ESTIMATION for THE FUNCTIONAL ACTIVITY of LIPO-OLIGOSACCHARIDE and N-ACETYL-D- GLUCOSAMINE on NODULATION of *Medicago* and *Trifolium*

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ABSTRACT

In this study, Lipo-oligosaccharide (LOS) nod factor isolated from *Sinorhizobium meliloti* and *Rhizobium trifolii* SU157 were stimulated (two and three) nodules formationon the root of their specific plants, alfalfa (*Medicago sativ*a) and white clover (*Trifolium repens*) respectively .This stimulation was clear on seedling grown on solid Nitrogen Free (NF) medium supplemented with (1.0 – 8.0 mg/L) Lipo-oligosaccharide . These nodules were small in size but became Larger with gradual increasing of LOS Concentration and increased in number on seedlings in inoculated with specific *Rhizobium* grown on solid NF medium supplemented with LOS from *S.meliloti* (11,10,11,12 Nodules/Plant) and *R. trifolii* (15,16,15,17 Nodules/Plant), comparing with grown on solid NF medium non- supplement with LOS molecule (8,13 Nodules/Plant). Where as the high concentration (8.0 mg/L) of rhizobial nod factor ;N-acetyl – D- glucosamine (GLcNAc) stimulated nodule formation on these Legume plants (1,2 Nodules/Plant).In contrast, these nod factors were unable, in the case of incompatable system , to form these nodules on the root of both clover and alfalfa seedlings. Light microscope study indicated that these nodules were empty and free from *Rhizobium* cells. Therefore this nod. factor are responsible for development of nodules on the host plant in the absence of rhizobial bacteria . The results of the statistical analysis by 3-way ANOVA of these values are significant at the level of potential alpha- 0.05 for alfalfa and clover plants.

INTODUCTION

The interaction between plants in the legume family and *Rhizobium* bacteria results in the development of a root nodule a new organ that is the site for bacteria and plant to cooperate in the fixation and assimilation of nitrogen(Mylona *et al*.,1995). In the case of alfalfa (*Medicago sativa*) *Sinorhizobium* cause initial developmental responses at least in the two distinct host cell types . Distortion of apical tips growth in epidermal hair cells which becomes sites of bacterial entry(Kijne,1992), and differentiation and mitotic activation of cells in the inner root cortex(Mylona *et al*.,1995).The ability of *Rhizobium* to cause these diverse host responses requires the function of nodulation (nod.) genes, which is required for plant invasion and host recognition(Long ,1996; Gage,2004).The nod genes direct the synthesis of noval signal molecules termed the lipo-oligosacchariedd (LOS) nod factors, which have a backbone of 3-5 N-acetyl glucosamine residues linked β-1,4. They also display host-specific modifications such as an N-acyl group on the non-reducing end residue and additional modifications on the reducing and non-reducing end residues or both(Denarie and Cullimore,1993). Nod factors can cause many plant responses such as root hair deformation penetration of the infection thread into

the cortical cells, expression of early nodulin genes, mitosis in

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the root cortex and the formation of nodule-like structures (Fisher and Long, 1992; Fraysse *et al*.,2002;2003). This study aimed to estimate the functional activity of LOS and N-acetyl-D- glucosamine (GLcNAc) on nodulation of alfalfa and clover legume plants .

MATERIAL AND METHODS

Plant material and growth medium: Seeds of *Medicago sative* (Alfalfa) and *Trifolium repens* (White clover) were soaked in sterile water, dehydrated for 30 sec. in 96% ethanol , and surface sterilized in 2% NaOCl for 10 min. (Petit *et al*.,1987),After 5-6 times washes in sterile water sterilized seeds were placed on the surface of agar solidified Nitrogen Free (NF) medium (Fahraeus,1957), and kept in dark (25˚C) for 24 h.and transferred to growth incubator conditions (25˚C,16h./ 8h, photoperiod 2000 Lux) for 24 h.

Bacterial strains and extraction of lipo-oligosaccharides: The fast – growing *Sinorhizobium meliloti* and *Rhizobium trifolii* SU157 (obtained from Prof. E.C. Cocking, Center for Nitrogem fixation, Univ . of Nottingham, Nottingham, UK.) were maintained on Yeast Extract Mannitol (YEM) (Vincent,1970)and Trifolii Mannitol Yeast (TMY), (Skotnicki and Rolfe, 1979)agar medium respectively. Bacterial suspension were prepared by inoculating liquid YEM or TMY medium with the rhizobial colony from an agar plate . *Rhizobium* suspensions were maintained in the dark for 3 day (27˚ C , 80 rpm) . These bacteria were harvested immediately by centerfugation (1500 rpm, 10 min.) and LOS molecules were precipitated from the supurnatant (Westphal and Jann , 1965).

Examination of Lipo-oligosccharide on plate material : The intact germinating seedlings of *Medicago sativa* and *Trifolium repens* (Two-day old seedlings) were transferred to NF medium plates (4 seedlings/ plate) supplemented with filter-sterilized rhizobial Lipo-oligosaccharides (LOS) molecules and N-acetyl-D-glucosamine (GLcNAc) From (BDH Chemical , Ltd poole England) . These materials were added before pouring (at about 50ْ C) at final concentration from : 0.0, 1.0, 2.0, 4.0, to 8.0 mg/L. In three separated experiments using each compound mentioned above, with compatable and incompatable symbiont. Plates were vertically incubated in raws at 25ْ C under a 16h. /8h. photoperiod and 2000 lux illumination .

Preparation of sections for light microscope: Specimens for light microscope examination were prepared as previously described(AL-Mallah *et al*.,1987) Thin sections (2.0 μm) of nodules were stained with 1.0% (w/v) methylene blue in 1.0% (w/v) sodium borate (5min, 22ْ C), and examined in olympus-IM photo-microscope .**Statical analysis:**The results are statical analysis by 3-Way Anova (1980، الراوي).

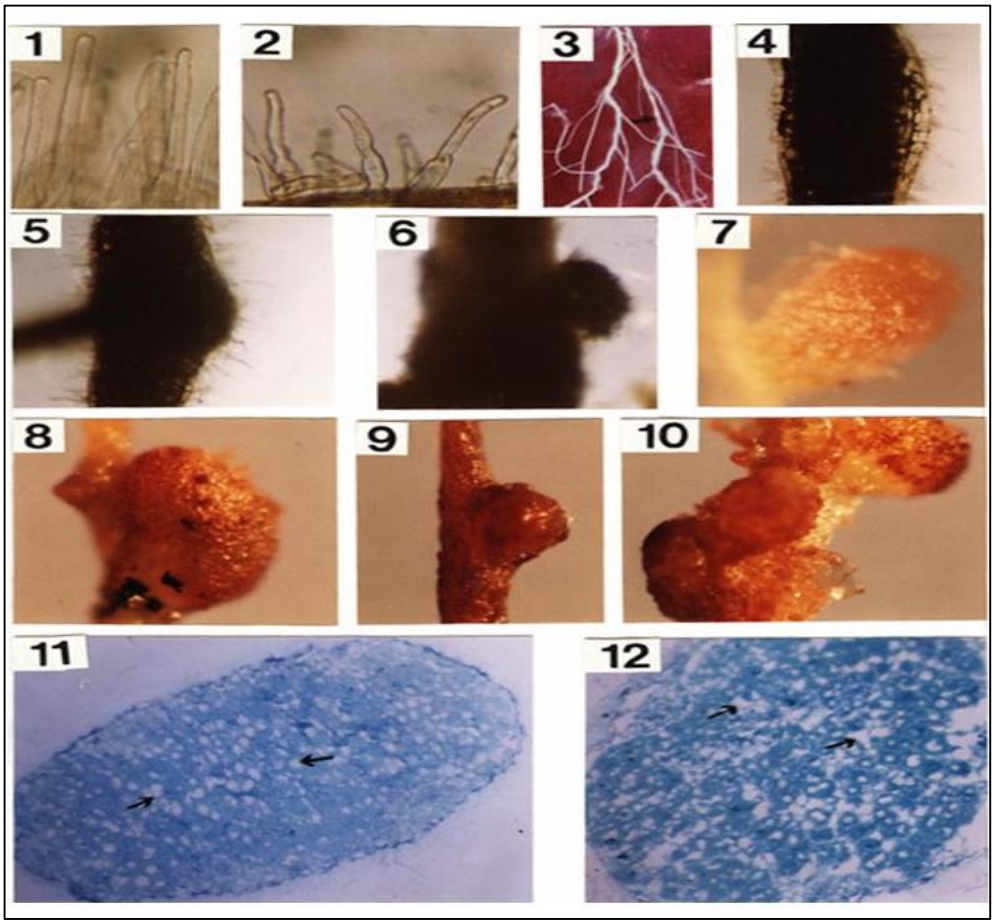
RESULTS And DISSCUSION

Within compatable system results indicate that Lipo-oligosaccharide (LOS); nod.factors are affective after five days at concentrations (1.0-8.0 mg/L) causing root hairs deformation such as curling, branching and distortion of these hair cells (Fig 1, 1&2). Thick roots are observed on seedlings of alfalfa

with the increasing concentration (Fig 1, 3&4) especially at (2.0-4.0 mg/L). And starting to form nodule morphogenesis at (1mg/L) on alfalfa seedlings by nod factor *S. meliloti* (Fig 1 , 5) at concentration (2mg/L), nodule development start on clover seedling by nod factor *R. trifolii* SU157 (Fig 1, 6). After ten days of the growth nodule primordia was small in size and became longer with gradual increasing of concentration (Fig 1, 7&8). These nodules were about 3mm in size and white in color and their number was ceased over 4weeks. Morover, supplementain of NF medium with LOS molecules derived from *S. meliloti* used to grow inoulated alfalfa seedlings was sustained the number of nodules developed this seedling and also on clover seedling cultivated at the same condition in comparsion with seedlings inoculated with specific *Rhizobium* grown on solid NF medium free from LOS, or with seedlings non-inoculated with specific bacteria grown on solid NF medium supplemented with LOS from *S. meliloti* for alfalf seedlings or from *R. trifolii* for clover seedlings (table 1). The core of rhizobial nod factor; N-acetyl - D – glucosamine (GLcNAc) tested as a control, only at higher concentration (8.0mg/L), stimulated nodules formation on these two legume plants. These nodules were developed on the same type of alfalfa and clover seedlings growing on solidified NF medium supplemented with GLcNAc (Fig 1, 9&10). On the other hand , this compound at concentration 8mg/L, increased the numbers of nodules (or nodule like structure) on alfalfa and clover seedlings after 5 days of inoculated with specific rhizobia (11 and 16 nodules/plants), respectively. In contrast , these nod factors (LOS), were unable in the case of incompatable system (non-host plants) to produce nodule even in concentration higher than the functional concentration for *S. meliloti* and *R. trifolii* SU157 signals molecule on alfalfa and clover seedlings , respectively . Light microscope studies indicated that these nodules were empty from rhizobial bacteria (Fig 1, 11&12). Furthermore, no growth of bacteria were observed when the nodules were crushed and plated on suitable medium. The above mentioned data provide that bacterial signal (nod. factor) are responsible for nodule-like response in plant host in the absence of the rhizobial bacteria. The results of the statistical analysis of the values after two and four weeks of incubation, significant at the level of potential alpha- 0.05 for plants (alfalfa and clover) in their growth on the medium of food supplemented with LOS or when inoculation with bacteria specialized for each of them and their development over the center support to this article as well as in their growth after inoculation on the medium supplementedwith GLcNAc material**.**

It seems clear that both the nod factors (from *S. meliloti* and *R. trifoliii* SU157) and the core of rhizobial nod factor (N-acetyl – D – glucosamine) molecules are the main responsible factors for eliciting nodule on legume host plants. In this invitro test system, the concentration of nod factors could affect plant response in compatable system. This may be explained by the possibility that : there are two distinct receptors; The first for low concentration which may be solely for root hairs deformation response. The second for high concentration that responsible for cortical cell division (Hirsch, 1992). The ability of nod factor at low concentrations to form nodule on their host plants was in contrast to the same concentration of N-acetyl –D- glucosamine which failed to induce nodule formation on their legume plants. This may due to the noval highly unsaturated lipid moiety and to the addition of acetyl modification

on the nod. factor molecule. Thus enhanced the formation of nodule and the production of new flavonoids invitro on the host plant(Recourt *et al.*,1991; Spaink *et al.*,1991; Geurts and Bisseling,2002). At the same time a study (Van Eijsden *et al.*,1994; Kalsi and Etzler, 2000) indicated that in such case, lectin



would coupled with the oligosaccharide part of nod.

Fig (1) **:** Functional effect of lipo-oligosaccharide and N- acetyl – D – glucosa-mine by light microscope on growth of root hairs and nodulation of axenic seedlings of *Medicago sativa* and *Trifolium repens* .

1-Normal root hairs of 4 days old alfalfa seedlings .
2-Deformation of root hairs growing on NF medium supplemented with 1 mg/L Los of *S. meliloti.*
3-Thick roots (arrow) of alfalfa seedlings growing on agar solidified NF medium containing 2 mg/L Los of *S. meliloti*
4-Close up shot of thick roots in (3).
5-Root nodule of alfalfa seedlings growing on agar solidified NF medium supplemented with 1 mg/L Los of *S. meliloti* .
6-Root nodules on clover seedlings growing on agar solidified NF medium supplemented with 2 mg/L Los of *S. trifolii* SU157.
7-Root nodule on alfalfa seedlings growing on agar solidfied NF medium supplemented with 8mg/L of los *S. meliloti* .
8-Root nodule on clover seedlings growing on agar solidified NF medium suplemented with 8 mg/L Los of *R. trifolii* SU157.

9-Root nodule on alfalfa seedlings growing on agar solidified NF medium supplemented with 8 mg/L N - acetyl –D- glucosamine.
10-Root nodule on clover seedlings growing on agar solidified NF medium supplemented with 8 mg/L N- acetyl – D – glucosamine.
11-Longitudinal section of nodules empty from *S. meliloti* (arrow) developed on alfalfa seedlings (Fig.1, 7) shown examined under light microscope.
12- Longitudinal section of nodules empty from *R . trifolii* SU157 (arrow) developed on clover seedlings (Fig.1, 8) shown examined under light microcope.

factor rather than to the lipid part. In contrast, these two compounds (LOS and GLcNAc), were incitement inoculated alfalfa and clover seedlings with specific bacteria to secrated hydrolysis and cellulose enzymes, which have a role in solving the cell wall of root hairs to enable and enter rhizobial bacteria to the root cells(Fraysse *et al*.,2002) and these increase the number of nodules formed on these legume plants. The two nod . factors produced from *S. meliloti* and *R. trifolii*, failed to form nodules on each other's host plant *Medicago sativa* and *Trifolium repens*, respectively. This appear to lie either in colonization specificity (Pellock *etal.*,2000), and in some cases, directed by the plant root signal which is only capable of inducing nod. factor in appropriate bacterial symbiont (Carlson *et al.*,1994). In the presence of the substitutent such as sulphate group on *S. meliloti* nod. factor appear to be critical for recongnition by an alfalfa receptor (Bohlool and Schmidt, 1974), not by other legume plant receptors. Moreover legume lectin possess a considerable variation in sugar binding specificity (Lerouge *et al.*,1990). Our results strongly suggest that back ground nodule of alfalfa by nod factor *S.meliloti* and white clover by nod factor *R. trifolii* , are due to the formation of empty pseudonodule, these result are similar to that result reported by some investigators (Hirsch, 1992). Further study on purification and chemical structures of these signals trigger the process of plant cell division and other responses are required.

Table (1): Effect of additions of lipo-oligosaccharide (LOS) and N-acetyl-D-glucosamine (GLcNAc) to nitrogen free medium on nodulation of alfalfa (*Medicago sativa*) and clover (*Trifolium repens*) seedlings with or without specific rhizobia.

Treatments	No.of nodules/plant (after 2 weeks)			Max. No. of nodules/plant (after 4 weeks)	
Rhizobium *	Concentration (mg/L)	Alfalfa	Clover	Alfalfa	Clover
	0.0	8	13	10	15
LOS	1.0	2	3	2	3
	2.0	2	3	3	3
	4.0	2	3	3	4
	8.0	2	3	4	4
**Rhizobium* + LOS	1.0	11	15	12	15
	2.0	10	16	12	16
	4.0	11	15	12	16
	8.0	12	17	15	18
GLcNAc	1.0	0.0	0.0	0.0	0.0

	2.0	0.0	0.0	0.0	0.0
	4.0	0.0	0.0	0.0	0.0
	8.0	1	2	1	3
**Rhizobium* + GLcNAc	1.0	9	12	10	13
	2.0	8	13	8	13
	4.0	9	12	11	15
	8.0	11	16	12	18

Rhizobium meliloti for inoculate alfalfa seedlings, *Rhizobium trifolii* SU157 specific for inoculate clover seedlings , LOS : Lipo-oligosaccharide , GLcNAc : N-acetyl - D – glucosamine.

تقديرالفعالية الوظيفية لعديد السكريات الدهنية ومركب الاسيتل كلوكوز أمين على تكوين العقد على نباتي الجت والبرسيم

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الخلاصة

لقد شجعت عوامل تكوين العقد Lipo-oligosaccharide (LOS) المعزولة من بكتريا *Sinorhizobium meliloti* وبكتريا *Rhizobium trifolii* SU157 علىتكوين عقدتين على جذور بادرات الجت *Medicago sativa* وثلاثة عقد على جذوربادرات البرسيم الابيض *Trifolium repens*النامية في وسط Nitrogen Free (NF) medium الصلب المدعم بالتراكيز (1.0-8.0 ملغم/لتر) من LOS . كانت العقد الناتجة صغيرة في احجامها وبدأت زيادتها بالحجم مع الزيادة التدريجية من LOS المضاف للوسط كما زادت اعدادها على هذه البادرات الملقحة بالبكتيريا المتخصصة لها والنامية على وسط NF الصلب المدعم بهذه المادة المعزولة من بكتيري *S.meliloti* **(** 11 ، 10 ، 11 ، 12 عقدة /نبات) والمعزولة من بكتيريا *Rhizobium trifolii* SU157 (15 ، 16 ، 15 ، 17 عقدة /نبات) .كما شجع(N-acetyl-D- glucosamine (GLcNAc عند التركيز العالي (8.0 ملغم/لتر) على تكوين العقد على جذور هذين النباتين البقوليين(1 ،2 عقدة /نبات) على التعاقب . وعلى العكس ، لم تتمكن عوامل تكوين العقد في الانظمة غير المتوافقة من تكوين العقد على جذور بادرات البرسيم والجت معاً . واظهرت فحوصات المجهر الضوئي عدم احتواء هذه العقد على بكتريا *Rhizobium* وهذا يؤكد مسؤولية هذه العوامل عن تكوين العقد في هذا العائل النباتي عند غياب بكتريا الرايزوبيوم .كما ظهرت فروق معنوية لهذه القيم عند مستوى احتمال الفا 0.05 عند تحليلها احصائيا ببرنامج تحليل التباين بثلاثة طرق.

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