Int.J.Curr.Microbiol.App.Sci (2020) *9*(10): 1150-1159

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 10 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.910.138

Bacterial Bioremediation of Imidacloprid in Mango Orchard Soil by *Pseudomonas mosselii* Strain NG1

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A B S T R A C T

Imidacloprid is used extensively in mango ecosystem to control hopper. It can persist in soil as residues for around 150 days. Many soil microorganisms have the ability to degenerate many pesticides by converting them to non-toxic compounds. The present study was undertaken to isolate imidacloprid degrading microbe from mango orchard soil and to test its utility in field conditions. Among the four isolated bacteria, *Pseudomonas mosselii* strain NG1 was found most effective in degrading imidacloprid. After spraying imidacloprid at 0.005% in mango trees, soil samples were treated with *Pseudomonas mosselii* strain NG1 immobilized in straw and as free form in broth. Immobilized *P. mosselii* degraded imidacloprid faster (from 0.606 μg g^{-1} in 0 day to 0.052 μg g^{-1} after 67 days of treatment – 91.42% degradation) in soil as compared to *P. mosselii* in free form (from 0.30 μg g^{-1} in 0 day to 0.043 μg g^{-1} after 67 days of treatment – 85.67% degradation) and control (from 0.216 μg g^{-1} in 0 day to 0.048 μg g^{-1} after 67 days of treatment – 77.78% degradation). The half-life values were calculated as 24.0, 15.5 and 8.0 days in soil without bacterial application, with *P. mosselii* in free form and with *P. mosselii* in straw, respectively. In conclusion, immobilized *P. mosselii* strain NG1 can be used to degrade imidacloprid in mango orchard soil. This is the first report of *P. mosselii* strain NG1 having imidacloprid degradation potential.

Keywords

Imidacloprid, Bacteria, Biodegradation, Mango orchard soil, *Pseudomonas mosselii*

Article Info

Accepted:
10 September 2020
Available Online:
10 October 2020

Introduction

Mango (*Mangifera indica* L.) is a commercially important fruit crop which is heavily infested by many insect – pests throughout its developmental stages requiring spraying of many insecticides for getting better yield. Hopper (*Amritodus atkinsoni* and *Idioscopus niveosparsus*) is one of the major insects of mango which attacks panicles/inflorescences resulting in less fruit set and finally low yield. Imidacloprid [1-(6-chloro-3-pyridinyl) methyl)-N-nitro-2-imidazolidinimine], a neonicotinoid insecticide, is extensively used to control mango hopper in India by spraying at pre-bloom stage of mango flowers. It is also used as seed dressing and as termiticide. Imidacloprid is a persistent insecticide and its persistence in soil is well reported. It is a polar compound with high solubility in water, relatively non-volatile and persistent in soil with a half life of about 156 days (Jeschke *et al.,* 2005; Baskaran *et al.,* 1997) have reported a persistence of 48-190 days for imidacloprid in soil. Different half-life values in different types of soils for imidacloprid have been reported earlier *viz.* 990-1230 days in sandy loam soil in Australia, 455-518 days in sandy loam soil and 233-366 days in silty clay loam soil in Spain and 29-48 days in alluvial, lateritic and coastal alkaline soils in India (Baskaran *et al.,* 1999; Fernández-Bayo *et al.,* 2009; Sarkar *et al.,* 2001). In a review it has been reported that imidacloprid is an extremely persistent insecticide in soil with half-life ranging from 28-1250 days depending on soil type (Goulson 2013). Even after spraying in trees some portion of insecticide will invariably come in contact with soil flora and fauna. Thus, it has the ability to contaminate both surface and ground water through leaching and thereby gets accumulated in the food chain.

Microbes play an important role in removing toxic insecticides from environment and microbial degradation can be considered to be a cost effective mechanism to detoxify the pesticide (Li *et al.,* 2012). In the recent decade many researchers in India and abroad have tested a number of microorganisms *viz. Leifsonia* sp., *Pseudomonas* sp., *Bacillus* sp., *Ochrobactrum* sp., *Enterobacter* sp., etc. for the degradation of imidacloprid isolated from various types of soil under different crop environment (Anhalt *et al.,* 2007; Pandey *et al.,* 2009; Sharma *et al.,* 2014; Hu *et al.,* 2013; Sharma *et al.,* 2014). However, information regarding microbial degradation of imidacloprid in mango orchard soil is very scanty. In one of our previous studies, it was noticed that one bacterium isolated from mango orchard soil and identified as a strain of *Pseudomonas* sp. was able to degrade imidacloprid under laboratory condition (Garg *et al.,* 2018). As imidacloprid is widely used in mango ecosystem, it is imperative to have its residue in orchard soil. Microbial bioremediation will help in improving the soil health by reducing the toxic effect of insecticide on soil microflora and fauna through conversion to non-toxic metabolites. It can also help in minimizing the leaching of pesticide residues to ground water. Therefore, the present study was undertaken to isolate different microbes from imidacloprid treated mango orchard soil and apply the most effective one to study its ability to degrade imidacloprid in soil under field condition.

Materials and Methods

The experiment was conducted at the mango field of ICAR-Central Institute for Subtropical Horticulture, Rehmankhera, Lucknow. Aseptic technique was followed during collection of soil samples to prevent any contamination.

For isolation and purification of culture having imidacloprid degrading potential, carbohydrate utilization broth and agar with 1 per cent imidacloprid formulation as carbon source were used. The broth was first autoclaved and then cooled and 1 per cent imidacloprid formulation (Media® 17.8 SL) and 1 mL of suspension from mango orchard soil were aseptically added. All composites were properly mixed and incubated for 3 days at 30 °C. One mL of culture broth was pour-plated on carbohydrate utilization agar having imidacloprid and the colonies were isolated. The isolates were further purified by streaking on same agar plates. The pure cultures were maintained on the Nutrient agar slants.

Sterile soil samples were inoculated with bacterial isolates and imidacloprid degradation pattern was observed under laboratory conditions after fortifying with 0.005% of its formulation. Soil samples were collected at 7 days interval, extracted with acetonitrile and analyzed by HPLC. All four bacteria were tested for beneficial effectiveness in soil including plant growth promoting rhizobacteria (PGPR) parameters like ammonia production, phosphate solubilization, siderophore production, indole acetic acid (IAA) production and hydrogen cyanide (HCN) production as per available methods (Ahmad *et al.*, 2008). They were also tested for degrading enzyme pectinase activity as per the method described in literature (Garg *et al.*, 2010).

The microbial isolates were observed microscopically after gram staining and catalase test was also performed. Four microbes were isolated from imidacloprid treated soil and all the microbes were found as gram negative bacteria. During an *in vitro* study, two isolates were found better in degrading imidacloprid in soil up to 28 days compared to other two isolates. Among them, culture no. 2 (CISH Bac-2) recorded maximum degradation (59.80%) of imidacloprid followed by CISH Bac-1 (55.68%). Again on the basis of plant growth promoting and enzymatic activities, CISH Bac-2 was found most active and selected for the present investigation. DNA was extracted from this culture and evaluated on 1.2 per cent agarose gel. Isolated DNA was amplified with *16S rRNA* Specific Primer (8F and 1492R) and a single discrete PCR amplicon band of 1500 bp was observed (Fig. 1). The PCR amplicon was enzymatically purified and sequenced further. Bi-directional DNA sequencing reaction of PCR amplicon was conducted with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit. The *16S rDNA* sequence was used to carry out BLAST alignment search tool of NCBI Gene bank database under accession number JX646650.1 as per the method described (Altschul *et al.*, 1997). Based on maximum identity score, distance matrix was generated using RDP database and the Phylogenetic tree was constructed using MEGA7 software package. To study the location of genes responsible for imidacloprid degradation, plasmids were cured from *Pseudomonas* cell using standard protocol; (Schwarz *et al.*, 1989).

On the basis of fast growth and better *in vitro* degradation record with the imidacloprid treated medium, the isolate CISH Bac-2 (bacteria) was used to degrade imidacloprid in the field condition. About 1 L of 48 h old culture of CISH Bac-2 was prepared in Nutrient broth in duplicate. One set of this broth was immobilized on sterilized wheat straw and another was kept as such. Straw was dipped in the medium to stabilize the bacterial cells. In the field six mango trees were selected for the degradation experiment (two trees for three treatments). Imidacloprid (Media® 17.8 SL) was sprayed to these trees during pre-bloom stage at 0.005 per cent dose to control mango hopper. The deposition of imidacloprid to rhizosphere soil after spraying was considered for residue analysis. Two trees were maintained as control and rhizosphere soil of other four trees were treated with immobilized bacterial culture (on straw) and free cell culture (in nutrient broth) separately (90 x 10^5 CFU g^{-1} soil). Soil samples were collected in triplicate at 0, 7, 15, 21, 37, 51 and 67 days after treatment for residue analysis of imidacloprid.

After drying, the soil samples were sieved to remove exogenous materials and crushed to powder with the help of pestle and mortar. Ten gram soil (four replications for each treatment on each day) was taken in 50 mL culture tube. To it 25 mL of AR grade acetonitrile was added and extraction was done by vortexing of samples for 5 min. Samples were then sonicated for 20 min in an ultrasonic cleaner and the supernatant was filtered through Whatman No. 42 filter paper. The process was repeated with 25 mL acetonitrile and pooled extract was evaporated in a rotary vacuum evaporator to near dryness. The residues were immediately dissolved in 5 mL of HPLC grade acetonitrile for HPLC analysis. A stock solution of 400 mg L^{-1} imidacloprid was prepared by dissolving accurately weighed 10 mg of technical standard of imidacloprid (Sigma-Aldrich, Switzerland; > 98% pure) in 25 mL of HPLC grade acetonitrile. Working solutions of 1, 2 and 4 mg L^{-1} were prepared by subsequent dilution in the same solvent.

A Shimadzu make HPLC (model LC 10 ATVP) coupled with a photodiode array detector and a reverse phase µBondapak™ C-18 column (300 mm × 3.9 mm id; with 125 Å porosity and 10 µm film thickness) was used for residue analysis of Imidacloprid (Bhattacherjee 2013). Acetonitrile:water (35:65, v/v) was employed as the mobile phase with a flow-rate of 0.8 mL min^{-1}. The detector wavelength, injection volume and retention time of imidacloprid were 270 nm, 20 µL and 5.856 ± 0.253 min, respectively. The samples were filtered through a nylon membrane filter (Millipore, 0.45 mm thickness and 13 mm diameter) held in a filter holder attached to a glass syringe before injection.

Results and Discussion

Isolation and identification of imidacloprid degrading microbes from soil

The general procedure for isolating pesticide degrading microbes is isolation of microbes either from soil of pesticides manufacturing site or from areas where constant application of pesticides is done. This ensures the adoption of microbes to increasing concentration of pesticides as well as use of pesticides as the only source of carbon. This protocol was followed in the present study. Four imidacloprid degrading bacteria were isolated from treated mango orchard soil. All these bacteria were gram negative rod shaped bacteria. Using *16S rDNA* gene sequencing technique culture no. 2 or CISH Bac-2 showed 99.9 per cent similarity with *Pseudomonas mosselii* strain NG1 based on nucleotide homology and phylogenetic analysis (Fig. 2). This bacterium was characterized as catalase negative bacterium and found effective in degrading imidacloprid in soil up to 28 days (59.80%) under laboratory condition. All bacteria were tested for several activities to verify their effectiveness against various plant growth promoting rhizobacteria (PGPR) parameters. The optimum pH and temperature for the growth of CISH Bac-2 were found to be 7.0 and 35 °C, respectively. The phosphate solubilization and siderophore production activities of *P. mosselii* strain NG1 were 88.46 and 72.41 per cent, respectively. IAA production activity was also highest in *P. mosselii* strain NG1. This bacterium also possessed good pectinase (0.735 unit mL^{-1}) activity.

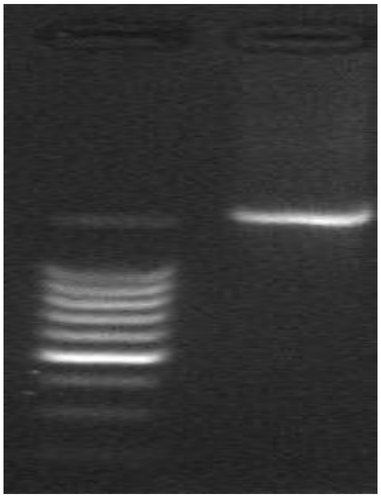
Leifsonia sp. strain PC-21 has been isolated with ability to degrade imidacloprid in the soil and identified by PCR amplification of a 500 bp sequence of 16S rRNA (Anhalt *et al.*, 2007). Similarly, *Pseudomonas sp.* strain 1G has been isolated from soil as imidacloprid degrading bacteria (Pandey *et al.*, 2009). *Ochrobacterium* sp. strain BCL-1, a gram negative rod shaped bacterium was identified from tea rhizosphere soil using 16S rRNA gene sequence with capability to degrade Imidacloprid (Hu *et al.*, 2013). Degradation of imidacloprid by *Enterobacter* sp. strain ATA1, isolated from paddy field soil at Punjab has also been reported (Sharma *et al.*, 2014). An aerobic bacterium, isolated from agriculture field soil by enrichment culture and capable of degrading imidacloprid, was identified as *Burkholderia cepacia* strain CH9 by 16S rRNA gene sequence method (Gopal *et al.*, 2011). Shetti and Kaliwal (2012) have isolated *Brevundimonas* sp. MJ15 (SP-1) as imidacloprid degrading bacteria from agricultural soil with a history of imidacloprid exposure. Three bacterial strains *Achromobacter* sp. GB 5, *Pseudomonas* sp. GB 35 and *Microbacterium* sp. GB 78 were identified to degrade imidaclopid in soil of agriculture fields of Uttarakhand, India by 16S rDNA blast method (Negi *et al.*, 2014). In clay loam soil, *Bacillus aerophilus* showed maximum potential to degrade imidacloprid (Sharma *et al.*, 2016).

Table.1 Degradation of imidacloprid by *Pseudomonas mosselii* strain NG1 in mango orchard soil (cv. Amrapali)

Days after treatment	**Immobilized *P. mosselii* in straw**	***P. mosselii* in nutrient broth**	**Control (without bacterium)**
	Residues (μg g^{-1})*	Residues (μg g^{-1})*	Residues (μg g^{-1})*
0	0.606	0.30	0.216
7	0.497	0.271	0.198
15	0.385	0.229	0.167
21	0.308	0.183	0.154
37	0.221	0.117	0.104
51	0.137	0.075	0.073
67	0.052	0.043	0.048
Regression equation	y = -0.090x + 0.678	y = -0.045x + 0.356	y = -0.029x + 0.253
R^2 value	0.996	0.990	0.982
DT_{50} (days)	8.0	15.5	24.0
Degradation (%)	91.42	85.67	77.78

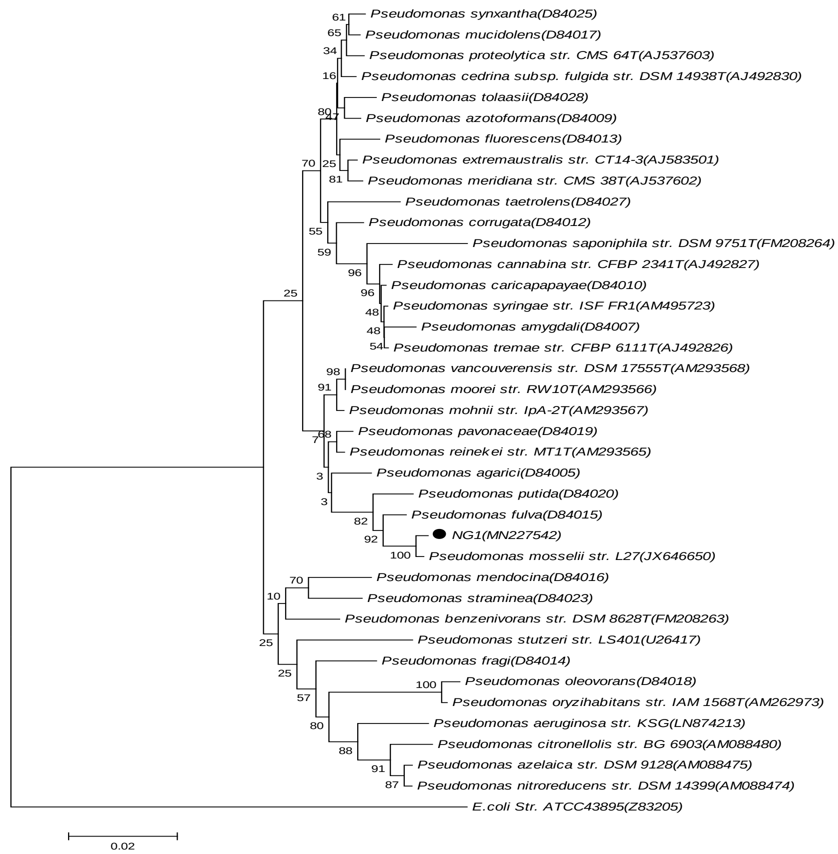
***The values are average of three replicates

Fig.1 1.2% Agarose gel showing single 1500 bp of *16S rDNA* amplicon



Lane 1: 100bp DNA ladder; Lane 2: *16S rDNA* amplicon

Fig.2 Phylogenetic tree constructed from the 16S rRNA gene of strains NG1 and related organisms constructed using neighbour-joining algorithm from an alignment of 1445 nucleotides. Accession numbers of corresponding sequences are given in parentheses, and scale bar represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1,000 replications. *E. coli* strain ATCC 43895 was taken as an out-group.



Effect of curing of plasmid on imidacloprid degradation potential of P. mosselii

To study the location of genes responsible for imidacloprid degradation, plasmids were cured from bacterial cell and the imidacloprid degrading potential was observed in cured cells. No degradation of imidacloprid was observed in *P. mosselii* cells without plasmid (5.40 µg/ml in both 0 and 15 days) and in control sample (5.50 µg/ml in 0 day to 5.40 µg/ml in 15 days – 1.82% degradation). However, slightly better degradation of imidacloprid was noticed in *P. mosselii* cells with plasmid (from 5.4 µg/ml in 0 day to 5.1 µg/ml after 15 days – 5.56% degradation). This suggests that imidacloprid degrading gene in *P. mosselii* strain NG1 is located in plasmid and not in nuclear DNA.

Microbial degradation of imidacloprid in mango orchard soil

After spraying imidacloprid at 0.005% to mango cv. Amrapali trees, rhizosphere soil (light sandy loam type) was treated with *P. mosselii* strain NG1 immobilized and or in free form to study its effect on the degradation of the insecticide. The data revealed that *P. mosselii* strain NG1 could help in degrading imidacloprid in soil in better ways. The degradation of imidacloprid was faster and higher in mango orchard soil when *P. mosselii* strain NG1 was added to it.

Residues of imidacloprid dissipated from 0.606 μg g^{-1} at 0 day (the day of application of bacteria) to 0.052 μg g^{-1} after 67 days of application resulting in 91.42 per cent degradation in soil where *P. mosselii* strain NG1 was applied with straw (Table 1). After 15 days of application the degradation was recorded as 36.47 per cent in soil, while it was 63.53 per cent after 37 days of application. When *P. mosselii* strain NG1 was applied in free form, the degradation of imidacloprid was slower (23.67, 61.00 and 85.67% after 15, 37 and 67 days of application, respectively) as compared to its application with straw. In this case imidacloprid residues dissipated from 0.30 μg g^{-1} at 0 day to 0.043 μg g^{-1} after 67 days of bacterial application. In case of control soil samples, where no bacterial culture was added, imidacloprid degraded from 0.216 μg g^{-1} at 0 day to 0.048 μg g^{-1} at 67 days after treatment (Table 1) resulting in only 77.78 per cent degradation which was much lower compared to bacterial treatment. The rate of degradation of imidacloprid followed first-order kinetics in all three cases. The half-life (DT_{50}) values, calculated from linear regression equations, were found to be 24.0, 15.5 and 8.0 days in mango orchard soil without bacterial application, with *P. mosselii* strain NG1 in free form and with *P. mosselii* strain NG1 in straw, respectively.

It was noticed from the present investigation that *P. mosselii* strain NG1 when applied in the soil as immobilized on wheat straw was more effective in degrading imidacloprid. This might be due to the stabilization effect caused by immobilization of *P. mosselii* strain NG1 in straw which helped in faster multiplication of bacterium as compared to normal condition where other soil microbes were competitive. Fibrous matrices of straw or wood chips provide adequate supporting surfaces for cell absorption due to their high specific surface area and void volume (Chu *et al.*, 2009). Better degradation rate of imidacloprid in immobilized bacterial cell might be due to the absence of internal and external mass transfer resistance. The advantage of the immobilization process included enhancing microbial cell stability, allowing continuous process operation and avoiding the biomass-liquid separation requirement as mentioned in the literature (Martins *et al.*, 2013). *Leifsonia* sp. strain PC-21 was able to degrade 35.8 per cent of imidacloprid in soil after 21 days of incubation as reported earlier (Anhalt *et al.*, 2007). In the present study, *P. mosselii* strain NG1 applied with straw and free form was able to degrade 49.17 and 39.0 per cent imidacloprid, respectively, in soil after 21 days of application. In another study, *Burkholderia cepacia* (strain CH 9) was found able to degrade 69 per cent of 50 μg g^{-1} of imidacloprid within 20 days of inoculation to a mineral-salts medium (Gopal *et al.*, 2011). *Ochrobacterium* sp. strain BCL-1 could degrade 67.67 per cent of 50 mg L^{-1} imidacloprid within 48 h of application as mentioned in literature (Hu *et al.*, 2013). The authors also noticed that the bioremediation rate of strain BCL-1 was significantly higher in tea soil from where it was isolated than in cabbage, potato and tomato soil. Degradation of imidacloprid in soil was 69.0 per cent by a consortium of 3 bacteria isolated from agricultural field soil of Uttarakhand, India after 20 days and in control 81 per cent imidacloprid remained undegraded after the same period (Negi *et al.*, 2014) which is at par with our study (77% imidacloprid remained undegraded in control soil after 21 day). Akoijam and Singh (2015) have observed that dissipation of imidacloprid followed pseudo first-order kinetics when applied at 50, 100 and 150 mg kg^{-1} in sandy loam soil amended with *Bacillus aerophilus* with respective half-life values of 14.33, 15.05 and 18.81 days. A consortium of *Bacillus aerophilus* and *Bacillus alkalinitrilicus* could degrade imidacloprid (50, 100 and 150 mg kg^{-1}) in clay loam soil at 56 days under autoclaved condition with half-life ranging from 13 to 16 days (Sharma *et al.*, 2014). The authors have also reported that *B. aerophilus* has maximum potential to degrade imidacloprid in clay loam soil after 56 days under autoclaved condition (93.45, 95.41 and 95.02% degradation from 50, 100 and 150 mg kg^{-1}, respectively) which was higher than that under unautoclaved condition (80.93, 87.57 and 85.95% degradation from respective doses) (Sharma *et al.*, 2016). In an *in vitro* study, imidacloprid was found degraded up to 97 per cent in mango orchard soil after applied at 8 mg kg^{-1} after 28 days by an unidentified strain of *Pseudomonas* sp. (Garg *et al.*, 2018).

From the present investigation it can be concluded that CISH Bac-2 or *Pseudomonas mosselii* strain NG1 has the potential to degrade imidacloprid in mango orchard soil under field condition preferably in immobilized state and therefore, can be used in farmers' fields to minimize its residues from contaminated soil. This is the first report of *P. mosselii* strain NG1 having potential to degrade imidacloprid in soil.

Acknowledgements

The authors are grateful to Council of Science & Technology, Uttar Pradesh, Lucknow, India for providing financial support of the present study in the form of a project grant vide Council's Letter No. CST/AAS/D-1542 dated 02/08/2017. The authors are also thankful to the Director, ICAR–Central Institute for Subtropical Horticulture, Rehmankhera, Lucknow for providing necessary facilities during the course of investigation.

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How to cite this article:

Anup Kr. Bhattacherjee, Neelima Garg, Pradeep Kr. Shukla, Balvindra Singh, Supriya Vaish and Abhay Dikshit. 2020. Bacterial Bioremediation of Imidacloprid in Mango Orchard Soil by *Pseudomonas mosselii* Strain NG1. *Int.J.Curr.Microbiol.App.Sci.* 9(10): 1150-1159.
doi: https://doi.org/10.20546/ijcmas.2020.910.138