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Modified Liquid Dual Culture Methodology for Screening Bacterial Endophytes Against Fungal Pathogens

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ABSTRACT

A modified methodology for screening bacterial endophytes against fungal pathogens was developed. This method is modified form of liquid dual culture assay which can provide rather appropriate information of antagonistic activity of the endophyte. Results indicated that the endophytes have shown significant suppression of pathogens by multiple mechanisms in the mixed culture. In this study, the modified methodology was compared with routinely followed screening methods in two soil borne pathogens *Rhizoctonia solani* and *Sclerotium rolfsii* of tomato. In modified method, lowest *Rhizoctonia solani* biomass (54.47mg) was observed from 6TH4b inoculation as compared to 218.52 mg in control. In *Sclerotium rolfsii* also, lowest biomass (18.84mg) was observed from 6TH4b inoculation as compared to 334.07 mg in control. Dual plate assay has shown maximum suppression of 70.81 per cent of *Rhizoctonia solani* and 78.02 per cent of *Sclerotium rolfsii* by endophyte 2PR9b. Host tissue extract enhanced the antagonistic potential of bacterial endophytes and the effect was pronounced in 3A, where pathogen biomass suppressed by 26.51 mg as compared to that of 130.43 mg without host tissue extract. Isolates 3A, 6TH4b, 1TH16, 4R4 AND 4PR19 performed poorly in routine dual culture assay but found to have excellent suppression of pathogens by modified method. This modification can be effectively used for primary screening of endophyte bacteria for biological control.

Keywords : Modified liquid dual culture, endophytes, *Sclerotium rolfsii, Rhizoctonia solani*

INDISCRIMINATE use of harmful agrochemicals in disease management is creating serious problems in human, plant and animal health. Financial load for huge lot of agrochemicals is another big issue for poor and marginal farmers. Use of microbial inputs for controlling plant pathogens is getting established as standard practice now a days. This has given choice to number of farmers who were aware of ill effects of harmful agrochemicals but didn't have choice to replace part of it.

Endophytes have served as great treasure for novel secondary metabolites and other antimicrobial compounds (Chung *et al.,* 2018). Screening of both rhizospheric and endophytic bacteria is done rigorously using different methods targeting various antagonistic features of an organism. Most commonly, dual plate assay is used for assessing antagonism as primary screening (Shen *et al.,* 2017). Dual plate method is further supplemented by other methods like siderophore production, inhibitory volatile compound production (ammonia, HCN and others), lipopeptide production etc. Since microorganisms are having variable potential for different biocontrol features, these studies generate different screening results. Therefore, the bacterial culture which qualifies most of these tests is selected as potential culture. The combined effect of different biocontrol features cannot be quantified for purpose of comparison.

Primary screening of biocontrol agents is usually done by dual plate assay wherein per cent inhibition is qualitatively judged by zone of inhibition. However, these methods are good only for those biocontrol agents which produces antifungal antibiotics or hydrolytic enzymes. In the recent years, there are several reports on the production of volatile organic compounds like HCN, ammonia and others (Bahroun *et al.,* 2017) which also need to be considered for screening. Some of the workers have reported liquid dual culture assay to maximize the interaction between pathogen and biocontrol agent (Broekaert *et al.,* 1990; Trivedi *et al.,* 2008). However, these methods could not include the volatile components; therefore, some modifications were still needed.

Therefore, there is a need to develop a suitable modification in screening method wherein the pathogen is in constant contact with biocontrol agent and two can interact in both spatial and temporal basis in a closed vessel providing quantitative figure. This method should also take into consideration multiple mechanisms which a biocontrol agent can exhibit these effects in the presence of host and thereby result in growth inhibition of pathogen.

Material and Methods

This study was conducted to design a modified methodology to screen endophyte for biocontrol. Here, production of antimicrobial primary metabolites, secondary metabolites, ammonia, HCN, other inhibitory volatiles, antifungal enzymes, competition for space and nutrients, surfactants, effect of host in producing these compounds etc. to provide a wider spectrum of biocontrol potential by single experiment. The bacterial endophytes under study were first screened by standard methodology as a check to validate the findings from modified methodology.

Detection of biocontrol traits by standard methodology

In vitro antagonism of bacterial endophytes against fungal pathogens was tested by assessing six different tests, *viz*., dual plate inhibition, volatile organic compounds, ammonia production, siderophore production, hydrogen cyanide (HCN) production and lipopeptide surfactant production.

In dual plate inhibition assay, circular disc (7 mm) of fungal pathogen was taken and put on the centre of PDA plate. Fresh bacterial culture was streaked equidistantly on two sides of pathogen disc and incubated. Per cent inhibition of fungal colony was calculated at the end of seven days incubation.

Production of VOCs by endophytes against these two soil borne pathogens was tested in petri plates with partition. PDA and NA were added in each of the two compartments of partition plates. Pathogen disc is added in PDA compartment and endophyte in NA compartment. The plates were sealed and incubated for one week.

Ammonia production was detected by growing cultures in peptone broth for 72 hours at 30 °C and then 1 ml of Nessler's reagent was added to it. Development of yellowish brown color indicated production of ammonia.

Siderophore production assay was done by spotting bacterial suspension on chrome azurol S (CAS) agar medium and incubated at 30 °C for one week. Development of contiguous orange halo with colony growth indicated positive for siderophore production.

HCN production was detected by growing cultures in King's B broth supplemented with 4.4 g/L glycine. A filter paper strip soaked in 0.5 per cent picric acid in 2 per cent sodium carbonate solution was placed hanging towards top of the tube and incubated at 28 °C for one week. Color change in filter paper from to light brown indicated HCN production.

Production of lipopeptide surfactant was detected by growing cultures in nutrient broth for a period of 16-18 hours. This broth is dropped on a hydrophobic film for spreading behavior. Laboratory parafilm was used as hydrophobic surface in this study.

Modified liquid dual culture assay

Inoculum used in the study was prepared freshly for each study. Endophyte cultures were isolated from tomato plants in earlier studies (Table I) and are tested against *Rhizoctonia solani* and *Sclerotium rolfsii.*

Table I

Endophyte cultures used for this study

Isolates	Identity
1PR7a	Bacillus wiedmannii
2P2	Bacillus haynesii
2PR9b	Bacillus altitudinis
3A	Stenotrophomonas maltophilia
4PH5	Bacillus wiedmannii
4PR8	Bacillus cereus
6TH4b	Bacillus zhangzhouensis
1TH16a	Lysinibacillus sp.
4R4	Bacillus fengqiuensis
4PR19	Lysinibacillus xylanilyticus

Endophyte bacteria and pathogen are inoculated in broth containing 1:1 ratio of potato dextrose broth and nutrient broth. The broth (100 ml) was taken in 250 ml capacity reagent bottles and autoclaved. Endophyte bacteria and target pathogen was inoculated into this media and kept in incubator at 28 °C for seven days. One set of this experiment is added with 2 ml filter sterilized host tissue extract (HTE) of tomato to see the effect of HTE on antagonistic potential. Inhibition in this modified liquid dual culture assay was measured based on biomass produced by fungus at the end of incubation. After complete growth, broth was autoclaved to kill the entire content so as to allow further safe handling of microbial biomass. The broth was then filtered using quantitative filter paper 420R equivalent to Whatman 42 filter paper. Supernatant along with filter paper was dried in oven at 60 °C till it gets stable dry weight.

The formula for determining the per cent inhibition keeping in mind the possible factors that influence the result, is proposed.

Weight of blank filter paper after drying (filter paper control) = f

Weight of filter paper + endophyte biomass after drying (endophyte control) = e

Weight of filter paper + pathogen biomass after drying (pathogen control) = p

Weight of interaction (filter paper + pathogen + endophyte) after drying = i

Weight of total biomass accumulated (pathogen + endophyte) in interaction = $Wi = i - f$

Weight of only bacterial endophyte in control = $We = e - f$

Weight of only pathogen in control = $Wp = p - f$

$$\% \text{ Inhibition} = \left[1 - \left(\frac{Wi - We}{Wp} \right) \right] \times 100$$

There are few reports of quantitative estimation in screening for biocontrol agent (Boekaert *et al.,* 1990; Trivedi *et al.,* 2008), but screening for bacterial endophytes needs some of the crucial factors to be included in these methods. First, this method can be improved by providing completely closed environment to account volatile inhibitory compounds produced by bacterial endophytes as endophytes are reported for having tremendous capacity of producing volatile organic compounds as a weapon against plant pathogens (D'Alessandro *et al.,* 2014). Addition of host tissue extract may help endophytes to perform well as reported by several workers (Arnold & Herre, 2003 and Thomas & Soly, 2009). Increasing incubation time can be useful for checking effects of secondary metabolites on fungal biomass (Madigan *et al.,* 2010).

Statistical analysis

The experiments were conducted precisely in lab conditions. Therefore, results were analyzed in complete randomized design (CRD) and means were separated by Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

Results and Discussion

Ten bacterial endophytes were taken in the study for comparing the screening methods (Table I). These ten endophytes represented three bacterial genera (*Bacillus, Lysinibacillus* and *Stenotrophomonas*) and nine different species. Using these test micro organisms from different genera and species was useful as it had given a broader genetic variability for comparing screening methodologies. These cultures were reported before for their biochemical activity and the selection of these endophytes for biocontrol studies is in line with findings of Trivedi *et al.* (2008), Shen *et al.* (2017) and Chung *et al.* (2018). The genera of endophytes used in the study were proven for production of siderophore, ammonia, hydrogen cyanide (HCN) and other inhibitory mechanisms on pathogens (Abdallah *et al.,* 2017).

Results indicated that the endophytes have variable potential for exhibiting the six biocontrol traits under study. In dual plate assay, bacterial endophytes 2PR9b and 2P2 have shown 78.02 per cent and 72.93 per cent inhibition of *Sclerotium rolfsii*, respectively. Highest suppression against *Rhizoctonia solani* was 70.81 and 70.15 per cent by 2PR9b and 1PR7a, respectively (Fig. 1). The suppression obtained against the pathogen in dual plate assay is might be due to diffusible compounds produced by the

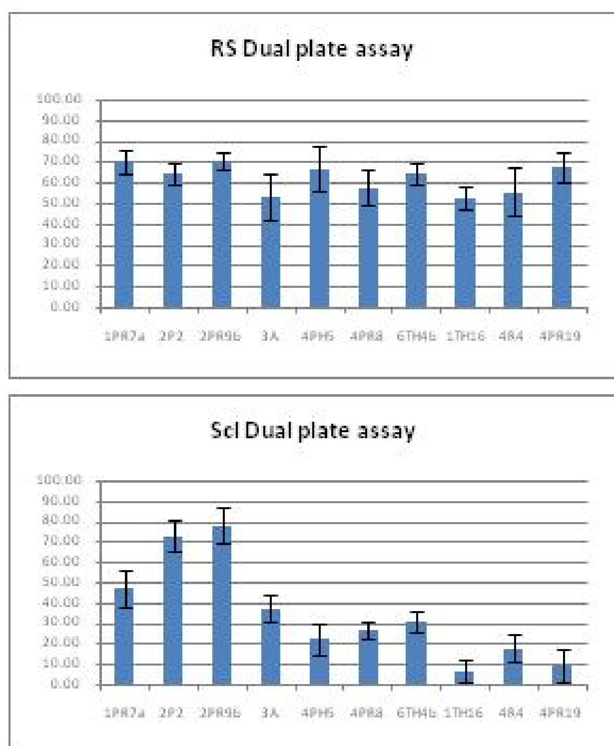

Fig. 1: Per cent inhibition of *Rhizoctonia solani* (RS) and *Sclerotium rolfsii* (Scl) by dual plate assay (semi-quantitative)

endophytes. Varied biocontrol ability of bacterial endophytes is reported by several workers and this is in accordance with earlier studies that *Bacillus* and *Stenotrophomonas* produce antifungal effects (Chung *et al.*, 2018).

The volatile organic compounds (VOCs) production studies indicated that the endophyte 4PR19 was potent in suppressing *Sclerotium rolfsii*. Endophyte 4R4 was also promising against *Sclerotium rolfsii* and *Rhizoctonia solani* (Table II). The pathogen suppressive results obtained in this study are in accordance with reports of Bahroun *et al.* (2017) which indicated different mechanisms for biocontrol of pathogens. The endophytes were also potent volatile organic compound producers which has caused suppression of pathogen in the plate. Similar was reported by D'Alessandro *et al.* (2014).

The cultures have separately shown their potential of producing ammonia, siderophore, HCN and surfactant lipopeptides. Ammonia production was assessed by looking for the change in color from pale yellow to light brown or dark brown. Based on this analysis, two isolates 4R4 and 3A were considered as most potent for ammonia production followed by 2P2 and 6TH4b. In siderophore production assay, the size of the orange halo zone around the colony was taken as an indication of siderophore production. Isolates 2P2 and 3A produced the biggest halo zone and were labeled as potent for siderophore production. Among the 10 isolates, 5 showed siderophore production. HCN production was found highest in 4PR19 and 2P2 which is supported by findings of Bahroun *et al.* (2017). In drop collapse assay, 2P2, 4PR8 and 3A found producing relatively higher quantity of surfactant and resulted in pronounced flattening on the hydrophobic surface. This result is in line with the finding of de Bruijn and Raaijmakers (2009). Among these biochemical tests, 2P2 was found most promising, but this type of inference could not be drawn for all the endophytes. Some of the endophytes exhibited variable results in these parameters (Table III).

TABLE II

Inhibition of two tomato pathogens by bacterial endophytes by production of inhibitory volatile compounds

Isolates	Inhibitory VOCs production	
	Rhizoctonia	*Sclerotium*
1PR7a	-	-
2P2	++	-
2PR9b	+	-
3A	+	-
4PH5	-	-
4PR8	-	-
6TH4b	-	-
1TH16a	+	++
4R4	++	+++
4PR19	-	+++

In modified method, the difference of results from standard methodology was apparent. In this, lowest *Rhizoctonia solani* biomass accumulation (54.47mg) was observed from 6TH4b inoculation as compared to 218.52 mg in control. In *Sclerotium rolfsii* also, lowest biomass (18.84mg) was observed from 6TH4b inoculation as compared to 334.07 mg in control. Addition of host tissue extract was found to

TABLE III

Assessment of biocontrol and plant growth promoting traits in bacterial endophytes

Isolates	NH_3	Siderophore production	HCN production	Surfactant production
1PR7a	-	-	-	+
2P2	++	+++	++	++
2PR9b	+	+	-	+
3A	+++	+++	-	++
4PH5	+	-	-	+
4PR8	-	-	-	++
6TH4b	++	+	-	-
1TH16a	++	-	-	-
4R4	+++	++	-	-
4PR19	+	-	++	+

+++ Prominent activity; ++ Moderate activity; + low activity; - no activity

enhance the antagonistic potential and the effect was pronounced in 3A, where the pathogen biomass was reduced to 26.51 mg as compared to 130.43 mg without host tissue extract (Table IV). Isolates 3A, 6TH4b, 1TH16, 4R4 and 4PR19 performed poorly in routine dual culture assay but found to have excellent suppression of pathogens by modified method (Fig. 1-3).The VOCs production results were not in line with dual plate assay but a clear effect of these VOCs can be seen in modified liquid dual culture method where host tissue extract is found to enhance the suppressive ability of these two isolates (Fig. 2 & 3).

Some of the workers had suggested liquid dual culture technique (Trivedi *et al.,* 2008) but the modifications made in the present investigation could add value to the screening of bacterial endophytes for biocontrol. Stringent selection based on standard tests may lead to chose 1PR7a, 2P2 and 2PR9b discarding other cultures but if we look at the results obtained from modified liquid dual culture method (Table IV; Fig. 2 & 3) there are other potential endophytes (like

TABLE IV

Quantitative estimation of holistic effect of endophyte on growth and biomass accumulation of Rhizoctonia solani *and* Sclerotium rolfsii *(mg of biomass)*

Treatments	*Rhizoctonia solani*		*Sclerotium rolfsii*	
	Without HTE	With HTE	Without HTE	With HTE
Control	218.52 [a]	205.75 [a]	334.07 [a]	351.10 [a]
1PR7a	90.05 [cd]	73.52 [d]	118.80 [bcd]	68.79 [cd]
2P2	90.74 [cd]	42.48 [e]	94.69 [cde]	57.95 [cde]
2PR9b	92.10 [cd]	56.73 [de]	90.11 [de]	83.86 [c]
3A	130.84 [bc]	26.51 [f]	77.94 [ef]	34.97 [de]
4PH5	132.43 [bc]	39.57 [ef]	95.60 [cde]	122.08 [b]
4PR8	165.46 [b]	143.38 [b]	64.63 [ef]	54.73 [cde]
6TH4b	54.47 [d]	40.79 [e]	18.84 [g]	23.22 [e]
1TH16a	110.24 [c]	83.23 [d]	57.33 [f]	64.43 [cd]
4R4	123.99 [c]	113.10 [c]	144.67 [b]	117.05 [b]
4PR19	119.81 [c]	80.01 [d]	124.70 [bc]	73.29 [c]

Note: Values with same superscript do not differ significantly at p £ 0.05

HTE= Host Tissue Extract

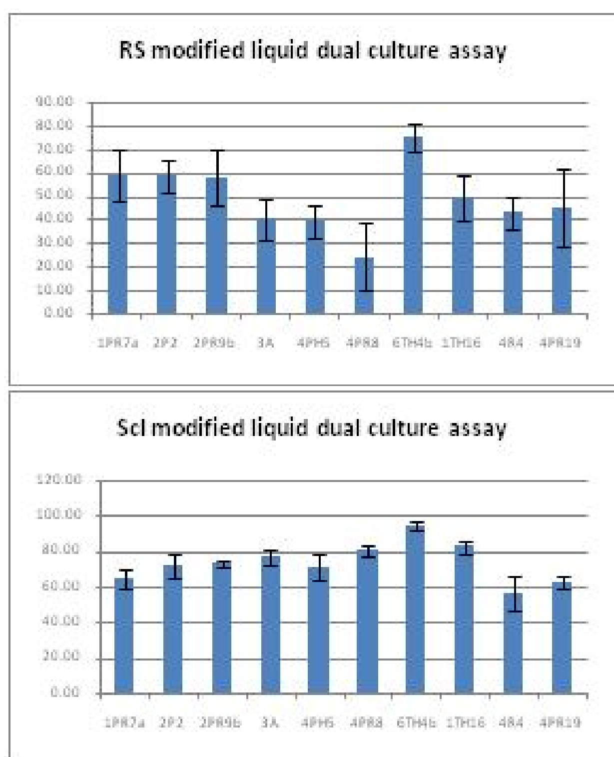

Fig. 2: Per cent inhibition of *Rhizoctonia solani* (RS) and *Sclerotium rolfsii* (Scl) by modified liquid dual culture method without HTE

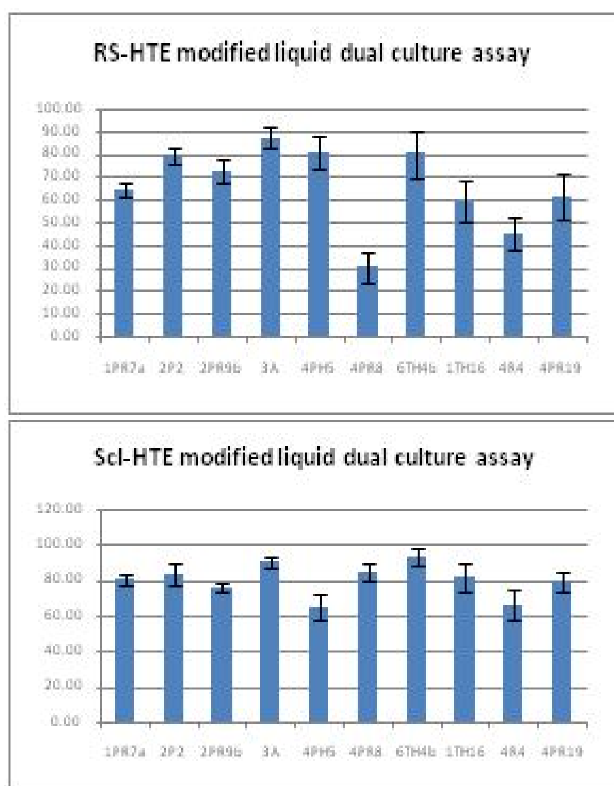

Fig. 3: Percent inhibition of *Rhizoctonia solani* (RS) and *Sclerotium rolfsii* (Scl) by modified liquid dual culture method with HTE

3A and 6TH4b) which suppress pathogens up to 94.36 per cent. There is induction of host tissue on performance of endophytes which is in line with findings of Arnold and Herre (2003).

It was evident from the study that the modification suggested could add value and it can be recommended for primary selection of a potent biocontrol agent against any pathogenic fungi as it provides quantitative data on percent inhibition. Performing this method for first screening could reduce the chances of losing a potent endophyte from primary population. In this method, one can rule out selection based on one mechanism and it can be used for rapid and reliable screening based on quantitative data. It is further recommended that the all possible mechanisms should only be studied after the selection of potent biocontrol agent based on this assay.

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