Int.J.Curr.Microbiol.App.Sci (2018) *7*(8): 1323-1331

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 08 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.708.150

Antagonism of *Trichoderma asperellum* Isolates against *Macrophomina phaseolina* Causing Soybean Charcoal Rot and Its Impact on Physiological Parameters of Crop

Ishwar Patidar[1*] and Vibha[2]

[1]Department of Plant Pathology, [2]Department of Plant Physiology, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur (M.P.) 482 004, India

**Corresponding author*

A B S T R A C T

Soybean (*Glycine max)* is a miracle crop due to its extraordinary qualities apart from known for rich source of vegetable protein and oil. Over the years, its production is stagnant or declining owing to climate change and also due to ever changing pathogenic behaviour of *Macrophomina phaseolina*. The present investigation was undertaken to utilize the dominant resident mycoflora of crop that are capable of adjusting to changing environment apart from its ability to combat the menace of its co-partner (pathogenic group) evolving with them. Dominant rhizosphere fungi viz. *Trichoderma asperellum* and its ten isolates were isolate d through dilution plate technique. Efficacy of *Trichoderma asperellum* isolates against *M. phaseolina* was established through dual culture and poison food technique. Under *in-vivo* conditions, their impact on physiological parameters and disease suppression ability has also been established. The isolates with higher mycelia growth suppressing ability under dual culture technique had not found inhibitory towards the test pathogen under poison food technique. Similarly, *T. asperellum* isolates tested under *in-vitro* showed their varying potential against pathogenic fungi under *in-vivo* conditions.

Keywords

Fungal isolates, Charcoal rot, Physiological parameters and disease incidence

Article Info

Accepted:
10 July 2018
Available Online:
10 August 2018

Introduction

Soybean is a miracle crop due to its extraordinary qualities as it contains about 40 percent protein, 29 percent carbohydrate and 20 percent oil. Its oil comprises of 85percent unsaturated cholesterol free fatty acid with essential nutrients (Aditya *et al.*, 2011). Charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goidanich is one of the most important diseases of soybean in tropical and subtropical regions of the world that results in reduced yield and poor seed quality. The pathogen is a cosmopolitan soil saprophyte and is well known facultative, opportunistic plant pathogen that infects plants exposed to certain stress conditions (Tesso *et al.*, 2005). This disease leads to early maturation, chlorosis and incomplete pod filling. Owing to higher variability among the

isolates of its pathogen, no commercial resistant soybean variety is yet available for effective management of this disease. The diversity among the genus *Trichoderma* is great and is considered as potential biocontrol and plant growth promoting fungi for many crops (Verma *et al.,* 2007; Bai *et al.,* 2008; Savazzini *et al.,* 2009). Effective biocontrol of Macrophomina *phaseolina* by *Trichoderma* species along with several other soil-borne fungi are widely established [1] besides their abilities to enhance systemic resistance system of plant against diseases [2]. Significant effect of defense enzymes such as peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase produced by *T. viride* alone or in combination with *Pseudomonas fluorescens* against *M. phaseolina* in green-gram plants has also been reported by Thilagavathi and co-workers (2007). Similarly, tomato plants primed with *T. arundinaceum* triggered the expression of defense-related genes belonging to the salicylic acid (SA) and jasmonic acid (JA) pathways in presence of *Botrytis cinerea* and *Rhizoctonia solani* (Malmierca *et al.,* 2012). The toxin (phaseolinone) produced by *M. phaseolina* (Tassi) Goidanich causes vascular blockage that ultimately led to destruction of plant organs. This pathogen is also considered as high-temperature pathogen because disease severity increases as the air and soil temperatures increase from 28 to 35°C *vis* à *vis* low soil moisture (Pearson *et al.,* 1984; Gary *et al.,* 1991). Management of charcoal rot disease is difficult due to nature of pathogen and rejection of chemical control methods owing to environmental and health issues. *Trichoderma* species are saprophytic, widely distributed, quick grower, with high population densities in soils and plant residues. They are easy to culture and can produce large amounts of conidia with long life time (Manczinger *et al.,* 2002). Hence, present investigation was undertaken with objectives (i) to establish variability among potential native isolates of *T. asperellum* on the basis of their qualitative attributes and (ii) to determine role of *T. asperellum* isolates against *Macrophomina phaseolina* under *in-vitro* and *in-vivo* conditions along with their impact on physiological parameters of crop.

Materials and Methods

Collection of diseased specimens and purification of the pathogens

Diseased soybean plants exhibiting typical symptoms of charcoal rot incidence levels were collected from the sick plots of AICRP on soybean experimental field of Jawaharlal Nehru Krishi Vishwa Vidyalaya (22°49'- 22^0 80'N; 78°21'- 80°58'E), Jabalpur in the Central India during 2015-16. The pathogen was isolate d and further purified through hyphal tip method and sub-cultured on PDA slants at 4 ºC for further use. Dilution plate method was used to isolate the *T. asperellum* isolates from soil samples of soybean plant showing different level of charcoal rot symptom, on Rose Bengal Agar medium (RBA). Plates with RBA medium was added with 0.1 ml (=10^{-4}) of suspension and incubated at 22 ± 2^{0}C for 15 days. The colonies were transferred to test tubes containing Potato Dextrose Agar (PDA) medium. The *T. asperilum* isolates were designated as TA1, TA2, TA3, TA4, TA5, TA6, TA7, TA8, TA9, and TA10, throughout the study.

Evaluation of antagonistic potential of beneficial fungi *in-vitro*

The antagonistic potentials of *T. asperillum* isolates were evaluated against the *M. phaseolina* through dual culture technique (Denis and Webster, 1971). A five mm disc of different fungal isolates was cut out from the seven days old culture and placed close to one end of the Petri-plate containing 20 ml solidified PDA medium. At the opposite end,

a similar disc from the culture of the pathogen *M. phaseolina* was placed simultaneously. The Petri-plates were incubated at 25±2ºC in a BOD incubator and the inhibition of the growth of the pathogen by the antagonistic fungi was measured after 48 hrs, 72 hrs. and 96 hrs. Of incubation till both occupies the entire space of Petri-plate.

Culture filtrate of TA isolates grown in PDA broth grown for 10 days were collected after passing it twice through Whatman filter paper No. 1. These filtrates were used to amend Petri-plates containing PDA at 5 per cent concentration and incubated at 25+2°C and observations were recorded after 48, 72, and 96, hours, respectively; an un-amended Petri-plate served as check (control). Each treatment was replicated thrice and the experiment was repeated twice.

The antagonism was measured on the basis of inhibition of the pathogen by the bio agent by the following formulae

$$\text{Inhibition} = \frac{\text{Radial growth in control (C)} - \text{Radial growth in the treatment (T)}}{\text{Radial growth in control(C)}}$$

Assessment of antagonistic potential of *T. asperillum* isolates under *in-vivo* conditions

The inoculum of fungus *M. phaseolina* was produced on sand + wheat flour mix (9:1), moistened with water and autoclave twice for 90 minutes on two consecutive days. Thirty days after the sowing of the seeds, the culture filtrate of individual beneficial fungi were added into the pots that were already containing the *M. phaseolina* inoculum spreaded on sand + wheat flour mix (@ 5 gm/ kg of potting mix). Two sets of experiments with three replicates for each treatment were maintained. The experiment was done in two sets in two different poly-houses. Ten soybean seeds were sown in each clean pot at the 2-3 cm deep in six pots for each strain of *T. asperillum* along with un-inoculated control.

Relative water content (RWC)

Measurements of RWC (Barrs and Weatherly, 1962) were performed on leaves collected from soybean plants. Individual leaves were first removed from the stem with tweezers and were weighed immediately (fresh mass, FM) to obtain minimum 0.5 gram from each sample.

In order to obtain the turgid mass (TM), leaves were floated in distilled water inside a closed Petri dish. At the end of the imbibition period, leaf samples were placed in a pre-heated oven at 80 ºC for 48 hr. to obtain the dry mass (DM). Values of FM, TM, and DM were used to calculate RWC, using the following equation:

$$\text{RWC (\%)} = [(\text{FM} - \text{DM})/ (\text{TM} - \text{DM})] \times 100.$$

Chlorophyll content index

Chlorophyll Content Index was estimated through the portable chlorophyll meter Peng *et al.,* (1992). Fully expanded leaf sample from three places of each plant of different treatments has been selected for estimation of chlorophyll content index. The mean of triplicate readings taken using SPAD-502 (SPAD-502, Minolta, Japan) around the midpoint near the midrib of each sample were recorded for different treatment of chickpea leaf and averaged.

Disease incidence

The percent of charcoal rot incidence of each treatment was calculated by using following formulae.

Disease incidence (%) = No. of plants exhibiting wilt symptom X 100

Results and Discussion

Evaluation of *T. asperellum* isolates against *M. phaseolina* under *in-vitro* conditions

The effects of ten isolates of *T. asperellum* were assessed for the inhibition of mycelial growth of *M. phaselina*. It is evident from the (Table 1) that all the isolates were highly suppressive towards the test pathogen. The suppression of mycelial growth of *M. phaselina* by different isolates of *T. asperellum* varied between 30.75mm to 39.34mm. The highest (30.75mm) inhibition was recorded with isolate 1 while least (39.34mm) with isolate 7. The isolate 1 (30.75mm), isolate 4 (30.96mm), isolate 5 (38.37), and isolate 10 (38.68) were equally suppressive towards the test pathogen and were next best to isolate 6 (31.56mm), 9 (33.11), 2 (35.51) and isolate 8 (37.33). There was retrogressive significant increase in growth of the pathogen was recorded at every interval of time. The highest (36.44mm) inhibition of *M. phaseolina* was recorded in cell free culture filtrate of isolate 6 followed by isolate 8 (38.43), isolate 3 (38.69mm), isolate 1 (41.87), isolate 7 (42.64) and isolate 2 (43.31) under poison food technique (Table 2).

Impact of *T. asperellum* isolates on physiological parameters and disease incidence under *in-vivo* conditions

The effects of culture filtrate of different isolates of *T. asperellum* were tested under *in-vivo* conditions on relative water content (RWC), chlorophyll content (SPAD-502) and on disease incidence (Table 3).

The highest (79.28 %) RWC was recorded in isolate 9 treated plants followed isolate 5 (75.69%), isolate 1 (67.32%) and isolate 3 (61.35%). Similarly, the lowest (16.66%) disease incidence was recorded in isolate 9 treated plants.

Identical but higher disease suppression was also recorded in isolate 5 (23.07) and isolate 1 (23.07%) followed by isolate 3 (28.57%).

Table.1 Evaluation of *Trichoderma asperellum* isolates against *M. phaseolina* through dual culture technique

S. No.	*Trichodrma asperellum*	Growth inhibition (in mm)		
		48 hours	72 hours	96 hours
1.	Isolate 1	17.0	27.0	35.7
2.	Isolate 2	24.0	35.0	43.0
3.	Isolate 3	29.7	37.3	46.3
4.	Isolate 4	16.3	26.0	38.7
5.	Isolate 5	30.7	39.3	46.0
6.	Isolate 6	18.0	30.3	35.0
7.	Isolate 7	31.7	41.0	48.3
8.	Isolate 8	30.7	37.3	42.7
9.	Isolate 9	21.0	32.3	37.0
10.	Isolate 10	30.0	40.3	47.3
	Control	**40.7**	**57.4**	**75.5**
	SE m±	0.54	0.50	0.43
	CD at (5%)	1.61	1.49	1.28
	CV	3.63	2.47	1.73

Table.2 Screening of *Trichoderma asperellum* isolates against *M. phaseolina* through poison food technique

S. No.	*Trichoderma asperellum*	Growth inhibition (in mm)		
		48 hours	**72 hours**	**96 hours**
1.	Isolate 1	34.7	41.7	57.7
2.	Isolate 2	37.7	42.3	61.3
3.	Isolate 3	25.3	37.3	55.7
4.	Isolate 4	36.0	44.0	62.7
5.	Isolate 5	37.3	45.0	64.0
6.	Isolate 6	18.3	39.3	50.3
7.	Isolate 7	33.0	43.0	62.3
8.	Isolate 8	22.7	40.7	54.0
9.	Isolate 9	33.3	45.7	64.7
10.	Isolate 10	32.0	47.7	65.0
	Control	**40.7**	**57.4**	**75.5**
	SE m±	0.42	0.46	0.37
	CD at (5%)	1.27	1.37	1.11
	CV	2.32	1.81	1.06

Table.3 Impact of *Trichoderma asperellum* isolates on physiological parameters and disease incidence of soybean

S. No.	*Trichoderma asperellum*	RWC (%)	Chlorophyll content (SPAD-502)			Disease incidence (%)
			BI	**AI**	**Loss (%)**	
1.	Isolate 1	67.32	32.0	27.2	70.0	23.07
2.	Isolate 2	47.27	30.9	28.9	69.1	44.44
3.	Isolate 3	61.35	27.3	27.9	72.7	28.57
4.	Isolate 4	55.87	28.5	18.9	71.5	33.33
5.	Isolate 5	75.69	27.0	24.2	73.0	23.07
6.	Isolate 6	42.48	29.0	29.1	71.0	50.00
7.	Isolate 7	47.48	27.8	25.5	72.2	42.85
8.	Isolate 8	54.41	31.3	25.2	68.7	35.71
9.	Isolate 9	79.28	30.0	32.2	70.0	16.66
10.	Isolate 10	45.47	28.7	27.0	71.3	45.45
	Control	**34.70**	**28.0**	**26.1**	**72.0**	**61.53**
	SE m±	0.40	0.34	0.72		0.55
	CD at (5%)	1.19	1.02	1.15		1.65
	CV	1.25	2.06	2.17		2.60

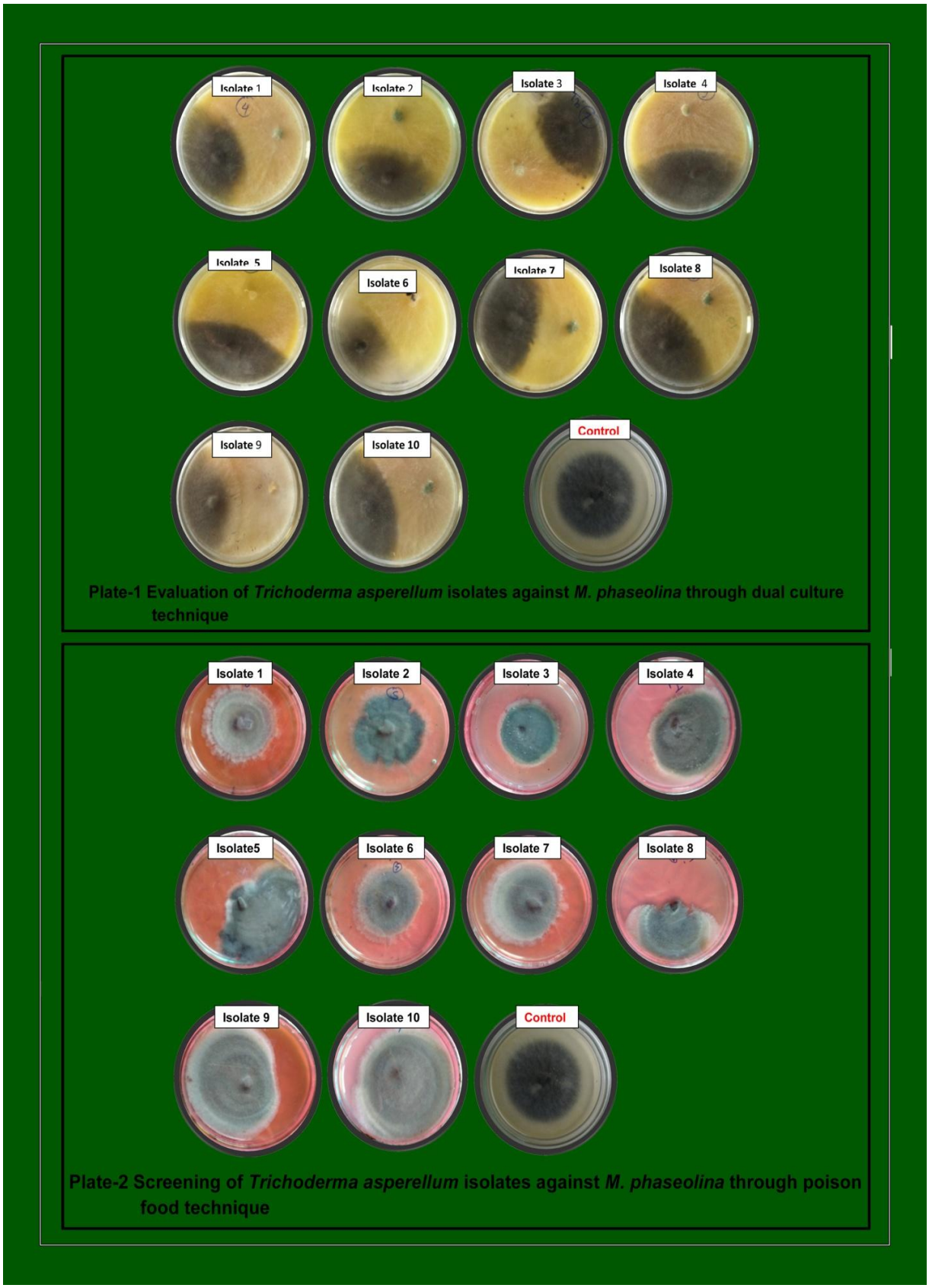

Plate-1 Evaluation of *Trichoderma asperellum* isolates against *M. phaseolina* through dual culture technique

Plate-2 Screening of *Trichoderma asperellum* isolates against *M. phaseolina* through poison food technique

The chlorophyll content of leaves ranged between 27.0 to 32.2 in culture filtrate inoculated plants of different isolates prior to *M. phaseolina* inoculation while 18.9 to 32.2 percent in *M. phaseolina* inoculation plants. The highest chlorophyll content was recorded in isolate 9 (32.2%) in *M. phaseolina* inoculated plants. Under the dual culture experiment, ten isolates of *T. asperellum* had shown strong antagonistic effect against *M. phaseolina*. The similar and the highest (16.3 mm and 17.0 mm) mycelia growth suppression of test pathogen was recorded with isolate 4 and isolate 1 followed by 6 (18.0 mm) at 48 hrs and 72 hrs but the highest (35.0 and 35.7 mm) suppression by isolate 6 and isolate 1 was recorded at 96 hours.

Such result could be due to faster growth and active parasitism by *T. asperellum* isolates. Member of *Trichoderma* species are known for their active hyperparasites of several soil fungi and hence they are used as a biocontrol agents (Ekefan *et al.,* 2009). Karthikeyan *et al.,* (2015) found that that *T. harzianum* and *T. viride* were effective against against *M phaseolina*. Maximum reduction in the growth of *Macrophomina* was observed in the presence of native *Trichoderma* isolate, TW17 to an extent of 62.2 per was reported by Nagamani and Reddi Kumar (2015).

In poison food technique, the highest inhibition (18.3 and 50.3 mm) was recorded in cell free culture filtrate of isolate 6 at 48 and 96 hours. The growth inhibition by isolate 8 (22.7 mm) was higher than isolate 3 (25.3 mm) at 48 hours but not at 72 hours. However, both isolates (isolate 8 and isolate 3) became equally inhibitory towards the *M. phaseolina* at 96 hours. Such result may be attributed to the availability of nutrient in the medium and its impact on biocontrol mechanism of bioagents. Competition for carbon, nitrogen and iron mechanism associated with biocontrol has been proposed by Couteadier (1992). Tapwal and associates (2011) reported that non-volatile metabolites of *T. viride* were more effective against *R. solani*, *Curvularia lunata* and *Alternaria solani*. Metabolite of *T. harzianum, T. viride* and *T. virens* has been found to inhibit the mycelial growth of *Fusarium oxysporum* f. sp. *ciceri* (Dubey *et al.*, 2014). *Trichoderma* strains inhibit the infections caused by plant pathogens using different biocontrol mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion (Hajieghrari *et al.,* 2008; Poovendran *et al.,* 2011).

Under *in-vivo* conditions, the maximum RWC was recorded in *T. asperellum* isolate 9 (79.28%) followed by isolate 5 (75.69%). The higher RWC in different bioagent treated plants could be due to their ability to mitigate the adverse effect of pathogen. The mitigation of water stress, caused by test pathogen, by inoculation of culture filtrate of bioagents could be attributed to such result. Increased stomatal resistance and reduced transpiration rates in *Vigna aconitifolia (*Yadava *et al.,* 1994*)* and sorghum *(*Pedgaonkar and Mayee, 1990*)* have been reported due to water stress and drought condition. Turkan *et al.,* (2005) observed that the water deficit result in decreased RWC in bean. Higher RWC in different bioagent treated plants could be due to their ability to mitigate the adverse effect of pathogen. Charcoal rot undergoes rapid development under strong water content depletion (Pedgaonkar and Mayee, 1990), therefore, cultivars that show reduced water depletion rates and a stable cellular turgor are resistant to charcoal rot (Mayek-Perez *et al.*, 2002). The minimum charcoal rot incidence was recorded in *T. asperellum* isolate 9 (16.66%), among *T. asperellum* isolates. *Trichoderma* is an effective mycoparasitic strains against fungal plant pathogens under a wide range of adverse environmental conditions (Manczinger *et al.,* 2002).

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How to cite this article:

Ishwar Patidar and Vibha. 2018. Antagonism of *Trichoderma asperellum* Isolates against *Macrophomina phaseolina* Causing Soybean Charcoal Rot and Its Impact on Physiological Parameters of Crop. *Int.J.Curr.Microbiol.App.Sci.* 7(08): 1323-1331.
doi: https://doi.org/10.20546/ijcmas.2018.708.150