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Effect of flavonoids and lipochitooligosaccharides on nodulation of rhizobia in black gram

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Abstract
In this study the impact of flavonoids on effective nodulation of rhizobia in black gram was assessed. For this study two synthetic flavonoids *viz.*, Quercetin, Naringenin and flavonoids extracted from black gram root exudate were used to induce nodulation along with rhizobial culture in black gram. Flavonoids were extracted and purified from root exudates of black gram and confirmed through HPLC. Chromatogram obtained from black gram root exudates were coherent with quercetin peak (standard) and confirms the presence of quercetin in black gram root exudate. Studies on interaction between flavonoids and rhizobial cultures depicted that treatment Quercetin + Naringenin + *Rhizobium* sp. Recorded highest nodulation (19 numbers/plant) compared to control (6 numbers/plant) and *Rhizobium* sp. alone (11 number/plant). From the results we concluded that flavonoids such as quercetin, naringenin which is naturally present in the root exudates of rhizobial host (Black gram) play a major role in effective nodulation and act as a signalling molecule in legume rhizobial symbiosis.

Keywords: legume-rhizobia, flavonoids, LCO, nodulation

Introduction

Flavonoids are a well-described group of plant specialized metabolites, consisting of more than 10,000 different compounds (Ferrer *et al.*, 2008) [6]. It can be aerobically metabolized by bacteria such as rhizobia and *Pseudomonas*. More than 4000 different flavonoids have been identified in vascular plants and a particular sub-set of them is involved in mediating host specificity in legumes (Perret *et al*., 2000) [12]. In legume rhizobial symbiosis nodulation was induced by specific flavonoids. In the pre-infection stage, specific flavonoids released by legume roots serve as chemoattractants for the rhizobial symbiont and also activate expression of rhizobial *nod* genes. In addition to their role as chemoattractants for rhizobia in the pre-infection stage, flavonoids produced by the host plant have long been suspected to play a direct role in nodule formation (Hirsch, 1992) [8].

Flavonoid biosynthesis is typically induced in response to both biotic and abiotic stressors (Abdel-Farid *et al*., 2009) [1], and while much is known, the profiling of flavonoids in response to allelopathy is still largely lacking. Many plant species also use flavonoids as signal molecules for beneficial microorganisms in the root rhizosphere, and as antimicrobial defense compounds in their interactions with pathogenic microbes. In legumes, flavonoids also play a critical role in promoting nitrogen- fixing symbiosis with rhizobia. Legume root-exuded flavonoids act both as chemo-attractants for symbiotic rhizobia, and signalling molecules for the activation of the expression of rhizobial *nod* genes, which are responsible for the synthesis of Nod factors,the bacterial signals that are necessary for the initiation of a new plant organ, the nodule.

The first signals to be exchanged between the host plant and its rhizobial symbiont are flavonoid compounds (2-phenyl-1.4-benzopyrone derivatives) produced by plants, which in addition to other functions are required for nod gene induction (Rossen *et al*., 1985) [13]. Production of Nod-factors is activated by release of plant phenolic signals. Predominantly flavonoids, where they activate a set of nod genes in the compatible rhizobial strain (Wasson *et al*., 2009) [16]. All flavonoids consist of two benzene rings linked through a pyran or pyrone ring. Specific substitutions on the ring produce flavonols, flavones, flavonones as well as isoflavonoids which are derived from shifting of B ring from position 2 to 3. Isoflavonoids are limited to legume family. Daidzein and genistein isoflavonoids produced by soybean are effective inducers of *Bradyrhizobium japonicum* nod genes. This specificity enables rhizobia to distinguish their host from other legumes. The specific flavonoid, not only induces gene expression, but also rhizobial chemotaxis.

The nature of both the flavonoid signal and the structure of Nod - factor are central to determining legume - rhizobial specificity, thus ensuring that plant accommodates only useful friendly bacterium. Nodulation in legumes appears to have absolute requirement for Nod-factors as rhizobia incapable of synthesizing Nod - factors fail to nodulate. Hence specific flavonoids are required to synthesize Nod – factors of particular rhizobia for effective nodulation. With this information in mind the current research was conducted to assess the impact of specific flavonoids (Quercetin and Naringenine) on nodulation of black gram with *Rhizobium leguminosorum*.

Materials and Methods

Standardization of Quercetin and Naringenine concentration for Effective Nodulation

Standardization of flavonoid concentration for effective nodulation was done with different concentrations of Quercetin and Naringenine using both pot culture and seedling agar method. For pot culture study black gram (*Vigna mungo*) variety CO 6 was taken and the crop was raised during February- March in a completely randomized block design with five replications at

green house, department of agricultural microbiology, Tamil Nadu Agricultural University, Coimbatore. The treatments were given as T_1 - Quercetin (20 ppm), T_2 - Quercetin (40 ppm), T_3 - Quercetin (60 ppm), T_4 - Quercetin (80 ppm), T_5 - Quercetin (100 ppm) for Quercetin standardization. Similarly naringenin was standardized with the treatments T_1 – Naringenin (20 ppm), T_2 - Naringenin (40 ppm), T_3 - Naringenin (60 ppm), T_4- Naringenin (80 ppm), T_5- Naringenin (100 ppm). From the above concentrations best treatment was used to produce LCO (Lipo Chito Oligosaccharide) for further rhizobia-legume symbiosis study. And also combined application of both the flavonoids were tested as T_1 – Quercetin +Naringenine (20 ppm), T_2 - Quercetin +Naringenine (40 ppm), T_3 – Quercetin+ Naringenine (60 ppm), T_4- Quercetin +Naringenine (80 ppm) and T_5- Quercetin+ Naringenine (100 ppm).

Both synthetic flavonoids as well as extracted flavonoids were used for the synthsize of LCO during Rhizobia-legume symbiosis. To assess the impact of flavonoid on induction of LCO and nodulation following treatments were given; T1 – Control, T2 - Rhizobium, T3 – Quercetin + Rhizobium, T4 – Naringenin + Rhizobium, T5 - LCO + Rhizobium,T6- Root exudates + Rhizobium,T7-Quercetin+Rhizobium +Naringenin.

Induction of LCO Production with Flavonoid and Analysis through HPLC

Bacteria were grown in YEMA medium for one day. For induction of LCO, quercetin (Sigma Chemical Co. St. Louis, MO) was added to a concentration of 2µM. LCO molecules were extracted from the cell cultures by centrifugation. For large scale purification, samples were redissolved overnight in acetonitrile/water (60:40, v/v) by vigorous shaking. Collected LCO were analyzed in HPLC along with flavonoids for assessing the conversion of LCO from flavonoid.

Extraction of Flavonoid from Black Gram and Analysis through High Performance Liquid Chromatography (HPLC)

Extraction and separation of flavonoids from black gram was done. Black gram seeds were sown in plastic pots (10" x 10") containing 250 g of pot mixture by providing with Hoagland's nutrient solution with twenty five replications. After 15 days of sowing, fully developed seedlings were transferred to test tubes (covered by a thick black paper to avoid direct sunlight) containing Hoagland's nutrient solution. The solution was given at 3 days interval to maintain normal growth of the plant. After 30 days the plants were taken for extraction of flavonoids.

Extraction process of flavonoids followed Kenjerić *et al*. (2007) with minor modification. 0.5 g of the dry shoots of the tested legumes were ultrasonicated in 70% methanol at room temperature for 45 minutes, and evaporated under reduced pressure. The dry residues were dissolved in 5 ml of distilled water and partitioned with ethyl ether (3 × 5 ml). The ether extracts were combined and ether removed under the reduced pressure. At the end of the extraction process, dry residues containing flavonoid fraction were re-dissolved in 0.5 ml of methanol and analyzed using high performance liquid chromatography-mass spectrometry (HPLC-MS/MS).

The flavonoids present in the black gram plants were analyzed with HPLC (Varian prostar) after 30 days of germination. Samples were run along with the pure standards of flavonoids (Quercetin and Naringenin) for analyzing the presence or absence of them in black gram. The mobile phase in the instrument was A: deionized water: acetic acid (98:2) and B: methanol with the flow rate ml^{-1} min^{-1}. In order to obtain a full absorbance spectrum of flavonoid compounds, detection was monitored over the interval of 200-600 nm, while quantitative determinations were carried out at two wavelengths: 310 and 380 nm.

Results and Discussion

Standardization of Quercetin Concentration for Effective Nodulation in black Gram

The results revealed that the maximum shoot length, root length and biomass was observed in (25.5 cm, 12.5 cm, 0.21 g respectively) T_3 (Quercetin- 60 ppm) followed by T_4 - Quercetin (80 ppm) with 22.45 cm, 11.2 cm, 0.18 g respectively. The present result has close conformity with Sreevidya *et al*. (2006) [14] and Veitch (2007) [15]. Both studied Flavonoid production in legumes. Nodulation was increased in T_3 followed by T_4 and T_5 with 17, 15, 13 numbers/plant respectively (Table 1). The result reveals that Quercetin @ 60 ppm concentration increases the nodulation efficiency of black gram. Similar results was obtained by Hungria and Phillips (1991), studied the bean nodulation by *Rhizobium* etli or *Rhizobium tropici* was enhanced by the addition of quercetin.

Table 1: Standardization of Quercetin concentration for effective nodulation in black Gram

Treatments	Shoot length (cm)	Root length (cm)	Biomass (g)	Nodulation (No./plant)
T_1	15.25	9.3	0.15	7
T_2	17.25	8.8	0.17	11
T_3	25.50	12.5	0.21	17
T_4	22.45	11.2	0.18	15
T_5	21.35	10.8	0.16	13
Sed	0.4299	0.1953	0.0140	
CD	0.9579	0.4351	0.0312	

T_1 - Quercetin (20 ppm), T_2 - Quercetin (40 ppm), T_3 - Quercetin (60 ppm), T_4 - Quercetin (80 ppm), T_5 - Quercetin (100 ppm)

Standardization of Naringenin Concentration for Effective Nodulation in Black Gram

Pre-treatment of *Bradyrhizobium japonicum* with genistein increased nodulation and grain yield of soybean (*Glycine max*) (Zhang and Smith, 1996) [17]; and pre-induction of *R. leguminosarum* with hesperetin and naringenin was found to stimulate nodulation and plant dry matter accumulation of pea and lentil plants (Begum *et al*., 2001) [3].

The results depicted that the maximum shoot length, root length and biomass was observed in (22.5 cm, 13.5 cm, 0.21 g respectively) T_3 (Naringenin- 60 ppm) followed by T_4 (Naringenin - 80 ppm) with 20.5 cm, 10.4 cm, 0.17 g respectively. Nodulation was increased in T_3 followed by T_5 and T_4 with 15, 13, 12 number/ plant respectively (Table 2).

Table 2: Standardization of Naringenin concentration for effective nodulation in black Gram

Treatments	Shoot length (cm)	Root length (cm)	Biomass (g)	Nodulation (No./plant)
T_1	13.25	9.7	0.13	6
T_2	14.25	8.9	0.15	10
T_3	22.50	13.5	0.21	15
T_4	20.50	11.2	0.19	12
T_5	19.25	10.4	0.17	13
SEd	0.3446	0.2721	0.0101	
CD	0.7679	0.6064	0.0225	

T_1 – Naringenin (20 ppm), T_2 - Naringenin (40 ppm), T_3 - Naringenin (60 ppm), T_4- Naringenin (80 ppm), T_5- Naringenin (100 ppm).

Standardization of Quercetin + Naringenine Concentration for Effective Nodulation in Black Gram

Mixtures of flavonoids and other compounds that are exuded by plant roots are thought to act as signals that influence the ability of rhizobia to colonize the roots, survive in the rhizosphere, and affect the competitiveness of rhizobia and symbiotic interactions with legumes (Cooper, 2004) [5]. Present results concluded that the maximum shoot length was observed in T_3 - Quercetin (60 ppm) with 27.50 cm followed by T_4 - Quercetin (80 ppm) with 23.45cm and T_5 - Quercetin (100 ppm) with 22.35cm. Similarly, the root length was recorded maximum at T_3 - Quercetin (60 ppm) with 14.5cm followed by T_4 - Quercetin (80 ppm) with 11.2 cm and T_5 - Quercetin (100 ppm) with 10.8 cm. Nodulation was increased in T_3 followed by T_5 and T_4 with 19, 16, 15 number/plant respectively (Table 3).

Table 3: Standardization of Quercetin +Naringenine concentration for effective nodulation in black Gram

Treatments	Shoot length (cm)	Root length (cm)	Biomass (g)	Nodulation (No./plant)
T_1	17.30	10.2	0.14	11
T_2	18.25	9.5	0.16	10
T_3	27.50	14.5	0.24	19
T_4	23.45	11.2	0.18	15
T_5	22.35	10.8	0.16	16
Sed	0.4428	0.2671	0.0145	
CD	0.9867	0.5952	0.0322	

T_1 – Quercetin +Naringenine (20 ppm), T_2 - Quercetin +Naringenine (40 ppm), T_3 – Quercetin+ Naringenine (60 ppm), T_4- Quercetin +Naringenine (80 ppm) and T_5- Quercetin+ Naringenine (100 ppm).

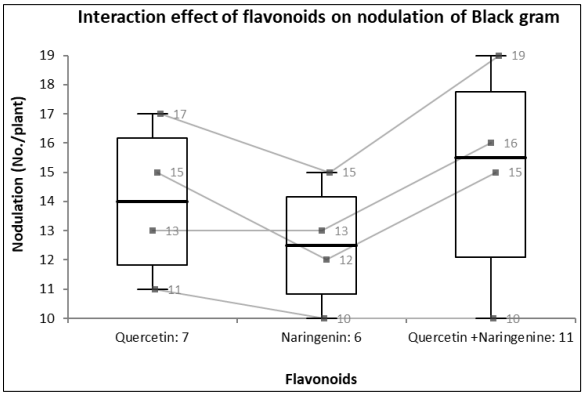

Fig 1: Single and dual application effect of synthetic flavonoids on nodulation of black gram

Above graphical representation explains that the combined application of Quercetin and Noringenin performs better compared to single application of Quercetin and Noringenin in terms of nodulation, which is the most assessing phenomenon for biological nitrogen fixation of leguminous plants

Influence of LCO for Effective Nodulation of Black Gram in Acid Soil

The results revealed that the maximum shoot length was observed in T_7 -Quercetin+*Rhizobium* + Naringenin with 16.8 cm at 15th DAS and 22.8 cm at 30th DAS followed by T_6 - Root exudates + *Rhizobium* with 15.8 cm at 15th DAS and 21.7 cm at 30th DAS. Similarly, the root length was recorded maximum at T_7 Quercetin + *Rhizobium* + Naringenin with 12.6 cm at 15th DAS and 25.1 cm at 30th DAS followed by T_6 - Root exudates + *Rhizobium* with 9.67cm at 15th DAS and 22.67 cm at 30th DAS. Recourt *et al*. (1992) [11] assess the flavonoids in uninoculated and inoculated roots of *Vicia sativa* subsp, *nigra* are four conjugates of the nodulation gene-inhibitor kaempferol. Inoculation of *Vicia sativa* subsp, *nigra* (V. *sativa)* roots with *Rhizobium leguminosarum* biovar, *viciae (R.L viciae)* bacteria substantially increases the ability of *V. sativa* to induce rhizobial nodulation *(nod)* genes.

Evidence exist that introduction of exogenous nod gene inducers increases nodulation of some legume species. Kapulnik *et al*. (1987) [10] studied the impact of luteolin on nodulation of alfalfa. The result suggested the addition of luteolin to certain alfalfa cultivars significantly increased nodulation. Maximum number of nodules was obtained from T_7 (Quercetin + Rhizobium + Naringenin) with 26 nodule /plant followed by T6- Root exudates + Rhizobium (21 nodule /plant) compared to control (6 nodule /plant) (Table 4). Because due to the addition of flavonoid, the nod D gene expression was induced and as a result nod factor LCO may be produced in the roots of legumes which is responsible for nodule formation and further morphogenesis of nodulation.

Table 4: Influence of LCO for effective nodulation of black gram in acid soil

Treatments	Shoot length (cm)		Root length (cm)		Biomass (g)		Nodulation (No./plant)
	15th	30th	15th	30th	15th	30th	30th
T_1	9.8	15.8	6.23	19.92	0.07	0.38	6
T_2	12.5	16.8	7.13	20.87	0.12	0.40	11
T_3	15.3	20.5	8.23	23.2	0.13	0.43	17
T_4	13.2	18.7	6.13	19.5	0.15	0.41	15
T_5	14.7	18.8	8.25	18.62	0.16	0.42	19
T_6	15.8	21.7	9.67	22.67	0.17	0.45	21
T_7	16.8	22.8	12.6	25.1	0.18	0.49	26
SEd	0.2431	0.3751	0.1492	0.4833	0.0133	0.0122	00
CD	0.5214	0.8046	0.3201	1.0367	0.0286	0.0262	00

T1 – Control, T2 - Rhizobium, T3 – Quercetin + Rhizobium, T4 – Naringenin + Rhizobium, T5 - LCO + Rhizobium,T6- Root exudates + Rhizobium,T7-Quercetin+Rhizobium +Naringenin

Analysis of Flavonoids and LCO through HPLC

Flavonoids extracted and separated from root leachate of black gram was analysed through HPLC. Standards of Quercetin and Naringenin were run at different concentrations. Retention time of Quercetin was near 2 minute and the sample (black gram leachate) peak also obtained near 2 minute, which confirmes the presence of Quercetin in black gram. There is no significant correlation between standard naringenin peak and black gram leachate peak indicates the absence of naringenin. The samples obtained from the LCO induction study also subjected to HPLC. The chromatogram peak was obtained before 2 minutes, indicates the convertion of LCO from flavonoids Gomaa *et al*. (2015) [7] conducted to estimate the allelopathic potential of both plant residue and root exudates of *S. oleraceus* on flavonoid composition and nodulation in a leguminous crop, *Trifolium alexandrinum*, and in two leguminous weeds, *Melilotus indicu*s and *T. resupinatum*. The results of high performance liquid chromatography-mass spectrometry (HPLC- -MS/MS) showed that all three legumes contained six flavonoid aglycones: apigenin, daidzein, kaempferol, luteolin, myricetin and quercetin; and seven flavonoid glycosides: daidzin, genistin, hesperidin, hyperoside, kaempferol-7-*O*- -glucoside, naringin and rutin

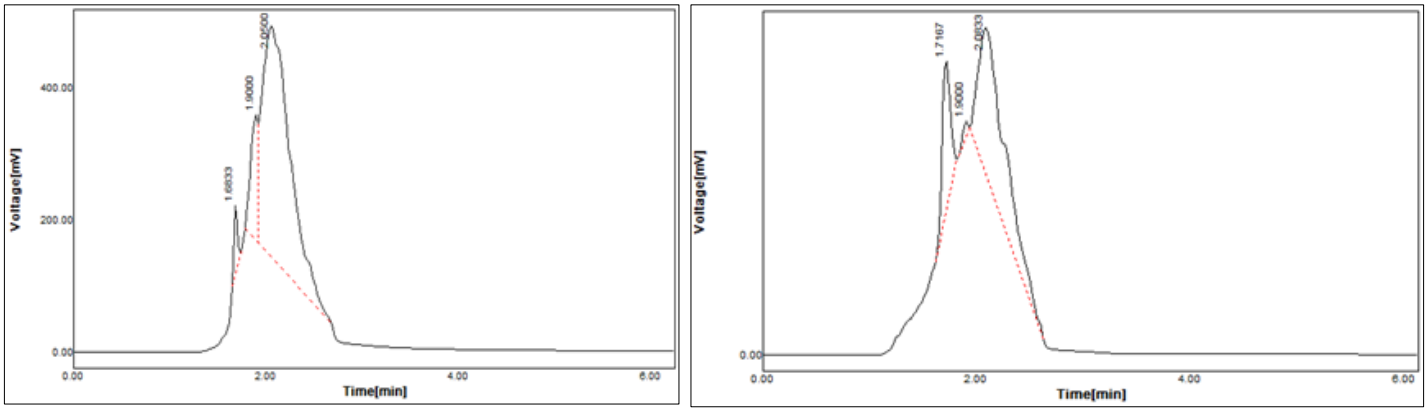

Fig 2: Chromatogram of quercetin (standard) 1b. (Black gram leachate)

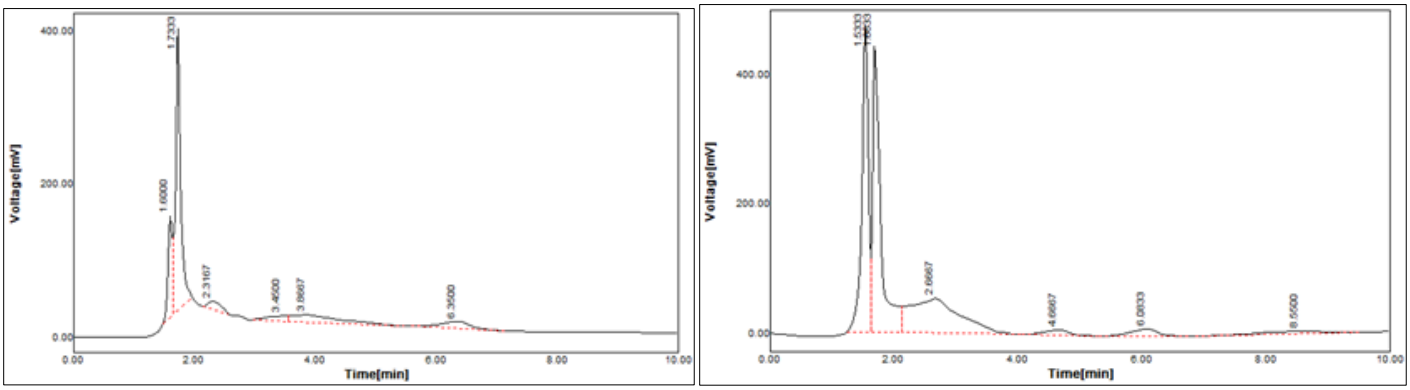

Fig 3: Chromatogram of Lipo Chito Oligosaccharide (LCO)

Conclusion

Many studies have determined types and concentrations of flavonoids in root exudates (Cesco *et al*., 2010) [4], although most of these were from plants grown in solution. The present study revealed that both plant residue and root exudates of black gram increases the nodulation efficiency of the test species. This observation is in agreement with Batish *et al*. (2007) [2], who noted the aggressive effect of *Ageratum conyzoides* residue on nodulation in chickpea. The interaction of legume plants with entire microbial communities is of particular interest for future research as we need to expand our understanding of the plant microbe interactions to the level that could help develop sustainable agriculture using the various functions of microbes on the soil. The nature of root exudation enables plants to actively regulate the rhizosphere microbial communities, and further research on legume root exudates could open the door to the possibilities of sustainable agriculture practices utilizing legume crops that actively secrete metabolites to recruit beneficial microbes and prevent pathogens.

Plate 1: Nodulation in T_7- Quercetin + Rhizobium +Naringenin

Plate 2: Nodulation in T_6- Root exudates + Rhizobium

Plate 3: Nodulation in T5- LCO + Rhizobium

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References

1. Abdel-Farid IB, Jahangir M, Hondel CAMJJ, Kim HK, Choi YH, Verpoorte R. Fungal infection-induced metabolites in *Brassica rapa*. Plant Science,2009:176:608-615.
2. Batish DR, Lavanya K, Singh HP, Kohli PK. Phenolic allelochemicals released by *Chenopodium murale* affect growth, nodulation and macromolecule content in chickpea and pea. Plant Growth Regul,2007:51:119-128.
3. Begum AA, Leibovitch S, Migner P, Zhang. Specific flavonoids induced nod gene expression and pre-activated nod genes of *Rhizobium leguminosarum* increased pea (*Pisum sativum* L.) and lentil(*Lens culinaris* L.) nodulation in controlled growth chamber environments. J.Exp.Bot,2001:52:1537–43.
4. Cesco S, Neumann G, Tomasi N, Pinton R, Weisskopf L. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant Soil,2010:329:1–25.
5. Cooper JE. Multiple responses of rhizobia to flavonoids during legume root infection. Adv Bot Res,2004:41:1–62.
6. Ferrer JL, Austin MB, Stewart C, Noel JP. Structure and function of enzymes involved in the synthesis of phenylpropanoids. Plant physiol biochem,2008:46:356-370.
7. Gomaa NH, Hassan MO, Fahmy GM, Gonzalez L, Hammouda O, Atteya AM. Allelopathic potential of *Sonchus oleraceus* L. on germination and seedling growth of crop and weed species. Acta Botanica Brasilica,2015:28:408-416.
8. Hirch AM. Developmental biology of legume nodulation. New phytologist,1992:122:211-237.
9. Hungria M, Phillips DA. Anthocyanidins and flavonols, major nod gene Inducers from seeds of a black-seeded common bean (*Phaseolus vulgaris* L.). Plant Physiol,1991:97:751–758.
10. Kapulnik Y, Joseph CM, Phillips DA. Flavone limitations to root nodulation and symbiotic nitrogen fixation in alfalfa. Plant Physiol,1987:84:1193-1196.
11. Recourt K, Verkerke M, Schripsema J, Anton AN, Ben VB, Lugtenberg JJ *et al*. Major flavonoids in uninoculated and inoculated roots of *Vicia sativa* subsp, *nigra* are four conjugates of the nodulation gene-inhibitor kaempferol. Plant Molecular Biol,1992:18:505-513.
12. Perret X, Staehelin C, Broughton WJ. Molecular basis of symbiotic promiscuity. Microbiol Mol Biol Rev,2000:64:180–201.
13. Rossen L, Shearman CA, Johnston AWB, Downie JA. The nodD gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the nod ABC genes. EMBOJ,1985:4:3369–73.
14. Sreevidya VS, Rao CS, Sullia SB, Ladha JK, Reddy PM. Metabolic engineering of rice with soybean isoflavone synthase for promoting nodulation gene expression in rhizobia. Journal of Experimental Botany,2006:57:1957-1969.
15. Veitch NC. Isoflavonoids of the Leguminosae. Natural Product Reports,2007:24:417-464.

16. Wasson AP, Pellerone FI, Mathesius U. Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and preventsauxin transport regulation by rhizobia. Plant Cell,2006:8:1617–29.
17. Zhang F, Smith DL. Inoculation of soy bean (*Glycine max*. (L.) Merr.) With genistein-pre incubated *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season condition. Plant Soil,1996:179:233–41.