



Full Length Article

Selection of *Rhizobium* spp. Isolates for Inoculation of Field Pea (*Pisum sativum*)

Çiğdem Küçük

Harran University, Science and Art Faculty, Department of Biology, Şanlıurfa, Turkey

For correspondence: cdmkucuk@yahoo.com

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Abstract

Rhizobium isolates from wild pea nodules were characterized on the basis of microbiological characteristics. P4, P7, P12, P14, P16, P19, P20, P22, P23 and P24 isolates grew at the 4.5 pH, P5, P6, P11, P12, P13, P14, P16, P17, P19, P20 and P21 isolates grew at 4% NaCl and P7, P8, P10, P11, P12, P14, P19, P20, P22, P23, P24 and P25 isolates grew at 40°C. Resistance to antibiotics ($\mu\text{g mL}^{-1}$) was investigated in a large proportion of isolates; streptomycin sulphate (80), rifampicin (40), erythromycin (30), chloramphenicol (100), Penicillin (40). In this study, local *Rhizobium* bacterial isolates were isolated from wild pea root nodules and their efficacy was investigated. Isolates significantly increased plant dry matter weight. The highest nitrogen fixation was achieved with P4 inoculation. Glutamine synthetase and leghemoglobin content of the nodules were determined in the inoculated with the highest P4 isolate. © 2021 Friends Science Publishers

Keywords: Glutamine synthetase; Nitrogen fixation; *Pisum sativum*; *Rhizobium* isolate

Introduction

Symbiotic nitrogen fixation occurs between bacteria and legumes (Bourion *et al.* 2018). These crops are an excellent source of protein and oils for human and animal consumption (Huang *et al.* 2017). In many studies, it has been explained that nodulation, yield and nitrogen content of the plant are increased with *Rhizobium* bacteria (Mishra *et al.* 2009; Rodriguez *et al.* 2014). *Rhizobium* bacterial inoculants as biofertilizers to improve soil nitrogen by biological nitrogen fixation have proven to be economically beneficial in all soils deficient of nitrogen (Riah *et al.* 2014). Appropriate isolates of *Rhizobium* bacteria to enhance effective symbiosis between the rhizobial isolates and plant on the use of amount of nitrogen fertilizer for early growth of the crops would help to improve yield (Riah *et al.* 2014). Approximately 80% of the nitrogen on land is provided by symbiotic nitrogen fixation (Fujita *et al.* 2014).

Nitrogen fixing root nodule bacteria are generally isolated from root nodules of legume plants (Peix *et al.* 2015; Bourion *et al.* 2018). The use of field pea as a forage legume in Turkey requires the introduction of the corresponding symbionts. This species has wide adaptation and produces high quality forage under grazing regimes (Huang *et al.* 2017). However, its use is currently limited by some problems. Biofertilizers are environmentally friendly, less expensive and hence lead to sustainable crop production. Field pea, being a legume crop responds to

inoculation with *Rhizobium* inoculation to meet the partial requirement of nitrogen (Mishra *et al.* 2009). Glutamine synthetase activity has an important role in nitrogen metabolism in legume root nodules (Gordon 1991). Leghaemoglobin, glutamine synthetase and sucrose synthetase contents are independent of plant growth and environmental conditions (Ceccatto *et al.* 1998; Dawood *et al.* 2019). However, very few reports are available regarding the effect of *Rhizobium* inoculation on field pea. In this study, *Rhizobium* bacteria were isolated from the root nodules of wild cowpea, the characterization and symbiotic effects of these isolates were investigated.

Materials and Methods

Experimental details and treatments

Isolation, purification and phenotypic features: *Rhizobium* isolates specific to pea were isolated from wild plants collected in non-cultivated fields of semi-arid regions of Turkey. The pink colored root nodules were selected, washed with tap water. Root nodules were then incubated in 15 s of 95% ethyl alcohol for 4 min in sodium hypochlorite for 3–5 min and then washed several times with sterile distilled water. The root nodules were crushed with forceps and the bacterial solution was inoculated into the Yeast Extract Mannitol (YEM) agar, were incubated at 28°C for 3–5 days (Jordan 1984). Selected cultures were purified on

YEM agar (Jordan 1984), colony.

Morphology, Congo red ($25 \mu\text{g mL}^{-1}$) absorption, acid reaction in YEMA containing bromthymol blue ($25 \mu\text{g mL}^{-1}$) and Gram reactions of the isolates were made and confirmed as *Rhizobium* bacteria. The isolates were transferred to the YEM broth and glycerol mixture and stored at -80°C . Sodium chloride tolerance of bacterial isolates was determined on YEM broth containing 0, 0.5, 1, 2, 3, 4 and 5% (w/v) NaCl (Küçük *et al.* 2006). Tolerance of the isolates to different temperatures (4, 15, 20, 25, 30, 37 and 40°C) was investigated in petri dishes containing Yeast extract mannitol agar (Küçük *et al.* 2006). The growth at the different pH values of the isolates was investigated. After incubation, bacterial growth was noted (Küçük *et al.* 2006). Bacterial isolates were inoculated into streptomycin sulphate (Str, $80 \mu\text{g mL}^{-1}$), rifampisin (Rif, $40 \mu\text{g mL}^{-1}$), erythromycin (Ery, $30 \mu\text{g mL}^{-1}$), chloramphenicol (Chl, $100 \mu\text{g mL}^{-1}$), Penicillin (Pen, $40 \mu\text{g mL}^{-1}$) containing Yeast extract mannitol agar (Küçük *et al.* 2006). Carbon (C) sources (10%), nitrogen sources (1%) and organic acids (1%) were added to the sterilized basal salts liquid medium by filtration ($0.45 \mu\text{m}$). The bacterial isolates were added to the medium and incubated for 5 days (Küçük *et al.* 2006). For the evaluation of carbon sources, the medium was added fructose, glucose, mannitol, sucrose, starch, dulcitol, citrate and rhamnose. For the nitrogen sources the medium was applied with mannitol and the following compounds were added L-asparagine, L-tryptophan, thymine and glycine (Küçük *et al.* 2006).

Pot experiment

The symbiotic effects of bacterial isolates on peas were evaluated in potted trials containing sterile soil. Some physical and chemical properties of soil used were analyzed according to Kacar and Katkat (2007). The healthy pea seeds were selected. The seeds were incubated in 1% sodium hypochlorite for 5 min, in 70% ethanol for 3 min and the surface sterilization was completed by washing several times with sterile distilled water. The isolates were incubated at 150 rpm, 28°C for 4 days in yeast extract mannitol broth medium. The sterilized seeds were treated with bacterial cultures (10^9 colony forming units mL^{-1}). Three seed was sown per plastic pot (20 cm diameter) and were filled with 500 g of sterilised soil.

Treatments included the 25 isolate isolated from wild pea, N application, uninoculated control (no inoculation or nitrogen) and reference isolate *Rhizobium leguminosarum*. Reference isolate was obtained from USDA. 4 kg da^{-1} NH_4NO_3 was given as nitrogen application. Triple super phosphate (as 8 kg da^{-1} P_2O_5) has been applied to all applications. Experiment was conducted under greenhouse conditions. The applications were made according to the trial design of randomized plots as three parallel. After flowering of plants, plants were harvested, nodule was determined. The plants were dried at 65°C for 24 h,

weighted and milled to determine N by Kjeldahl method (Jordan 1984). The nitrogen content (shoot dry weight x N concentration) in the plant and the fixed nitrogen (plant N content in inoculated pots) were calculated (Beck *et al.* 1993). Symbiotic activity (%SE) calculated with the following formula (Beck *et al.* 1993).

$$\text{SE (\%)} = (\text{Plant N content in inoculated pots} / \text{Plant N content in N applied}) \times 100$$

Leghaemoglobin, glutamine synthetase and nodule protein

Root nodules were homogenized with 25 mM phosphate buffer (pH 7.0) and 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The content was centrifuged for 30 min at 20 000 rpm at 4°C . The samples were then analyzed for leghaemoglobin and glutamine synthetase (Gordon 1991). Reaction mixture for glutamine synthetase 0.2 mL 250 mM Tris HCl, pH 7.2; 0.2 mL of 30 mM ATP, pH 7.0; 0.2 mL 500 mM MgSO_4 ; 0.2 mL of 300 mM glutamate (pH 7.0) and 0.25 mL of supernatant were formed. The content was read in the spectrophotometer at 540 nm (Ceccatto *et al.* 1998; Dakora *et al.* 1991). The data were expressed in $\mu\text{mole of } \gamma\text{-glutamyl-hydroxamate min}^{-1}\text{mg}^{-1}$. To determine the leghemoglobin content, the supernatant was extracted in the Drabkins solution and centrifuged at 20 000 rpm for 30 min. Then the supernatant was read on the spectrophotometer at 540 nm and the results were given as mg leghaemoglobin mg protein^{-1} (Ceccatto *et al.* 1998; Küçük 2011).

Statistical analysis

All varieties were analyzed by using JMP11 statistical program. Comparisons were done using student's t-test. Mean comparisons has been made using a least significant difference test ($P < 0.05$).

Results

Twenty-five isolates from the pea roots were isolated. The isolates showed different colony characteristics. The colony diameters vary between 0.4–3.3 mm, and they were circular, either opaque. All of the isolates were catalase positive. All retrieved isolates were Gram negative, moderately motile. The isolates varied in tolerance of pH, NaCl sensitivity and heat tolerance (Table 1). All of the isolates showed growth in media containing 0, 0.5, 1, and 1.5% NaCl (w/v). All isolates were tested for their tolerance to antibiotics (Table 2). The soil used in the study has a clay texture. The soil was slightly alkaline (pH 7.74) and other soil chemical properties are as follows; soil organic matter content (1.71%), available K (97.2 kg da^{-1}), total N (0.18%) and EC (2.45 dS m^{-1} at 25°C).

The symbiotic efficacy of local twenty-eight isolates was confirmed by plant nodulation testing (Table 3). In this study, inoculation with different local isolates showed differences in some plant characteristics such as plant height, dry nodule weight, root weight, root length biomass, total dry matter, total nitrogen and symbiotic yield. In this

Table 1: Colony size, pH, temperature and NaCl tolerance of *Rhizobium* spp. Isolates

Isolate	Colony size (mm)	Growth at pH range	Max. tolerance to		Isolate	Colony size (mm)	Growth at pH range	Max. tolerance to	
			Heat (°C)	NaCl (%)				Heat (°C)	NaCl (%)
P1	3.2	5.5-10.0	37	3.5	P14	2.1	4.5-10.0	40	4
P2	2.1	5.5-10.0	37	3.5	P15	0.6	5.5-10.0	37	3.5
P3	2.0	5.5-10.0	37	2	P16	2.9	4.5-10.0	37	4
P4	1.9	4.5-10.0	37	2	P17	2.2	5.5-10.0	37	4
P5	3.1	5.5-10.0	37	4	P18	2.3	5.5-10.0	37	1.5
P6	1.3	5.5-10.0	37	4	P19	0.4	4.5-10.0	40	4
P7	2.4	4.5-10.0	40	2	P20	1.8	4.5-10.0	40	4
P8	3.1	5.5-10.0	40	3	P21	2.1	5.5-10.0	37	4
P9	2.8	5.5-10.0	37	3	P22	1.3	4.5-10.0	40	1.5
P10	3.1	5.5-10.0	40	3.5	P23	0.8	4.5-10.0	40	2.5
P11	1.1	5.5-10.0	40	4	P24	1.0	4.5-10.0	40	1.5
P12	0.8	4.5-10.0	40	4	P25	1.3	5.5-10.0	40	1.5
P13	3.3	5.5-10.0	37	4					

Table 2: Antibiotic resistance pattern of root nodule isolates

Resistance pattern	No. of resistance isolate	%
Str, Rif, Ery, Chl, Pen	P6, P12, P14, P20, P1, P18	24
Str, Rif, Ery, Chl	P17, P5, P7, P11, P13	20
Str, Rif, Ery, Pen	P2, P4, P15, P10	16
Str, Rif, Chl, Pen	P19, P21	8
Str, Rif, Ery	P3, P7	8
Str, Rif	P8	4
Rif, Ery, Chl, Pen	P1, P9	8
Rif, Chl	P16, P24	8
Rif, Pen	P22	4
Ery, Pen	P23	4
Ery, Chl, Pen	P25	4

study, all isolates tested provided N to the plant as indicated by dry weight and total nitrogen content. All the isolates that used in the study were effective on plant dry matter compared with the control pots. The lowest values were obtained from the control treatment. However, the total dry weight was taken from native isolated isolates (P4, P10, reference isolate and P14, respectively). The glutamine synthetase and leghaemoglobin contents in the nodule extracts are shown in Fig. 1. The variance analysis of the obtained data showed that the isolates had significantly effects on shoot dry matter, total nitrogen, total symbiotic efficiency, and efficiency rates. In the application of nitrogen and inoculation with local bacteria, glutamine synthetase activity was found to be higher compared to control. Glutamine synthetase and leghaemoglobin level was found to be different among the *Rhizobium* isolates with inoculated applications (Fig. 1). Symbiotic activities of the isolates were statistically different.

Discussion

The isolated bacteria were grown in YMA medium and colony morphology was examined. All of the isolates were examined to be mobile, gram negative and rod shaped. Different between isolates were verified using some morphological properties (Küçük *et al.* 2006) and ten isolates were high produced of mucus. Küçük *et al.* (2006) explained the importance of produced mucus by bacterial

isolates in plant growth and nodulation formation. There is no salt problem in the soil from which the bacteria are isolated, whereas the isolates were tolerated in media containing 3.5 and 4% (w/v) NaCl (Table 1). The tolerance of local isolates to some stress conditions was investigated. According to the results, increased NaCl concentration (5%) adversely affected the growth of bacterial isolates. Similar observations were reported with isolates from different legumes, including field pea (Küçük *et al.* 2006; Borucki and Sujkowska 2008).

High salt tolerance is very important property for survival under the hot and dry conditions. None of the isolates grew in the medium containing 5% NaCl. These results are similar to those of Cordovilla *et al.* (1994). Abi-Ghanem *et al.* (2013) and Wielbo *et al.* (2015) found that there was a difference in the growth of *Rhizobium* isolates in salt containing environments and they reported that *Rhizobium* isolates were more tolerant to salt than the host plant. *Rhizobium* isolates were found to be more resistant to osmotic stress than legume plants (Cordovilla *et al.* 1994). It was observed in studies that show different responses to salts of different *Rhizobium* species (Abi-Ghanem *et al.* 2013). In this study, 42.8% of the isolates grew at the 4.5 pH, 39.2% of the isolates grew at 4% NaCl and 42.8% of the isolates grew at 40°C. These results are similar previous reports for different *Rhizobium* bacteria (Singleton *et al.* 1982). Although the soil temperature in semi-arid areas exceeds 40°C at 5 cm from the surface, the temperature tolerant

Table 3: Total N, percent N derived from N₂ fixation, shoot dry matter and nodulation score of field pea, inoculated with different isolates of *Rhizobium* spp.

Isolate	Shoot dry matter (g plant ⁻¹)	N %	Total N	N Fixed	Nodulation score [#]
P1	0.26g-i	3.19a	8.34e-k	4.47hi	2
P2	0.24g-i	2.23c-f	5.22kl	5.22h	2
P3	0.27f-i	2.38c-f	6.51i-l	6.51gh	1
P4	0.58ab	2.63a-e	15.26ab	15.26ab	1
P5	0.53ab	2.87a-c	15.09ab	15.09a-c	2
P6	0.35d-f	2.79a-d	9.72d-h	9.72d-g	2
P7	0.31e-h	2.33c-f	7.16g-l	7.16f-h	1
P8	0.43cd	2.57a-f	10.92d-g	10.92d-f	1
P9	0.42cd	2.68a-d	11.28c-f	11.28c-e	1
P10	0.57ab	2.61a-e	14.84a-c	14.84a-c	3
P11	0.33ef	1.49c-f	5.06f-l	7.58e-h	3
P12	0.50b	0.19c-f	0.94m	0.94 i	2
P13	0.29e-h	1.53c-f	5.00f-l	7.50e-h	1
P14	0.55ab	2.24c-f	12.36a-d	12.36b-d	2
P15	0.28e-h	1.98c-f	5.54i-l	5.54gh	3
P16	0.22h _i	2.27c-f	5.09kl	5.09h	1
P17	0.49bc	2.47c-f	12.24a-d	12.25b-d	2
P18	0.43cd	2.6a-d	11.61a-e	11.62b-d	1
P19	0.37de	2.32c-f	8.65d-i	8.65d-g	3
P20	0.22h _i	2.11c-f	4.64lm	4.64h _i	2
P21	0.32e-g	1.90c-f	6.16i-l	6.16gh	2
P22	0.23h _i	2.38c-f	5.35i-l	5.35h	1
P23	0.43cd	2.71a-d	11.58a-e	11.58b-d	3
P24	0.19 _i	1.18c-f	2.60lm	3.90 _i	2
P25	0.27g- _i	2.53a-f	6.75h-l	6.75gh	1
<i>R. leguminosarum</i>	0.57ab	2.86a-c	16.34a	16.34a	1
N	0.59a	3.13ab	18.61a	18.61a	0
Control	0.18 _i	2.11c-f	3.86lm		0
LSD	0.087	0.686	3.809	3.885	

N: NH₄NO₃, #1: very well nodulated, 2: moderately nodulated, 3: less nodulated on the roots. Different letters indicate means separation within columns.

Rhizobium bacteria have not affected the ability of nodule formation (Wielbo *et al.* 2015). High temperature resistance between *Rhizobium* bacteria is important in the selection of local isolates (Wielbo *et al.* 2015). On the other hand, Meghvansi *et al.* (2010) reported that the high temperature adversely affected the viability of *Rhizobium* bacteria. The isolates isolated from arid and warmer regions were more tolerant to temperature and drought. It was thought that the tolerances of the isolates to the drought and temperature could be related to the geographic origin (Mutch and Young 2004; Wielbo *et al.* 2015). *Rhizobium* spp., isolated from pea roots grown in West Africa, was reported to grow at 40°C (Muniz *et al.* 2017).

In general, the Turkish isolate were able to use several compounds as sole carbon sources, as reported for other *Rhizobium* bacteria (Riah *et al.* 2014). 25% of the isolates showed multiple resistances to five different antibiotics. The multiple antibiotic resistances was associated with plasmids and this was more common in Gram-negative bacteria (Küçük *et al.* 2006). This result is consistent with literature showing that *Rhizobium* bacteria are resistant to high concentration of antibiotics (Küçük *et al.* 2006). The antibiotic resistance of bacteria in genetic studies is extremely important (Riah *et al.* 2014). There are three known determinants of bacterial permeability to an antibiotics; hydrophobicity, electrical charge and size of the antibiotics (Abaidoo *et al.* 2002). Phenotypic characterization based on intrinsic antibiotic resistance has

been used both with a view to isolate identification and taxonomic classification (Riah *et al.* 2014). There are also no reports of *Rhizobium* isolates being isolated from field pea nodules in Turkey. Moreover, other studies with native isolates and pea have shown that significantly increased nodulation and symbiotic efficiency (Erman *et al.* 2009; Mishra *et al.* 2009). The interaction between bacteria and host plant is particularly important (Muniz *et al.* 2017). The results of our study revealed that the native isolates had significant effect on the plant total weight according to control (noninoculation and non-fertilizer). Besides, the isolates had significant effects on symbiotic efficiency. On the other hand, the uninoculated controls did not form any nodules. Shoot dry weight was greater with local

isolate P4 (580 mg plant⁻¹) than in the control treatment, while field pea plants inoculated by reference isolate *R. leguminosarum* gave root dry weight of 560 mg plant⁻¹. The shoot dry weight increased by 222.2% with inoculation of P4 isolate and 216.7% with inoculation of P10 isolate. It was determined that there were differences between isolates by analysis of variance. Similar results have also been investigated in studies (Mishra *et al.* 2009; Abi-Ghanem *et al.* 2013). The symbiotic performances of local isolates were compared with the reference isolates in greenhouse conditions. Inoculation with different isolates resulted in plant N accumulation of more than 180 mg N plant.

It was determined that all isolates increased the total

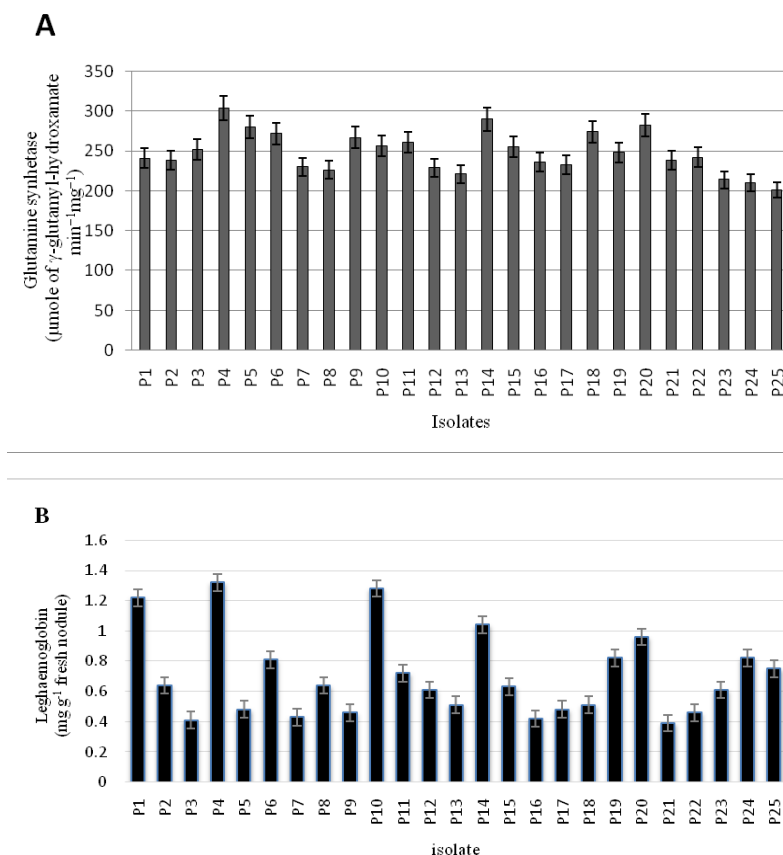


Fig. 1: Glutamine synthetase and leghaemoglobin determined in nodules of field pea

nitrogen content and dry weight of the plant and provided nitrogen to the plant. Similar evidence of increase in plant growth with *Rhizobium* inoculation has been reported (Borucki and Sujkowska 2008; Rodriguez *et al.* 2014). These results could be attributed to the development of efficient symbiosis of field pea with local isolates. These results also show that effective *Rhizobium* local isolates are more tolerant than standard isolate as observed by Bourion *et al.* (2018).

The glutamine synthetase activity (304 μmol -glutamyl-hydroxamate h^{-1} per g fresh weight) was highest in isolate P4 inoculation. Similar results were obtained by Ceccatto *et al.* (1998) for bean plants with inoculation. In a study on root nodules, it was determined that NH_3 was assimilated with the help of glutamate synthetase (GS) and NADH-dependent glutamate synthase (NADH-GOGAT) enzymes in amino acids (Gordon 1991). Cordovilla *et al.* (1994) explained that GS functions as a positive inducer of nitrogenase and those *nif* genes are controlled by GS. The control of enzyme synthesis was thought to activate the transcription of operons involved in the use of nitrogen source (Cordovilla *et al.* 1994). Leghaemoglobin levels differed between inoculations. Isolate P4, P10, P1 and P14 with inoculation showed a maximum activity (1.32, 1.28, 1.22 and 1.04 mg leghaemoglobin mg protein^{-1} , respectively).

Conclusion

In this study, leghaemoglobin, glutamine synthetase and plant growth were associated with bacterial inoculation. This can help to determine the effective isolate in nitrogen fixation similar results have also been reported by various investigators (Cordovilla *et al.* 1994; Küçük 2011). It was determined that bacterial inoculation in peas affected nodulation, dry matter weight, % nitrogen content and fixed nitrogen per plant compared to the control plant. The activities of the isolates differed from each other. However, the leghemoglobin and glutamine synthetase activities determined in root nodules of peas inoculated with different isolates were also found to be different. Therefore, the use of mineral nitrogen fertilizers, which are important in soil, water and air pollution, can be reduced by the use of *Rhizobium* isolates effective in plant cultivation. In conclusion, our *Rhizobium* isolates have an ability to fix nitrogen. In similar environmental conditions, bacteria isolate to be inoculated with peas may increase the protein content and yield of peas. However, it can be recommended to the farmer. In later studies, the bacteria will be genetically identified and the genes that play a role in the symbiotic interaction will be identified.

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Author Contributions

The author of this work has been involved in the entire work, which includes research, design, analysis and final presentation of the study.

Conflict of Interest

I declare no conflict of interest of any kind

Data Availability Declaration

All data reported in this article are available with the author and will be produced on reasonable demand

Ethics Approval

Not applicable

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