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Nitrogen Assimilation Diversity among *Ramulariopsis* Isolates Collected from Cotton Fields in Odisha: A Comparative Analysis

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ABSTRACT: In this study, we developed and optimized a growth medium using various nitrogen sources for the cultivation of *Ramulariopsis* spp. a potential threat to cotton growing areas of Odisha. The composition of basal Richard's liquid medium was modified and the nitrogen content was replaced by alternative nitrogen sources *viz*., urea, ammonium chloride, potassium nitrate, ammonium nitrate, sodium nitrate, calcium nitrate, magnesium nitrate and diammonium hydrogen orthophosphate. Results showed that fungal growth was significantly higher when the nitrogen sources incorporated were potassium nitrate, sodium nitrate and diammonium hydrogen orthophosphate. Least growth and sporulation was recorded in medium with urea as nitrogen source. The findings indicated that the isolates exhibited varying degrees of utilization for different nitrogen sources. RGKN1 demonstrated the highest average dry biomass weight, followed by RPGGNPR1. Moreover, the research unveiled the existence of cultural diversity among the *Ramulariopsis* isolates concerning their nitrogen utilization patterns. The main challenges during the research was the standardisation of isolation procedure for the pathogen. So this research will clearly contribute towards knowing the exact nutritional requirements for the fungus to grow properly.

Keywords: Variability, *Ramulariopsis*, nitrogen sources, grey mildew, cotton.

INTRODUCTION

Ramularia leaf spot, also known as grey mildew, is a common fungal disease that affects cotton plants worldwide. It is caused by the fungus U. Braun (sin. = *Ramularia areola* GF Atk), *Ramulariopsis gossypii* (Speg.) with the teleomorph *Mycosphaerella areola*. It was used to be seen of as a secondary and late-season disease in many countries, including Brazil and India, but recently it has gained significant importance because of its infectivity during the entire crop cycle (Girotto *et al.*, 2013). When compared to the prevalence over the previous five years, it has intensified by 10–30% in India. Up to 60% of the crop might be lost owing to disease-related yield losses in countries like Madagascar and India (Kirkpatrick & Rothrock 2001). Among the fibres the productivity of cotton has increased at a CAGR of 3.35 during the last two decades in Odisha (Odisha Economic Survey, 2021-22). The threat posed by grey mildew has grown in importance in recent years, particularly in western Odisha's cotton-growing regions. In the recent years, it has been observed that this disease is destructive enough to cause roughly 50% of premature defoliation in susceptible varieties, leading to significant low productivity. As the economic significance of this disease and the difficulty in culturing and maintaining *Ramulariopsis* spp. in artificial media has been realised in the recent past, a very limited research has so far been done in several aspects involving the disease and the causal fungus. Comparative analysis of geographically distinct fungal strains and the development of optimum *in vitro* growth conditions for the fungus are two instances. In our analysis, we took into account nutritional variation, with a focus on nitrogen substrate utilisation. Fungi require a variety of components for growth and reproduction, and C and N sources are commonly found in fungal growth media. According to Gao *et al*. (2007), fungi are versatile in their use of C and N sources due to their capacity to secrete enzymes that break down polymers into smaller compounds. Lee *et al*. (2007) found that, although only trace amounts are needed, micronutrients including iron, zinc, and copper are crucial for both the initiation and maintenance of infection (Vicentefranqueira *et al.*, 2015; Wiemann *et al.*, 2017). In addition, carbon and nitrogen compounds must be acquired in large quantities as they sustain cellular biosynthetic processes (Ramachandra *et al.*, 2014). In this research paper, we examined the nitrogen source utilization patterns of *Ramularia* isolates collected from the major cotton growing regions of Odisha. To achieve our objective of establishing the link between nitrogen source assimilation with variability among different strains, *Ramulariopsis* isolates were collected from cotton plants in different locations across the state, cultured on media with different nitrogen sources and their dry biomass weight and sporulation were measured. Our research findings will contribute in the direction of better understanding of the fungal metabolism and nutritional requirement which will aid in developing optimal culture conditions leading to in depth research and eventually advanced effective strategies to manage this disease and safeguard cotton yield and fibre quality in major cotton-producing regions.

MATERIALS AND METHODS

A study was conducted to examine how different nitrogen sources influence the growth and sporulation of *Ramulariopsis* isolates (RGBP1, RPGGNPR1, RGBLGR1, RGNP1, RGKN1, RPGKG1). Eight nitrogen sources were tested and the utilization of these sources by the fungus was analyzed in liquid Richard's medium. Each nitrogen source, including urea, ammonium chloride, potassium nitrate, ammonium nitrate, sodium nitrate, calcium nitrate, magnesium nitrate and diammonium hydrogen orthophosphate was individually substituted in the basal medium. The C/N ratio was maintained constant and the quantities of nitrogen compounds were adjusted to match the nitrogen content of 10 g of potassium nitrate in one liter of Richard's medium, based on their molecular weight (Table 1). The control used a basal medium without any nitrogen source.

Mycelial suspension (2 ml) was inoculated into 75 ml of liquid medium in 100 ml Erlenmeyer flasks, which were then incubated for 25 days at a temperature of 19 ± 2°C. The dry biomass weight was measured to evaluate mycelial growth, with three replicate flasks for each combination of isolates and nitrogen source. Harvested samples from three replicate flasks of each treatment were filtered, oven-dried at 60°C and the fungal dry weights were recorded. The average mycelial growth was calculated and subjected to statistical analysis using analysis of variance.

Table 1: Different nitrogen sources along with their amount to be used in basal medium.

Nitrogen Source	Molecular Formulae	Molecular weight(g/mol)	% Nitrogen	Weight(g) per litre of medium
Urea	CH_4N_2O	60.06	46.62	2.98
Ammonium Chloride	NH_4Cl	53.49	26.17	5.31
Potassium Nitrate	KNO_3	101.10	13.84	10.00
Ammonium Nitrate	NH_4NO_3	80.04	34.98	3.95
Sodium Nitrate	$NaNO_3$	84.99	16.47	8.43
Calcium Nitrate	$Ca(NO_4)_2$	164.08	17.06	8.11
Magnesium Nitrate	$Mg(NO_3)_2$	148.30	18.88	7.33
Diammonium hydrogen orthophosphate	$(NH_4)_2HPO_4$	132.06	21.20	6.53

RESULTS AND DISCUSSION

In liquid media condition after 25 days of incubation at 19 ± 2°C temperature, dry weight of mycelium and sporulation were recorded and are presented in Table 2 and 3. The fungus utilized all the nitrogen sources tested for growth and sporulation but the extent of utilization differed with the type of nitrogen sources. Significantly maximum fungal growth with respect to dry biomass weight (607.00 mg) was obtained by potassium nitrate followed by sodium nitrate (571.32 mg) and diammonium hydrogen orthophosphate (562.03 mg). While other nitrogen sources like calcium nitrate (514.38 mg), ammonium nitrate (470.31 mg), magnesium nitrate (456.74 mg) and ammonium chloride (403.24 mg) yielded moderate dry mycelial weight. The least dry biomass weight was produced by the remaining one nitrogen source, urea with 274.78 mg dry biomass weight. In control i.e. without nitrogen source 167.31 mg dry biomass weight was produced. All the nitrogen sources produced good growth of fungus compared to control (Fig. 1). Abundant sporulation was observed in potassium nitrate, sodium nitrate and diammonium hydrogen orthophosphate, whereas calcium nitrate and ammonium nitrate supported good sporulation. Magnesium nitrate, ammonium chloride supported moderate sporulation of the fungus. There was very little growth and sporulation recorded in nitrogen free medium (control) which was just below urea. It clearly established the essentiality of nitrogen source for metabolic activities of the fungus. Similar to the trend noted in carbon source utilisation, RGKN1 (527.72 mg) and RPGGNPR1 (480.98 mg) surpassed the other isolates in terms of mean dry biomass weight and sporulation. The next best performing isolates were RGBLGR1 (453.64 mg) and RGNP1 (423.23mg). RGBP1 (402.17mg) and RPGKG1 (396.99mg) were the least efficient of all with respect to nitrogen utilisation and did not show any substantial variation in mean dry biomass weight and sporulation. In a nut shell, in terms of nitrogen sources, potassium nitrate, sodium nitrate, and diammonium hydrogen orthophosphate supported maximum fungal growth in our study. Calcium nitrate, ammonium nitrate, and magnesium nitrate yielded moderate growth, while ammonium chloride and urea showed relatively lower growth. Similar results were reported by Dasarathabhai (2005), who found that potassium nitrate was the best nitrogen source, followed by sodium nitrate and calcium nitrate. Our findings also aligned well with the results obtained by Ayed *et al.* (2020) who evaluated the effect of different nitrogen and carbon sources on the mycelial growth of *Sclerotium rolfsii* isolates, in which sodium nitrate and potassium nitrate were found effective nitrogen sources, supporting good radial growth of all isolates tested.

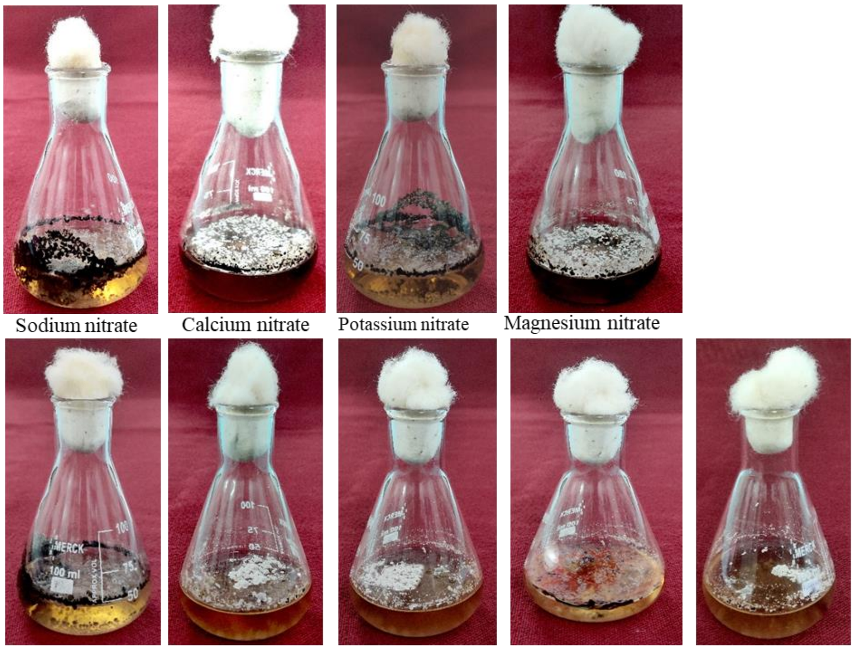

Fig. 1. Variability of isolate RGKN1 basing on nitrogen source utilisation.

Table 2: Effect of different nitrogen sources on sporulation of six different isolates of *Ramulariopsis* spp.

Nitrogen source	RGBP1 (mg)	RPGGNPR1 (mg)	RGBLGR1 (mg)	RGNP1 (mg)	RGKN1 (mg)	RPGKG1 (mg)	Mean (mg)
Urea	245.10	289.39	279.16	258.11	335.68	241.30	274.79
Ammonium chloride	365.38	431.81	416.56	385.56	460.73	359.42	403.24
Potassium nitrate	544.55	643.49	620.74	574.53	722.97	535.69	607.00
Ammonium nitrate	428.58	510.24	476.98	450.29	534.09	421.70	470.31
Sodium nitrate	512.74	610.45	572.61	536.67	691.00	504.49	571.33
Calcium nitrate	461.26	558.21	519.79	491.03	598.66	457.35	514.38
Magnesium nitrate	411.09	497.49	463.46	430.82	530.01	407.60	456.75
Diammonium hydrogen orthophosphate	505.77	612.12	570.04	530.02	652.78	501.48	562.03
Control	145.14	175.63	163.51	152.11	223.59	143.91	167.32
Mean(mg)	402.18	480.98	453.65	423.24	527.73	396.99	
	N		**I**		**N x I**		
SE(m)±	**6.333**		**5.172**		**15.515**		
CD(0.01)	**17.78**		**14.51**		**43.55**		
N- Nitrogen source, I- Isolate							

Table 3: Effect of different nitrogen sources on sporulation of six different isolates of *Ramulariopsis* spp.

Nitrogen source	RGBP1	RPGGNPR1	RGBLGR1	RGNP1	RGKN1	RGKG1
Urea	++	+++	+++	++	+++	++
Ammonium chloride	++	+++	+++	+++	+++	++
Potassium nitrate	++++	++++	++++	++++	++++	++++
Ammonium nitrate	+++	++++	+++	+++	++++	+++
Sodium nitrate	++++	++++	++++	++++	++++	+++
Calcium nitrate	+++	++++	+++	++++	++++	+++
Magnesium nitrate	+++	+++	+++	+++	++++	+++
Diammonium hydrogen orthophosphate	++++	++++	++++	++++	++++	+++
Control	+	++	+	+	++	+
++++ - Excellent sporulation, +++ - Good sporulation, ++ - Moderate sporulation, + - Poor sporulation						

CONCLUSION AND FUTURE SCOPE

Overall, this study highlighted the significance of nitrogen sources in supporting fungal growth and sporulation. Potassium nitrate, sodium nitrate, and diammonium hydrogen orthophosphate were identified as the most favorable nitrogen sources for promoting the growth of *Ramulariopsis* isolates. These findings have the potential to be valuable in enhancing the culture conditions for the fungus and advancing its subsequent investigation.

Future studies should focus on considering other attributes like colony morphology, pH, temperature and photoperiod requirement, differential reactions on cultivars and genetic constitution in order to obtain a clearer picture regarding the basis of variability among the fungal isolates collected from different geographical locations. Further research could also explore the underlying mechanisms by which different nitrogen sources influence the growth and sporulation of *Ramulariopsis* isolates, providing insights into the fungal physiology and optimizing cultivation conditions for potential applications.

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Conflict of Interest. None.

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