytic microorganisms have ability to increase their number on healthy leaves (Dik *et al.* 1991, Fokkema, 1973, Hirano *et al*. 1982, O'Brien & Lindow, 1989). They utilize carbon, nitrogen source and certain inorganic molecules as feed (energy) for growth. It is reported that epiphytic bacterial communities are limited in size by the presence of available and utilizable carbon and/or nitrogen sources, as suggested by Wilson & Lindow (1994), so it might be expected that the availability of utilizable nutrient sources would vary greatly among leaves of the same plant and also among leaves of different plant species. Some Aphid honeydew and pollens have been reported which dramatically increase the microbial carrying capacities of some leaves (Fokkema *et al.* 1983, Warren 1972). However, in the common absence of such obvious and abundant nutrient sources, plants are still usually colonized by high numbers of bacteria under favourable environmental conditions, such as when high relative humidity or free

water is present (Hirano & Upper 1989, 1990). Molecules leached from plant leaves include a variety of organic and inorganic compounds such as sugars, organic acids, amino acids, methanol and various salts (Corpe & Rheem 1989, Fiala *et al.* 1990). Partitioning of these nutrients varies with leaf age and growing conditions (Wildman & Parkinson 1981). These carbon and nitrogen compounds were shown to be limiting factors for bacterial and their population on leaves (Bashi & Fokkema 1977, Wilson & Lindow 1994).

There has been no detailed examination of the variation in leaf surface nutrient availability among leaves that would enable us to determine their contribution to bacterial and fungal populations. In this study we tested the efficacy of sugar and amino acids (as feed for pathogen growth) on the velocity/development of hypersensitive response (HR) in tobacoo leaves when infiltrated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Aspergillus* species. We further investigated the impact of nitrogen fertilizer (nitrate vs ammonium) on the development of HR to ascertain their role on microbial growth i.e., to associate such nutrients with differential colonization potential.

MATERIALS & METHODS

Plant cultivation—Tobacco (*Nicotina tobaccum*) Kanchan variety seeds (obtained from CTRI, Rajahmundry) were grown on petriplate containing moistened sterilized filter paper for 4-5 weeks, then transferred in tray containing sand for next 5-6 weeks. Plants were established in plant growth chamber with day/night cycle of 16/8 h of light of photosynthetic photon flux density of 350-400 µmol $m^{-2}s^{-1}$ in growth chamber at 22°C/20°C day/night temperature and relative humidity of 70%. The NO_3^- and NH_4^+ nutrient solutions were continuously used as nitrogen source according to Planchet *et al.* (2005). Eight to nine weeks old tobacco leaves were used in each experiment.

Pathogen culture preparation for infiltration—Bacterial culture of *Xoo* was procured from CCMB, Hyderabad (Courtesy: Prof. Ramesh Sonti) and *Aspergillus* sp. from Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, India. *Xoo* and *Aspergillus* species cultures were maintained on peptone sucrose ager (pH 7.2) and Potato Dextrose Agar (pH 6.8) media, respectively. Suspension cultures (0.05 $OD_{60;}$ 1×10^2 cells/ml) from both strains were prepared for each experiment in sterilized 10 mM $MgCl_2$.

Sugar and amino acids preparation as a feed—To evaluate the growth of pathogens and their impact on velocity/development of hypersensitive response in plant leaves, 10 µM amino acids (Aspartic acid, Glutamic acid, Alanine, Cystein and Methionine) and 20 mM sucrose were used along with the suspension culture of pathogens.

Pathogen infiltration and colonies count—Suspension cultures of pathogens were infiltrated in the tobacco leaves. HR region developed in the leaves were excised with the help of 0.76 cm^2 cork borer after 6 and 24 h and crushed in 10 mM $MgCl_2$ solution (Figure 1). Serial dilution of crush suspension solutions of pathogenic strain were pore plated on respective media and incubated at 28±2°C. Colony forming units (CFU) were counted after seven days.

RESULTS & DISCUSSION

Effect of sucrose and amino acids on growth of *Xoo* and *Aspergillus* apecies—A test of utilization of sugar and amino acids as a feed was investigated in plants for growth of pathogens as well as their impact on HR development. Pathogens use sucrose and amino acids for multiplication on the surface of leaves. CFUs were counted for *Xoo* and *Aspergillus* sp. pathogens in comparison to the conditions with and without exogenous supplements of sucrose and amino acids. We found that maximum CFU increased with increasing time from 6 to 24 h with sucrose supplement in comparison to amino acids. Colonization was increased by 82 to 729% in sucrose while in amino acids increased by 27 to 157% upon *Xoo* infiltration (Table 1). However, in combination of amino acids and sucrose, colonization was drastically enhanced by 473 to 900% in nitrate medium or grown tobacco leaves (Table 1). We also investigated colonization by these pathogens outside of HR region; it spread out side with time after 24 h towards periphery of leaves. However, ammonium ions favoured increased colonization of *Xoo* since it was increased by 1689 to 2809% when sucrose was supplemented as a

feed from at 6 to 24 h. But when *Xoo* used amino acids as a feed supplement, colonization was enhanced by 1163 to 2809% from 6 to 24h. Same result was also found with combination of sucrose and amino acids as a feed at the end of 24 h (Table 1). One consequence of nitrogen mediated resistance was observed against *Pseudomonas syringae* pv. *phaseolicola* in tobacco when programmed cell death in terms of hypersensitivity response occurred when fed with NO_3^- and NH_4^+ (Gupta *et al.* 2013); it was observed that speed of cell death was faster in NO_3^- fed compared with NH_4^+ fed plants, which correlated respectively with increased and decreased resistance. One of the possible mechanisms to influence the HR formation in plants is nitric oxide (NO) generated by nitrate reductase (NR). NO generation was reduced in NH_4^+ fed plants where N assimilation bypassed the NR step. Our result indicates that NO_3^- nutrient containing plants resist more spread of *Xoo* than NH_4^+ nutrient containing plants. This NO_3^- nutrient solution participated in activation of defence mechanism in plants that is responsible for preventing the spread of bacterial pathogens. In this experiment colonization of *Xoo* was increased in ammonium form in tobacco plants. We can also predict from above results that CFU increment was directly proportional to less HR development and plants were more susceptible to disease.

Similarly, colonization of fungal pathogen was increased by 100 to 283% from 6 to 24 h in nitrate growing plant. However, in ammonium, it increased from 111 to 1200%. Utilization of amino acids as feed is slow process; therefore, colonization on the leaf surface by *Aspergillus* species was less as compared to sucrose. It increased by 19 to 33% in nitrate and 61 to 517% in ammonium growing plant in presence of amino acids as feed. However, the combined effect of both sucrose and amino acids led to increased CFU i.e., 88 to 333% in nitrate and 128 to 1033% in ammonium growing plant after 6 and 24 h (Table 2). We also tried to investigate the penetration and spread of pathogens towards outside from the site of HR regions. The present data indicated that the pathogen continuously spread toward periphery with increasing time; this means they are using sucrose and amino acids more efficiently for their growth (Table 2). On the basis of carbon and nitrogen source on the leaf surface, spread of pathogens varies from plant to plant. Brodie & Blakeman (1976) reported that conidia formation was increased when *Botrytis cinera* used carbon source as feed. Our findings indicate that carbon and nitrogen are inevitably required for the growth of pathogens. Further, NO_3^- nutrient grown plants resist more than NH_4^+ nutrient to disease, possibly through nitrate reductase mediated NO generation, leading to increased HR development (Gupat *et al.* 2013).

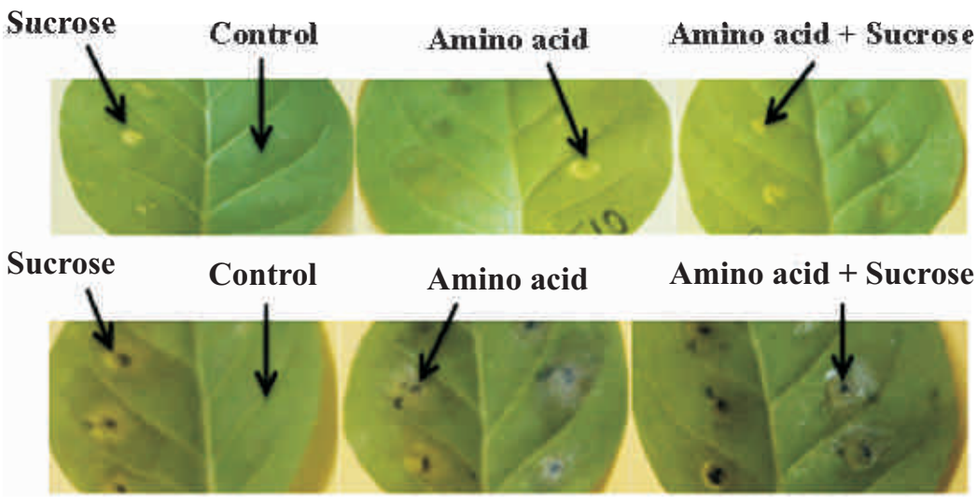

Fig. 1- Infiltration of sucrose and amino acids as an exogenous supplement with *Xoo* (upper lane) and *Aspergillus* sp. (lower lane) pathogens suspension. Treatment without any supplement serves as control. Black arrows represent the HR region develop after infiltration.

Table 1—Colony forming units per 0.76 cm^2 leaf sample after treatments with chemical supplements; 10 µM amino acids (Gluatamic acid, Aspartic acid, Alanine, Cystein and methionine) and 20 mM sucrose along with pathogen *Xoo* with control.

Treatment	CFU x 10^3							
	Nitrate growing leaves				Ammonium growing leaves			
	6 hpi		24 hpi		6 hpi		24 hpi	
	within cork borer	outside cork borer (1mm)	within cork borer	outside cork borer (1mm)	within cork borer	outside cork borer (1mm)	within cork borer	outside cork borer (1mm)
Control	110	80	70	30	19	11	11	5
Sucrose	190	120	580	130	340	440	320	160
Amino Acid	130	160	180	10	240	180	320	240
Sucrose + Amino acid	620	100	700	180	326	219	234	1010

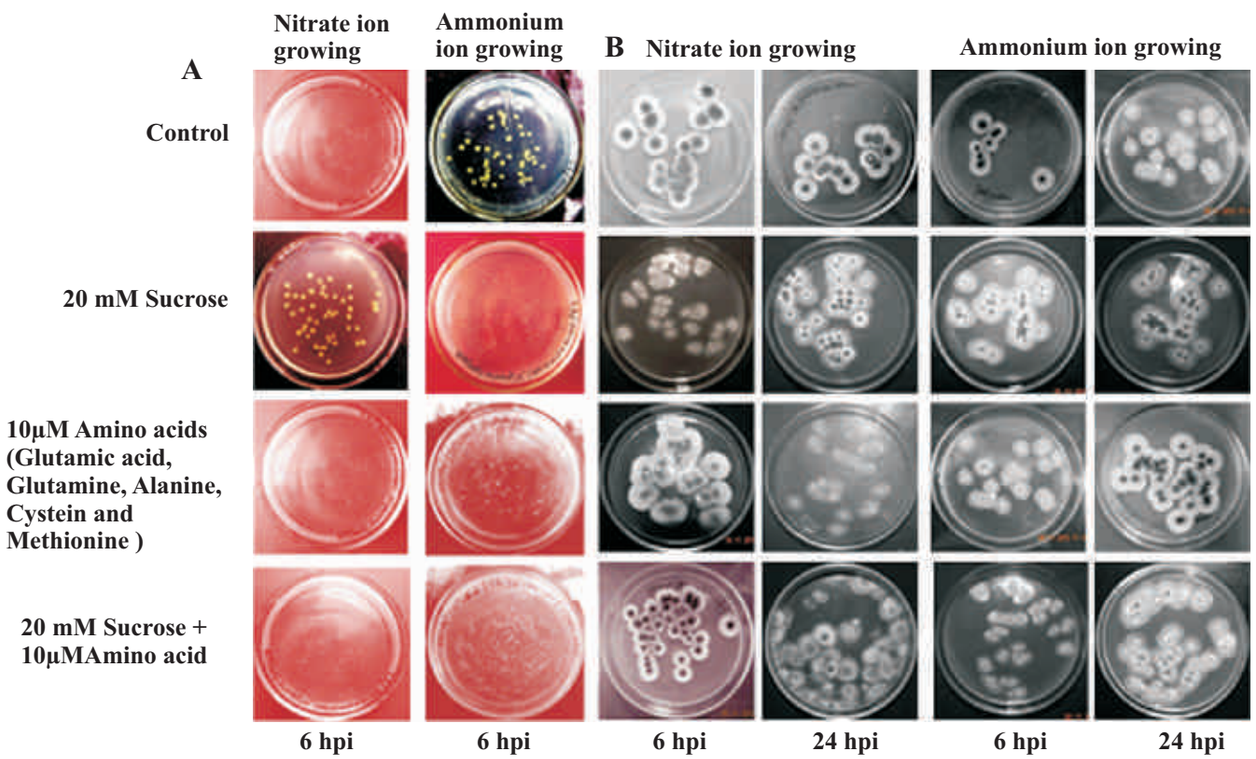

Fig. 2— Colony forming units with exogenous supplement (10 µM amino acid and 20 mM sugar) at 6 and 24 hpi. Figure 2A shows the yellow colonies of *Xoo* and black colonies of *Aspergillus* sp. (Fig. B) after pour plate on their corresponding media. Data from 24 hpi in both nitrogen nutrients growing tobacco with bacteria are not shown.

Table 2— Colony forming units per 0.76 cm^2 leaf sample after treatments with chemical supplements such as 10 µM amino acids (Glutamic acid, Aspartic acid, Alanine, Cystein and Methionine) and 20 mM sucrose along with pathogen *Aspergillus sp.* in comparison to control.

Treatment	CFU (Number of colony x 10^3)							
	Nitrate growing leaves				Ammonium growing leaves			
	6 hpi		24 hpi		6 hpi		24 hpi	
	within cork borer (1mm)	outside cork borer	within cork borer (1mm)	outside cork borer	within cork borer (1mm)	outside cork borer	within cork borer (1mm)	outside cork borer
Control	16	8	12	5	18	14	6	7
Sucrose	32	10	46	18	38	16	78	32
Amino Acids	19	10	16	6	29	11	37	22
Sucrose + Acid acids	30	15	52	21	41	18	68	24

CONCLUSION

The availability of major carbon-containing compounds such as sugars would place constraints on the population size of bacteria and fungus that could be attained on plants, assuming that the physical environment on leaves was not limiting. It appears that abundance of nutrient source of sugar and amino acids assist in pathogen's growth and multiplication. Nutrient accumulation and microbial colonization are not static processes but probably occur discontinuously at a rate influenced by environmental factors (Timmer *et al*. 1987). The factors that influence the availability of nutrients on the leaf surface and, in turn, microbial populations may be rather complex. Further insight into this research to utilizing the nutrient availability by bacteria and fungus *in situ* will require the development of tools such as biological sensors that are responsive to such compounds.

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