Libyan Journal of Ecological & Environmental Sciences and Technology
(LJEEST)

LJEEST
ISSN: 2710-5237
www.srcest.org.ly/jou

DOI: https://doi.org/10.63359/get3w403

Nodule Morphogenesis of Various Libyan Herbaceous Legumes

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ARTICLE INFO

Vol. 3 No. 1 June, 2021
Pages (17 - 24)

Article history:
Revised form 5 March 2021
Accepted 30 March 2021

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Keywords:
Medicago polymorpha, *Medicago minima*, *Ononi*s *viscosa*, Plant roots, Nodulation, N_2-fixation, leguminous family.

ABSTRACT

This study was conducted through spring 2020 to prove the ability of various most common leguminous herbs; *Medicago polymorpha*, *Medicago minima* and *Ononis viscosa*, to observe symbiotic relationship via N_2-fixation by rhizobial bacteria. *Rhizobium meliloti* associating with *M. polymorpha* forming fan shaped nodules and *M. minima* forming finger-like shape nodules, their nodules were distributed on adventitious roots. While *Rhizobium trifolii* was associated with *O. viscosa* in rounded nodules attached to the tap root. Larger nodule number was in *O. viscosa* with average (19.3) nodules/root, while the least number was in *M. polymorpha* with an average (4.43) nodules/root, with regard to the nodule length, the longest was for *M. minima* with (2.99 cm), and the least length was for *M. polymorpha* with average (1.59 cm). Bacteroid forms resulted here were varied between bacterial species inside nodule cells. To test the nodule activity, sections of *M. polymorpha* nodules were in black color, and sections of *M. minima* nodule were in light brown color, while *O. viscose* nodule sections appear in pink color. This study proved the existence of two types of nodules (determinate and indeterminate); indeterminate nodules in *M. polymorpha* and *M. minima* elongated with apical meristem and containing different central tissues. Other type was determinate nodule in *O. viscose* lacking the existence of active meristem leading to spherical nodules containing a single, homogeneous central N_2-fixation zone.

الملخص العربي

عند دراسة تشكل وأنماط العقد المتكونة أمكن ملاحظة الشكل المروحي للعقدة عند إرتباط البكتريا *Rhizobium meliloti* مع النبات البقولي *M. polymorpha* ، وعقدة بشكل أصابع اليد في حالة النبات *M. minima* وتنشأ على الجذور الجانبية، بينما لوحظ الشكل المستدير للعقدة في حالة إرتباط البكتريا *Rhizobium. trifolii* مع النبات البقولي *O. viscosa* وتتصل بالجذر الوتدي مباشرة. إتضح أن أكبر عدد للعقد الجذرية في النبات *O. viscosa* بمتوسط 19 عقدة/للجذر ، وأقل عدد للعقد المتكونة في النبات *M. polymorpha* بمتوسط 4.43 عقدة/ للجذر، كما وجد أن أطول عقدة في النبات *M. minima* بمتوسط 2.99 سم ، وأقصر عقدة في النبات *M. polymorpha* بمتوسط 1.59 سم. وقد تباينت أشكال البكتيرويد حسب نوع البكتريا داخل خلايا العقدة. ولقياس نشاط العقد تباينت ألوان مقاطعها من الأسود الداكن إلى البني الفاتح إلى الوردي. أثبتت هذه الدراسة وجود نوعين من العقد غير محددة النمو في نباتي *M. polymorpha* و *M. minima* وكلاهما كانت متطاولة الشكل ولهما نسيج مرستيمي قمي وتحتويان على عديد من الأنسجة المركزية ، ومحددة النمو في نبات *O. viscosa* والتي تفتقر للمرستيم النشط فتظهر العقدة مستديرة تحتوي على منطقة مركزية متجانسة ذات نسيج واحد يقوم بعملية تثبيت النيتروجين.

INTRODUCTION

Fabaceae Lindy (1836: 544) or Leguminosae De Jussieu (1789: 345) is the third largest family of flowering plants, with ca. 19,500 species in 770 genera. Both names Leguminosae and Fabaceae are preserved by The International Code of Botanical Nomenclature (ICBN) (Olwey, 2019). In Flora of Libya the family is represented by 201 species and 42 genera with a few more cultivated species (Jafri & El-Gadi, 1980). The family is economically important, with food plants providing extremely nutritious protein sources and micro-nutrients that can benefit health and livelihoods and are considered to be important sources of non-animal protein, a number of species are also weedy pests in different parts of the world (Stevens, 2008). Clovers (*Trifolium* and *Medicago* spp.) are commonly cultivated as forage plants or to improve soil fertility. In addition to their medicinal value for some species and related species are the source of rotenone, which is used as a poison and insecticide. The legume family also is rich in timber species and also planted as ornamentals (Ali *et. al.* 2019). These plants in Libya are distributed in four main regions: Jabal Al Akhdar, Jabal Nafusa, the Coastal belt and in the Fezzan region, in the form of annuals and few perennials. They play an important role by supplying plant nutrients specially nitrogen, to preserve the biological diversity of the Libyan desert, fixing sand dunes, protecting the soil from erosion and preserving the vegetation cover of degraded lands, in addition to its use as fodder and pastoral crops for livestock by Libyan herders (Khalefah, 2013).

The genus *Ononis* Linnaeus (1753: 716, 1754: 321) comprises 86 species worldwide, distributed in temperate regions (Turini *et al.* 2010). Later, Willkomm (1877) subdivided *Ononis* into four sections and six subsections based on habit, leaf composition, presence or absence of spines. The *Medicago* genus, contains 83 species, is distributed mainly around the Mediterranean basin, and is a summer annual, bi-annual or perennial plant. This plant can be seen through the old world: all of Europe, North and South Africa, the islands of the Atlantic (the Canaries, Madeira) and throughout the United States (Izadpanah & Jafari, 2012). *Medicago polymorpha* L., the most common of the annual medics, grows in widely different edaphic and climatic conditions. Its distribution is limited by extreme climatic conditions prevailing in deserts, tropics and high mountainous areas. It is found throughout the Mediterranean basin, extending to the Himalayas and the highlands of east Africa. It is widely adapted to soil types surrounding the Mediterranean basin (Loi *et. al.* 1995).

The most important interrelationship between species, is the symbiotic relationship between legumes and rhizobacteria, where the plant takes advantage of N_2 fixed by bacteria while the organic carbon of the host plant transmitted to the bacteria, despite this, there is little knowledge about the type of compounds that transmitted between plants and bacteria, as well as the foundations that control the specialty of infection and the ways in which bacterial nodules form on the roots of plants (Alexander, 1982). Atmospheric nitrogen fixation is intended to convert it from the inert free-state to a bound state in a chemical compound. Biological fixation of atmospheric nitrogen is limited to prokaryotic organisms which is capable to fixing it in its cells, because they possesses nitrogenase enzyme. (Khalefah, 2013). It is estimated that 44 to 66 million tonnes of atmospheric nitrogen is fixed annually by legume symbioses which is almost half of the amount that is used in agriculture (Giller, 2001).

The symbiosis of rhizobium and legumes results in the formation of N_2-fixing root nodules, which can have determinate or indeterminate nodule growth. Determinate nodules lose their meristem at an early stage of development. By contrast, indeterminate legume nodules have a persistent meristem at their apices by which they add cells to the different nodule tissues throughout their lifetime (Hadri et al., 1998). Nodule formation as well as the infection process is controlled by specific lipochito-oligosaccharides (Nod factors) that are secreted by rhizobia (Lerouge *et al.* 1990).

Rhizobium is a rod shape, motile, gram negative bacteria, non-spore forming live in the soil, grows well in the laboratory on Manitol yeast extract medium, utilizes many carbohydrates as a source of carbon often produces acid but not gas. From the agricultural point of view, the rhizobium is divided into two types: fast-growing and slow-growing, fast-growing rhizobium with a generation time of 2-4 hours, while slow-growing rhizobium has a generation time of more than 6 hours (Khalefah, 2013). Hence, the importance of symbiotic relationships between leguminous plants and rhizobium bacteria in the soil, this research was conducted to observe the ability of some leguminous herbs to form root nodules and their patterns of root formation and development of those nodules under growth conditions prevailing in the karestic lakes region in Benghazi, Libya.

MATERIALS AND METHODS

Study area

During spring 2020 from February to April, plant materials were taken from the edges of six karestic lakes in the district of Budzira, east of Benghazi. The study area formed a polygon with a perimeter of 2772 m^2 and an area of about 3.96 km^2, its coordinates between 32° 10` 23.52" - 32° 09` 5 5.76" N, and 20° 07` 13.44" - 20° 08` 57.12" E. (Fig. 1) on the coastline.

Fig 1. Destination of study area located east between Benghazi city and Kuwayfiyah in form of polygon about 3.96 km^2

Legume plants:
Three herbaceous legume plants were selected in this study and grown around the edges of karestic lakes in the study area. A total of 10 samples from each type were taken carefully, three plants from each were mounted on herbarium sheets, dried and compressed for further identification depending on the description of Libyan Flora (Jafri & El-Gadi, 1980). Rest of samples were collected in plastic bags to perform the necessary examinations for root nodules.

Nodule recognizing and examination of bacteroids
Recovering nodules from the leguminous plants, delivery of the root nodules to the laboratory, distribution of the nodules on the root system and examining nodules and bacteroids under microscope were conducted according to the method described by Somasegaran & Hoben (1985).

Etection of active N_2-fixing nodule:

The thin sections of the nodules cut off with a razor blade and floated on a drop of water on a microscope slide; a cover glass used and examined under low power (10x) and high power (40x) objectives (Somasegaran & Hoben 1985). Sections for light microscope were viewed by using a BLP 4000 Hamilton microscope. Images were photographed by a digital camera (IT-305WC).

Isolation and incubation of Rhizobacteria
A loopful of the nodule suspension streaked on a yeast-mannitol agar (YMA) plate or loading the loop with inoculums directly from nodule section to examine rhizobia for cell morphology using a simple stain (carbol fuchsin) and the Gram stain as were done by (Woomer *et al.* 2011).

Identification of Rhizobium species
Pure cultures were made and then subjected to Gram reaction. The Gram negative bacteria were further subjected to biochemical tests including catalase, oxidase, voges-Proskauer and indole tests for confirmation.

Statistical analysis

Experiment was conducted in complete randomized design (CRD), with 30 replicates, average of nodule numbers and nodule dimensions were calculated, data were subjected to ANOVA (Gomez & Gomez, 1984), Tukey's-Kramer multiple comparison test was used to assess differences between treatments ($P \leq 0.05$). The package used for analysis was NCSS 2020 Data analysis.

RESULTS

Identification of legume plants

Compressed samples of leguminous plants were compared to the description given by Libyan flora and the plants were diagnosed as follows: *Ononis viscose*, *Medicago minima* and *Medicago polymorpha* "Fig. 2,".

Identification of Rhizobium species
Diagnostic tests have demonstrated the existence of three species of nitrogen-fixing bacteria; *Rhizobium meliloti* on *M. polymorpha* and *M. minima,* while, *Rhizobium trifolii* on *O. viscose*.

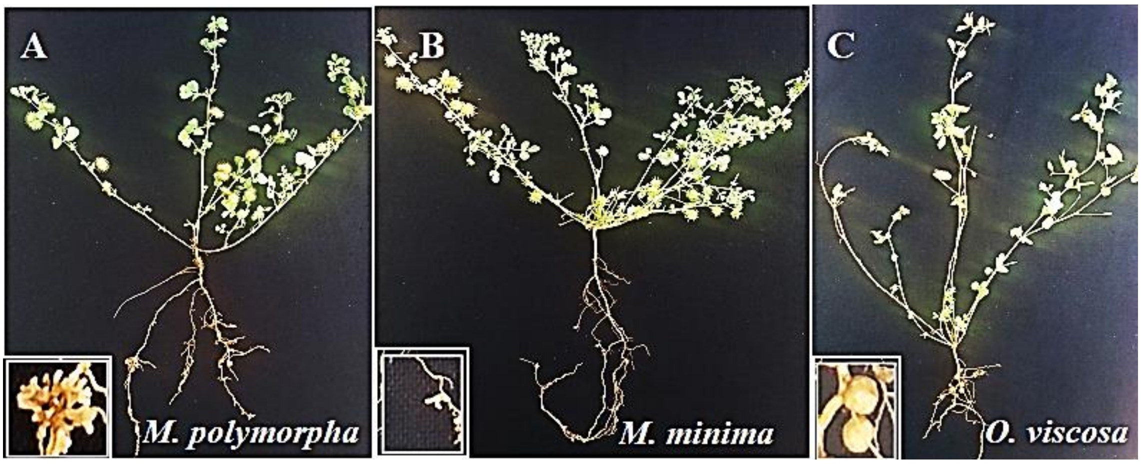
Description of nodule shape and distribution on root system
After carefully cleaning the roots of the leguminous plants, the shape and distribution of the root nodules were varied between the studied plants as following: *Medicago polymorpha* nodules were in more width takes fan shape (coralloid), lobular with whitish grey to light brown color (Table 1) "Fig. 2A,", they were distributed only on lateral roots and root hairs in multiple nodules "Fig. 2A,". *Medicago minima* nodules were observed as in finger-like shapes elongated and lobed (dichotomous) with whitish grey color (Table 1) "Fig. 2B,", nodules were distributed in a few numbers with small size on lateral roots and root hairs "Fig. 2B,". However, in the case of *O. viscose,* nodule appears completely in rounded shape with brown to olivaceous brown color (Table 1), smooth with a soft touch "Fig. 2C,", it is partly distributed on the adventitious roots and further in huge numbers on the tap root "Fig. 2C,".

Nodule number and size and bacteroid forms

Results related to the shapes and measurements of the nodes are shown in Table 1. From data below, we found that the size of nodules were vary among the various leguminous plants. The mean of nodule numbers for *M. polymorpha* (4.43) was the lower significantly compared to the nodule numbers of the other two plants. Measurements of nodule length of this specie was with average (1.59 cm) and without significant differences with *O. viscose,* but for *M. minima* the mean of nodule numbers (10.66) was higher than *M. polymorpha* significantly. Consequently, nodule length 2.99 cm for *M. minima* was the highest compared to others with significant differences. Furthermore, *O. viscose* nodule numbers were the larger with 19 nodules, it was superior significantly compared to the others. On the contrary, nodule diameter was low with average 1.69 cm insignificantly with *M. polymorpha*.

Bacteroid stage which formed after successive symbiotic relationship between rhizobial bacteria and plant cells inside nodules were detected under light microscope.

Table 2. exhibited a variation to some extent between bacteroid forms in the three legume plants. It appears that both species of *Medicago* showed a different forms, in case of *M. polymorpha* three forms (V-Y-X) have been seen, similarly *M. minima* has three bacteroid forms also (T-V-Y). On the contrary, bacteroids of *O. viscosa* was confined only to the usual forms of *rhizobium* cells; bacilli forms were observed "Fig. 3,".



"Fig 2,". Three herbaceous legume plants (*Medicago minima*, *Medicago polymorpha*, *Ononis viscosa*) grown beside Karestic lakes in the study area. Nodule distribution on; lateral roots and root hairs for *M. polymorpha* with fan shape and *M. minima* with finger-like shape, while *O. viscosa* nodules on tap root and advantage roots with rounded shape.

Table 1. Mean of nodule distinctive characteristics (shape, color, number and dimensions) for the three legume herbaceous plants (*M. polymorpha*, *M. minima* and *O. viscose*).

Legume plants	Shape	Color	average of Nodule numbers	Nodule dimensions/cm
M. polymorpha	Dictyomus	Whitish grey	4.43*	1.59 (L)*
M. minima	Lobular fan-shaped	Dark brown	10.66*	2.99 (L)**
O. viscosa	Rounded	Olivacious brown	19.3*	1.69 (D)*

*** Significant according Tukey-Kramer test, ** High significant, L- Length, D- Diameter**

Table 2. Bacteroid forms seen under microscope for *Rhizobium* spp. from legume suspension, from the three legume plants.

Legume plants	Bacteroid Forms			
	T	V	Y	X
M. polymorpha	-	+	+	+
M. minima	+	+	+	-
O. viscosa	-	-	-	-

T,V,Y,X: The observed shapes of bacteroid under microscope.

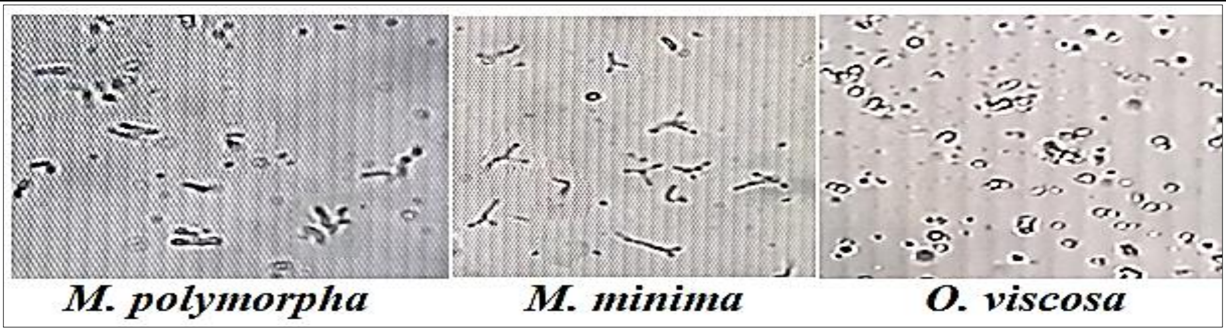


Fig 3. Bacteroid forms of *Rhizobium meliloti* bacteria were seen under microscope; (V, Y, X) forms for *M. polymorpha*, (T, V, Y) forms for *M.minima* and regular cell shapes of *Rhizobium trifolii* for *O. viscosa*.

Nodule section color as indicator to activity

Examination of nodule sections that prepared as mentioned above, achieved to prove the activity of nodules for N_2-fixation. Sections of *M. polymorpha* nodules were in black color with light pink color at the middle areas, whereas, sections of *M. minima* nodule were in light brown color, and it turned out to be that sections of *O. viscose* nodule appear in pink color in most parts "Fig. 4,".

Determinate and indeterminate nodules

When we focused on histological studies of nodule tissues for the three leguminous plants, with reference to the previous studies, longitudinal sections were described as following: there were two types of nodules; indeterminate nodules in *M. polymorpha* and *M. minima* and determinate nodule in *O. viscose*. In indeterminate nodules, as the infection threads proliferate below the meristem and enter these active cells, the infection process and differentiation of symbiotic cells occur constantly in the apical nodule region. The differentiation of the infected cells leads to regeneration of N_2-fixing cells and this increase in cell numbers leading to the enlargement of infected cells. The indeterminate nodules are elongated and contain different central tissues (zones) "Fig. 5,". These are: (i) apical meristem zone; (ii) a zone of infection and differentiation; and (iii) fixation zone; a region of plant cells filled with N_2-fixing bacteroids. The central tissues are surrounded by a cortex, which contains the nodule vasculature connected to the root stele. Other type of nodules is the determinate nodule that is distinguished *O. viscosa*; all cell division stopped rapidly and no meristem is formed. In the infected cells, bacteria divide repeatedly and host cells enlarge to host them. N_2 fixation begins in all infected cells, before cell enlargement is complete. Growth of determinate nodules is mainly dependent on the enlargement of the infected cells. When nodule differentiation is terminated, growth discontinue, leading to spherical nodules containing a single, homogeneous central N_2-fixation zone "Fig. 5,"

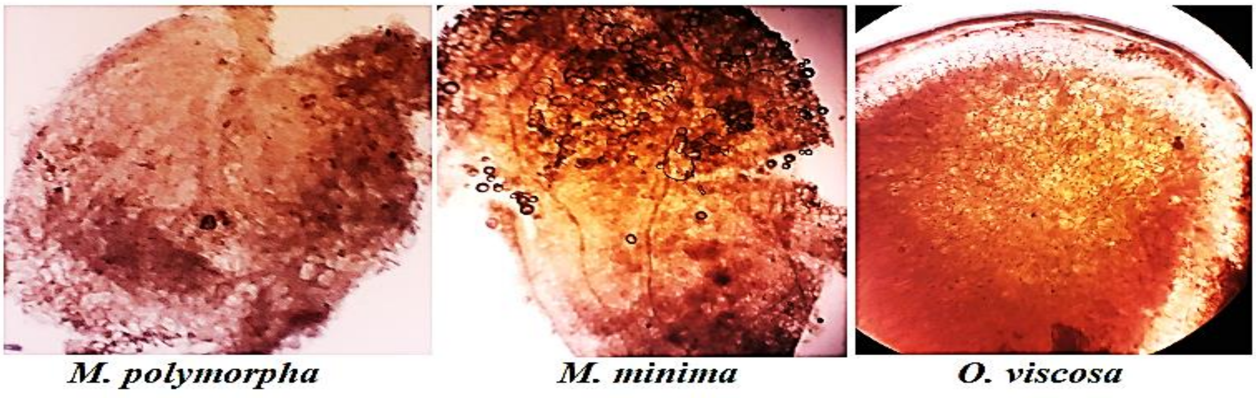


Fig 4. Nodule sections of legume plants *M. polymorpha, M. minima* and *O. viscose* under light microscope (10x), (40x) objectives.

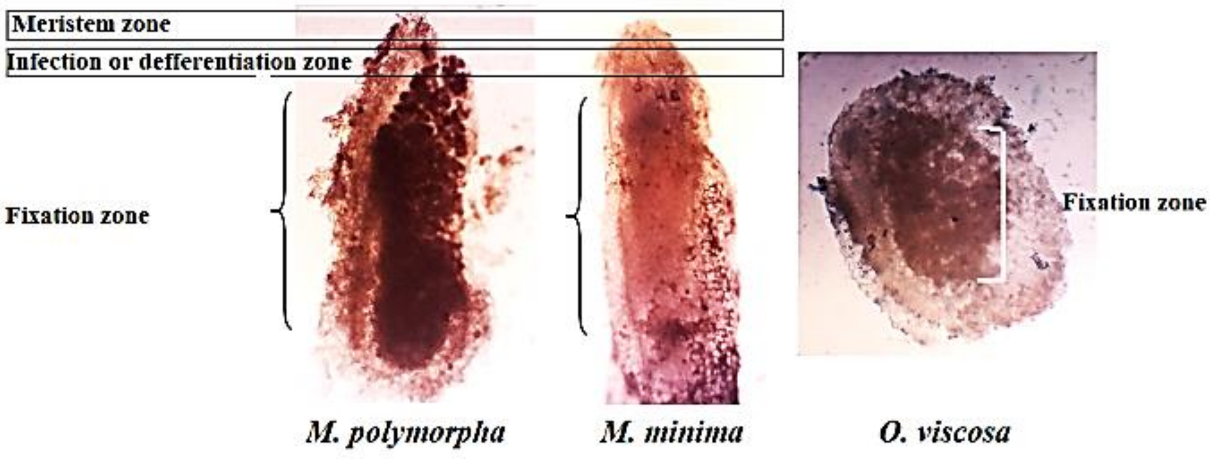


Fig 5. The two types of nodules; indeterminate elongate nodules in *M. polymorpha* and *M. minima* divided in 3 zones; meristem zone, infection zone and fixation zone. Determinate nodule in spherical shape; distinguish *O. viscose* with central fixation zone.

DISCUSSION

This study concerned three herbaceous wild plants belonging to Fabaceae, which were growing near the karestic lakes, represented in two genera *Medicago spp.* and *Ononis spp.* Ali, *et al.* (2019) pointed out in an analytical study of Fabaceae that *Medicago* genus containing 21 species and *Ononis* genus containing 12 species, among of them are the species mentioned in this study. This study demonstrated the ability of the three species to form root nodules by nitrogen-fixing *Rhizobium*.

The distribution of the nodules on the root system is dependent on the legume species and rhizobial strain as well as soil structure and composition (Woomer *et al.* 2011; Mohamed *et al.* 2018). This study proved the pivotal role of the plant species in nodule distribution, as the nodules were distributed on the tap root in *O. viscosa* and distributed on the lateral roots in *M. polymorpha* and *M. minima*. Likewise, the shapes and sizes of nodules differed by plant species, in terms of being large round, small finger or fan-shaped as mentioned in current study, this was agreed with Pommeresche & Hansen (2017) where they found that nodule size and shape can differ from one plant species to another. Large, round nodules are usually found on soybean (*Glycine max*) and faba bean (*Vicia faba*). Smaller, more elongated nodules are present on clover species (*Trifolium* spp.) and alfalfa (*Medicago*).

As a result, there were few nodules that formed on the roots, perhaps this was due to all the tested plants have tap roots, and it was found that most of the nodules were in *O. viscosa* with an average of 19.3 per plant, this was in harmony with Alexander (1982) findings where he reported that, fibrous legumes contain root nodules in greater numbers than legumes that have a tap roots.

Rhizobia inside the plant cell are called bacteroids; though located in the cytosol, their location is equivalent to the extracellular space, which are capable of N_2 fixation (Dilworth *et al.* 2008). This study proved the detection of three types of bacteroid forms inside legume cells; x-y-t and the fourth are usual forms of bacterial cells (rod shape). Based on their morphology, three distinct bacteroid morphotypes have been described (Oono *et al.*, 2010; Bonaldi *et al.*, 2011; Czernic *et al.*, 2015). These are elongated bacteroids (E-morphotype), spherical bacteroids (S-morphotype) and unmodified bacteroids (U-morphotype). The latter have a morphology similar to that of free-living bacteria (Ren, 2018).

It is useful to mention that the color of nodules can be an indication of their activity, this property was studied by examining sections of nodule tissues. The results showed that nodule colors in the leguminous plants varied from dark color to light brown to pink (Figure 4). An active N_2-fixing nodule contains a protein called leghaemoglobin. Its presence in the nodule can be noted by the characteristic pink, red, or brown coloration. Active nodules may also be black. Black nodules are not very common. They have been reported on *Lablab purpureus*, *Dolichos biflorus*, & *Vigna unguiculata* when inoculated with some strains of rhizobia (Somasegaran & Hoben, 1985). Dark coloration of the nodules and roots is due to anthocyanins present in some individuals of *Robinia pseudoacacia* species (Lotocka *et al.* 2012).

A major difference of the indeterminate nodule and determinate nodule type is the absence of a non-infected meristem in the determinate nodules. Further, the primordium cells that are penetrated by infection threads and contain released bacteria in their cytoplasm remain mitotically active during determinate nodule formation. However, they stop dividing at an early stage of development and therefore these nodules have a determinate growth type and obtain a spherical shape. In contrast, in the indeterminate nodule, primordium cells that are penetrated by an infection threads cease dividing.

The meristem is first formed from a middle cortical cell layer when the infection threads have traversed this layer. This meristem remains active throughout the lifespan of the nodule, by which they have an indeterminate growth and obtain an elongated shape. This apical meristem adds cells to all nodule tissues, including the peripheral tissues (Ren, 2018). Sharma *et al* (2020) proposed the role of exopolysaccharides in nodule function based on type, where exopolysaccharides is essentially required for the establishment of nitrogen-fixation and symbiosis in legumes with an indeterminate type of nodule, whereas plants that develop determinate nodules do not have such a requirement. The activity and also the very existence of an indeterminate meristem is controlled by multitude of factors that may be roughly divided into endogenous (i.e. transcription factors, signal peptides, phytohormones) and exogenous (growth conditions: light, temperature, nutrients availability, herbivores etc) (Lotocka *et al.* 2012).

According to Pommeresche & Hansen (2017) findings, only *Ononis viscosa* has more than 15 nodules that is often indicate higher nitrogen fixation rates, while *Medicago polymorpha* and *Medicago minima* has lower than 15 nodules. Nodules were in the upper and lower root zones in the three leguminous plants, which indicate sufficient air in deeper soil layers that may induced nodule development leading to successive nitrogen fixation. Nodules of *Ononis viscosa* were on the tap or main root only, may indicate that the bacteria try to compensate low fixation by increasing their number inside the nodules.

CONCLUSION

Our findings proved the existence of three nodule shapes; fan, finger-like, and rounded shapes they were distributed on adventitious and tap roots of the herbaceous legumes. Given the number of nodules *M. polymorpha* had fewer nodules, but *O. viscosa* had the greatest number, whilst nodule dimensions were close to each other. Sections color of nodules as indicator of N_2-fixation showed high variation between legumes due to the presence of leghaemoglobin protein; Its presence in the nodule can be noted by the characteristic pink, red, or brown coloration, active nodules may also be black. Histological studies of nodule tissues proved the presence of two types of nodules; indeterminate nodules in *M. polymorpha* and *M. minima* however, determinate nodule in *O. viscose*, their detailed description provided. In view of the low cost of these plants, as they are wild plants that are easy to propagate and grow, we need more studies to assess their efficiency by measuring the fixed nitrogen under optimal conditions to benefit from them as bio-fertilizers.

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