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Herbicides effects on symbiotic nitrogen-fixing bacteria

Učinak herbicida na bakterije simbiotske fiksatore dušika

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ABSTRACT

Rhizobia are an important component of sustainable agricultural production. In symbiosis with legumes, they provide adequate amounts of nitrogen for their growth and leave nitrogen in the soil for crops that follow in the rotation. One of the major threats to symbiotic nitrogen fixation are herbicides. The effect of herbicides on symbiotic nitrogen fixation can be positive or negative. The positive effect is manifested in the stimulation of growth and development of rhizobia. When symbiosis is negatively affected, the efficiency of nitrogen fixation is reduced, resulting in lower legume growth and yield. Herbicides can contact rhizobia immediately after application or, in the case of persistent herbicides, later in the growing season. Herbicides may affect the rhizobia, the host plant, or the establishment and development of the symbiosis. This paper reviews previous research on the effects of herbicides on rhizobia.

Keywords: herbicides, rhizobia, symbiotic nitrogen fixation

SAŽETAK

Kvržične bakterije važan su dio održive poljoprivredne proizvodnje. Uspostavom simbiotskog odnosa, osiguravaju leguminozama, kao i kulturama koje slijede u plodoredu, dostatne količine dušika za rast i razvoj. Jednu od najvećih prijetnji uspješnog uspostavljanja simbiotskog odnosa predstavljaju herbicidi. Učinak herbicida na simbiotsku fiksaciju dušika može biti pozitivan ili negativan. Pozitivan učinak očituje se u poticanju rasta i razvoja kvržičnih bakterija. U slučaju negativnog djelovanja herbicida na simbiozu, fiksacija dušika biti će reducirana što će rezultirati reduciranim rastom i prinosom leguminoza. Herbicidi mogu doći u kontakt s kvržičnim bakterijama odmah nakon primjene ili u slučaju perzistentnih herbicida, kasnije u vegetaciji. Herbicidi mogu utjecati na same kvržične bakterije, biljku domaćina ili na uspostavu i odvijanje simbioze. U radu je dan pregled dosadašnjih istraživanja utjecaja herbicida na kvržične bakterije.

Ključne riječi: herbicidi, rizobije, simbiotska fiksacija dušika

INTRODUCTION

The world population continues to increase and agriculture must keep pace with the increased demands for food and feed production. It is projected that the world population will reach about 9 billion by 2050 (UNEP, 2007). There are many challenges that pose a threat to adequate crop yields such as lack of arable land, phytophagous insects, microorganisms that cause plant diseases, weeds and other pests whose occurrence or risk of occurrence often requires the use of pesticides. Despite their usefulness, the excessive and irrational use of pesticides can pose a risk to human health, the environment and the whole organism (Özkara et al., 2016). The use of chemical fertilizers, especially nitrogen, which is the key element of plant nutrition in the combat against inadequate and reduced soil fertility, is costly. It can also lead to environmental and groundwater pollution from nitrates and air pollution from nitrous oxide (N_2O) (Kumar et al., 2019). Most legumes such as soybean (*Glycine max* L.), bean (*Phaseolus vulgaris* L.), chickpea (*Cicer arietinum* L.),

groundnut (*Arachis hypogaea* L.), and legume forage crops such as alfalfa (*Medicago sativa* L.) and clover (*Trifolium* sp.) are capable for symbiosis with their proprietary rhizobia and symbiotic nitrogen fixation, thereby obtaining fixed atmospheric nitrogen (N_2).

Rhizobia are a group of free-living soil bacteria that possess the enzyme nitrogenase, which fixes atmospheric nitrogen into ammonia (NH_3) - a form of nitrogen that can be used by plants by forming a symbiotic relationship with compatible legumes. It may be naturally present in the soil or introduced through seed inoculation treatment. Although plants are practically surrounded by nitrogen from the atmosphere, they cannot utilize N_2 directly because the triple bond in N_2 is highly resistant to changes in oxidation state. The enzyme nitrogenases are the only proteins capable of reducing N_2 to NH_3, and they are found only in nitrogen-fixing bacteria such as rhizobia (Newton, 1999). In this context, it is important to gain knowledge about the effects of herbicides on non-target organisms, especially those useful for agricultural production. If symbiotic nitrogen fixation is negatively affected, it will also negatively affect crop yields. As noted by Zahran (1999), herbicides are among the potential limiting factors that can affect symbiotic nitrogen fixation. In previous studies, herbicides were found to have the greatest impact on disrupting symbiotic fixation compared to other pesticides (Mårtensson, 1992; Madhavi et al., 1993; Zahran, 1999).

Therefore, the purpose of this paper is to review previous research that has examined the effects of herbicides on symbiotic nitrogen-fixing bacteria.

PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

Bacteria that inhabit the rhizosphere and have the ability to colonize the root system in the presence of a competing soil microflora are called rhizobacteria (Kloepper et al., 1991). They can be classified according to their effect on the roots and hence the nature of their interaction with the plant. Some exert beneficial effects on the plant while others are pathogenic (Saharan and Nehra, 2011), i.e. harmful rhizobacteria (Prashar et al., 2013). Rhizobacteria that have a beneficial effect on the plant are grouped under the name Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth, 1978; Saharan and Nehra, 2011; Prashar et al., 2013; Prasad et al., 2019). Approximately 2 - 5% of rhizospheric bacteria belong to PGPR (Antoun and Prevost, 2005). Bacteria reported to significantly enhance plant growth include various species of *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Streptomyces*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Flavobacterium*, *Burkholderia*, *Bradyrhizobium*, *Mesorhizobium*, *Rhodococcus*, and *Serratia* (Tilak et al., 2005; Prashar et al., 2013). The term PGPR was coined by Joe Kloepper in the late 1970s and defined by Kloepper and Schroth (1978) as "the soil bacteria that colonize the roots of plants by subsequent inoculation onto the seed and improve plant growth". PGPR directly or indirectly enhance plant growth and development by either releasing and/or modifying endogenous phytohormones for plant growth, improving the availability and uptake of essential nutrients through fixation and mobilization, or reducing the harmful effects of pathogenic microorganisms on plants (Hayat et al., 2010; Prasad et al., 2019; Singh et al., 2019). They provide an economically and environmentally favorable pathway for successful agricultural production with less use of chemical inputs. They have great potential to contribute to sustainable agricultural systems (Schippers et al., 1995). As noted by Dilnashin et al. (2020), the implementation of PGPR in agriculture can help reducing pollution, urea consumption, and soil depletion because PGPR have a critical function in soil formation and development, organic matter transformation, ecosystem balance, and bioremediation (Toal et al., 2000).

Depending on their position in the soil or roots, PGPR are divided into extracellular PGPR (ePGPR), which are located in the rhizosphere, on the rhizoplane, or in the intercellular spaces of the root cortex. They can also occur within root cells as intercellular PGPR (iPGPR), which are known for their specialized nodular structures (Gray and Smith, 2005). They promote plant growth primarily by increasing the uptake of nutrients that are difficult for the plant to absorb on its own and converting them into

absorbable and usable forms (Dobbelaere et al., 2003). Therefore, iPGPR have a unique function in an important process in soil known as symbiotic nitrogen fixation.

Symbiotic nitrogen fixation

Rhizobia or root nodule bacteria are common names for a group of medium-sized, rod-shaped, Gram-negative bacteria with the ability to form nodules on the roots of legumes (Somesagaran and Hoben, 1994), which are the best known iPGPR (Singh et al., 2019). Rhizobia are unique among soil microorganisms and other PGPR species in their ability to form N_2-fixing symbioses with legumes as symbiotic diazotrophic soil bacteria. They are facultative microsymbionts that live either symbiotically within the root nodule of the host legume (symbiotic nitrogen fixers) or outside the root nodule as part of the free-living soil microbial population (associative or free-living nitrogen fixers) (Somesagaran and Hoben, 1994; Prashar et al., 2013). Outside the root nodule, rhizobia may be present on the root surface (rhizoplane) or in the soil around and near the root surface (rhizosphere). In addition, rhizobia may be introduced by seed or soil inoculation if they are not present in the soil (Somesagaran and Hoben, 1994). The legume plant forms a symbiotic relationship with certain compatible species of rhizobia (Singh et al., 2019). An association between rhizobia and legume is highly specific, such that each strain of rhizobia has a definable host range (Perret et al., 2000). Rhizobia are a polyphyletic group of Proteobacteria (Kumar et al., 2019) and are represented by species of Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria (Beukes et al., 2019). These three classes are informally referred to as α-, β-, and γ-rhizobia (Moulin et al., 2001). According to the current taxonomic classification, 14 genera and 98 species have been identified in rhizobia belonging to different groups such as α-rhizobia (*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Ensifer*, *Phyllobacterium*, *Microvirga*, *Ochrobactrum*, *Methylobacterium*, *Devosia* and *Shinella*), β-rhizobia (in Burkholderia, Cupriavidus), and γ-rhizobia (*Pseudomonas*) (Berrada and Fikri-Benbrahim, 2014). Most legumes are compatible for symbiosis with species of α-rhizobia. However, some legumes, especially those of the genus *Mimosa*, are nodulated mainly by β-rhizobia (Aoki et al., 2013).

The symbiotic relationship manifests itself primarily in the roots of legumes, where the formation of root nodules occurs. In the nodules, the fixation of molecular atmospheric nitrogen (N_2) takes place with the help of the enzyme nitrogenase, which converts the nitrogen into a form (ammonia, NH_3) that is more usable by plants (Mylona et al., 1995). In return, the plants transport carbohydrates into the nodules, which provide energy for the rhizobia. The rhizobia use some of the carbohydrates as a source of hydrogen to convert atmospheric N_2 to NH_3 (Morrison et al., 1988).

The symbiotic relationship between rhizobia and legumes is highly specific and is controlled by the exchange of specific chemical signals between the two partners in the symbiosis. The symbiosis is controlled at the molecular level by the presence of appropriate genes (Mylona et al., 1995). Rhizobia contain specific groups of genes called nodulation genes or Nod genes, which are responsible for the formation of the bacterial signaling molecules Nod factors, which play a key role in the mutual recognition of a particular bacterial species and legumes. Activation and expression of Nod genes is critical as it enables the initiation of reactions and processes in the plant that are necessary for successful infection, nodulation and the actual symbiotic fixation of nitrogen. Two main groups of nodulation genes are required to establish effective symbiosis: nodulation genes and genes involved in nitrogen fixation. Nodulation genes (nodABC) encode enzymes responsible for Nod factor biosynthesis and secretion, while genes responsible for nitrogen fixation (nodNIF and nodFIX) contain structural genes for nitrogenase (nifHDK) - an enzyme responsible for nitrogen fixation (Laranjo et al., 2014). There are early and late Nod genes; early Nod genes encode products that are expressed before the onset of nitrogen fixation and are involved in infection and nodulation processes, while products of late Nod genes are involved in host plant interaction and nodule metabolic specialization

(Mylona et al., 1995).

Legumes as host plants produce phytochemical signals or a unique mixture of flavonoids that are released into the rhizosphere via the root system. The function of phytochemical signals is to attract compatible bacterial species and repel incompatible bacterial species present in the rhizosphere. The nodulation gene D (nodD) recognizes plant phytochemical signals and induces transcription of other Nod genes (Mylona et al., 1995). It also has the ability to bind to specific flavonoids released by host plant roots. After binding to a flavonoid, it becomes an activator of the transcription of other Nod genes that encode enzymes involved in the synthesis of the Nod factor (Fisher and Long, 1992). For example, alfalfa (*M. sativa*) produces specific flavonoids luteolin and apigenin to recruit the rhizobia *Sinorhizobium meliloti* (Peters and Long, 1988). Luteolin interacts with the constitutively expressed NodD factor, leading to the transcription of a Nod gene crucial for symbiosis. Therefore, luteolin-NodD signaling is necessary to initiate the events that lead to nitrogen-fixing symbiosis, which is beneficial to both the host plant and the bacteria (Spaink et al., 1987).

Nod factors represent the response or reaction of bacteria to plant phytochemical signals. Specialized receptors on the root of the host plant recognize bacterial Nod factors, which triggers the development of nodules on the root system (Mylona et al., 1995).

However, it has been found that herbicides can inhibit production and/or reduce total phenolics (Daniel et al., 1999; Fox et al., 2001; Fox et al., 2004; Fox et al., 2007). This is an important finding considering that the amount of phenolics produced directly correlates with the host plant's ability to signal and attract compatible rhizobia (Peters and Long, 1988; Daniel et al., 1999). Any negative change in the process of signaling inhibits symbiotic nitrogen fixation or reduces its efficiency (Fox et al., 2004). To establish host specificity, the host plant secretes a unique mixture of flavonoids into the rhizosphere that serves a dual purpose: to attract compatible species of rhizobia while opposing unfavorable species of rhizobia (Peters and Long, 1988). Phytochemical signals (flavonoids) released by host plants are specifically recognized by rhizobial nodD receptors, which are transcriptional regulators that bind DNA response elements and thus control the transcription of rhizobial nodulation (Nod) genes. The whole process is thus dependent on flavonoids.

The end products of Nod genes are Nod factors, which are response signals sent back to the host plant by the rhizobia. When specialized receptors in the root of the host plant recognize the Nod factors, root nodules begin to develop. Therefore, the temporal and chemical specificity of symbiotic signaling is critical for the establishment of symbiosis. However, in the dynamic environment of the rhizosphere, these molecules are exposed to a mixture of antagonistic and agonistic chemicals, such as herbicides. The extent to which NodD receptors are able to combat these signals in locating their symbiotic partner will most likely determine the efficiency of symbiotic nitrogen fixation (Fox et al., 2004). Only rhizobia possess these genes, whereas their expression is absent in normal soil bacteria.

After bacterial infection, the tips of the root hairs twist and trap the bacterial cells in this part (Mylona et al., 1995) and at this point the plant cell wall is decomposed (Morrison et al., 1988). After the decomposition of the cell wall, the twisting of the plasma membranes and the accumulation and incorporation of new materials into the membrane begins (Morrison et al., 1988). This leads to the formation of an entirely new structure called the infectious filament, which the bacteria use to invade the root of the host plant (Mylona et al., 1995). The infectious filament is formed by the root cells in response to bacterial infection. It first develops and spreads through the root hair cells, then invades other root cells where it begins to branch in all directions and spread the infection through the root tissue. The bacteria within the infectious filament divide intensively and constantly produce Nod factors that stimulate cell division within the root, resulting in the formation of new structures – nodules or tubercles on the root. Mylona et al. (1995) state that the onset of nodule formation is visible as early as 1 hour after Nod

factor formation. The bacteria are released into the cytoplasm of the plant cell, where they are surrounded by a peribacteroid membrane formed by the plant cell. They divide intensively and transform into a bacteroid, a form that has the ability to fix atmospheric nitrogen (Oke and Long, 1999). Synthesis of the enzyme nitrogenase takes place in these forms.

Peribacteroid membranes are involved in creating anaerobic conditions within nodules that are necessary for the nitrogen fixation process. They are also involved in preventing host responses of legumes to the presence of bacteria in the nodule cells of the root system, which is critical for the successful establishment of a symbiotic relationship between the host plant and the bacteria (Oke and Long, 1999). The process of nodulation ends with the formation of nodules on the root of legumes. Ammonia, as a product of fixation of atmospheric nitrogen, is transported by the nodules to the plant, which uses it for its own needs, while in return the host plant supplies the bacteria with 20-30% of the products of photosynthesis via the nodules (Lindemann and Glover, 2003). Rhizobia infect the root a few days after germination, and nodulation begins 12 - 14 days after sowing. Nitrogen fixation begins 18 - 21 days after infection. The period of intense fixation occurs 28 - 37 days after infection, when nodules reach their maximum size (3 - 6 mm) (Vratarić and Sudarić, 2000). The nodules are active for 50-60 days, after which they die (Vratarić and Sudarić, 2000). Lindemann and Glover (2003) indicate that small nodules are visible in the field 2 to 3 weeks after planting, depending on the legume species and conditions for germination and emergence.

For symbiotic nitrogen fixation to be effective, it is important that no part of the process described be disturbed. However, since soil is the medium for both plant growth and soil microorganisms, it can affect rhizobial populations by directly affecting their growth and indirectly affecting the host plant, thus inhibiting symbiosis (Moradi et al., 2011). If one or more factors in the soil are unfavorable, this can lead to a reduction in rhizobia, and in extreme cases even to a complete absence of activity, i.e. their disappearance from the soil. As a result, legumes can no longer use the fixed nitrogen from their symbiotic relationship with the rhizobia, but like other crops, rely on nitrogen from the soil obtained through fertilizers (Zahran, 1999). Therefore, it is important to provide favorable soil conditions for rhizobia, so that inexhaustible quantities of free atmospheric nitrogen are available to the legumes.

Abiotic or biotic stresses that reduce plant activity also reduce nitrogen fixation, i.e. rhizobial activity (Zahran, 1999; Lindemann and Glover, 2003). This may be due to salinity, unfavorable soil pH, soil nutrient deficiency, temperature fluctuations, excessive or inadequate soil moisture and the cause of plant diseases as well as fertilizer and pesticide use and other management practices such as irrigation, cultivation, tillage which may also negatively affect rhizobial activity (Zahran, 1999; Moradi et al., 2011). Herbicides, as the most commonly used pesticides (Oerke, 2006), pose a threat to the establishment and successful development of a symbiotic relationship between rhizobia and the host plant (Zahran, 1999).

INTERACTIONS BETWEEN HERBICIDES AND RHIZOBIA

Legumes often have low initial growth rates, making them poor competitors for weeds (Knott and Halila, 1988). Although herbicides are intended to protect the crop, they can be potentially phytotoxic to legumes and pose a threat to rhizobia and thus to symbiotic nitrogen fixation (Mårtensson, 1992). Excessive and improper use of herbicides can contaminate the soil environment and affect non-target organisms, especially soil microflora. Soil microorganisms are the first biota exposed to the direct and indirect effects of toxic substances introduced into the soil. Herbicides are able to penetrate the bacterial cell, disrupt metabolism and often cause the death of sensitive species of soil microflora (Cycon and Piotrowska-Seget, 2009). They can have a phytotoxic effect on plants by depriving the microorganisms of rhizodeposits.

Exposure of herbicides to legumes could be either intentional through application (weed control) or unintentional through residues of persistent molecules left over from previous treatments or drift from other crops (Mårtensson, 1992). Therefore, rhizobia may come into contact with herbicides either at the time of planting or later in the season (Malik and Tesfai, 1983). Most of the applied herbicide, in both foliar and soil applications, is retained in the top soil layer (0-15 cm) (Cycon and Piotrowska-Seget, 2009), where the number and activity of soil microorganisms is greatest (Parkin et al., 2002). In addition, seed inoculation treatment of legumes overlaps with the timing of soil herbicide application.

After sowing inoculated seeds, bacterial cells already multiply on the surface of the seed (Vratarić and Sudarić, 2000). In the same period, when the nitrogen-fixing ability of rhizobia is most needed, soil herbicide application occurs. Certainly, soil application poses a greater risk to effective symbiosis, as initial infection and nodulation may be impaired, but foliar application also appeared hazardous (Mårtensson and Nilsson, 1989; Mårtensson, 1992). For example, one of the most widely used groups of herbicides are sulfonylurea herbicides, which are sufficient for weed control at low doses (Boldt and Jacobsen, 1988; Rose et al., 2016). The mode of action of sulfonylureas targets enzymes in plants involved in the synthesis of the amino acids valine, leucine, and isoleucine, and therefore should not be toxic to organisms that do not synthetize these amino acids, i.e. mammals. However, other non-target organisms, such as soil microorganisms and rhizobia, which synthetize amino acids from carbon and nitrogen sources similar to plants, may be harmed (Boldt and Jacobsen, 1998).

Most studies state that the nature of herbicide effect depends on the physicochemical properties of herbicides, the application dose, the type of microorganisms present, the mineral and organic matter content of the soil, the mechanical composition of the soil and its moisture, temperature and pH reaction (Radivojević et al., 2007; Trimurtulu et al., 2015). Also, it is not always clear whether the herbicide directly affects the survival and function of rhizobia or whether the observed effects occur indirectly as a consequence of the general effect on plant growth and development (Walley et al., 2006).

According to Eberbach (1993), there are four mechanisms by which herbicides can affect the rhizobia-legume symbiosis: (i) directly affecting the host plant by reducing the growth and/or photosynthate supply to the rhizobia, i.e. bacteroids; (ii) affecting the growth, reproduction, and survival of the rhizobia in the soil; (iii) affecting the efficacy of the rhizobia, i.e. their ability to nodulate and form an effective symbiosis; and (iv) inhibition of the enzymes or biochemical pathways directly involved in N_2 -fixation in the bacteroids. Herbicides can negatively affect the species diversity, abundance, activity, and reproduction of rhizobia (Johnsen et al., 2001) or positively affect the growth and development of rhizobia and/or host plants directly or indirectly (Bouqard et al., 1997; Trimurtulu et al., 2015).

Rhizobia can degrade/mineralize herbicides, reducing potential phytotoxic effects on the host plant, and produce phytohormones that stimulate plant growth and help plants overcome stress caused by herbicide application. In a study by Khan et al. (2006a), some of the *Bradyrhizobium* sp. strains showed considerable production of IAA (3-indole acetic acid), which was also found by Frah et al. (2005) with *Azotobacter* sp. and *Pseudomonas* sp. This effect may be reflected in an improvement in soil fertility due to better conditions for the host plant together with an adequate amount of photosynthates and thus more effective nitrogen fixation (Trimurtulu et al., 2015). Some species of rhizobia are capable of degrading herbicides and using them as a source of energy for metabolic activities and physiological processes (Trimurtulu et al., 2015). For example, a strain of *Rhizobium* sp. isolated from an agricultural soil was found to actively degrade atrazine, providing a source of energy other than photosynthates alone (Bouquard et al., 1997). In the case of a negative effect, herbicides can reduce growth (Anderson et al., 2004) and rhizobial survival (Singh and Wright, 2002), reduce host plant recognition and signal exchange between rhizobia and host plant (Daniel et al., 1999; Fox

et al., 2001; Fox et al., 2004; Fox et al., 2007), nodulation and root hair formation (Mårtensson and Nilsson, 1989; Mårtensson 1992; Musarrat and Haseeb, 2000; Singh and Wright, 1999; Singh and Wright, 2002; Zaidi et al., 2005) and nitrogenase activity (Mårtensson and Nilsson, 1989; Mårtensson 1992; Hernandez et al., 1999; Anderson et al., 2004). In addition, herbicides can affect host plant growth through phytotoxicity by reducing its growth and development and/or its photosynthetic rate (Rennie and Dubetz, 1984; Bertholet and Clark, 1985; Sprout et al., 1992; Vidal et al., 1992; Abd-Alla et al., 2000; Singh and Wright, 1999; Singh and Wright, 2002; Zaidi et al., 2005), resulting in a reduced energy source (carbohydrates) for rhizobia and thus reduced nitrogen fixation (Zaidi et al., 2005).

The effects of herbicides depend primarily on the plant and rhizobia species, soil type, environmental conditions, and applied dose of herbicide (Singh, 2005). For this reason, research is often conducted both in the laboratory (*in vitro*) and in the field and greenhouse (in vivo), resulting in significant differences (Bollich, 1985). In the field experiment, it is possible to consider the influence of soil properties and weather conditions on the behavior of herbicides, while in the greenhouse these conditions can be controlled by specific humidity and temperature. Various parameters are used to measure performance such as dry mass of nodules, number of nodules, plant growth and development, nitrogenase activity, nitrogen content in the plant and leghemoglobin content by visual assessment of nodule cross section (Bollich, 1985).

Although field studies provide reliable results, it is important to include laboratory experiments as they provide a clearer picture of direct contact between herbicides and rhizobia. Under field conditions, many factors such as sorption to soil particles and solubility, concentration and half-life (DT_{50}) affect the bioavailability of herbicides to rhizobia (Abdel-Mallek et al., 1994).

The most common test methods in the laboratory are the paper-disc method, incorporation of herbicides into the solid nutrient medium (nutrient agar), and inoculation of rhizobia in liquid broth with the addition of herbicides (Bollich, 1985).

However, Mårtensson (1992) found that the results of studies conducted with liquid broth experiments without agar addition differed greatly from those of agar-based methods. It was concluded that the presence of agar may affect the mode of action of the herbicide under study, probably due to the complex composition, constituents and pH in agar. On the other hand, in experiments conducted with soil, the persistence and behavior of the herbicide is influenced by the adsorption and absorption of the soil and its physiochemical properties, along with many other factors such as volatilization, leaching, photodegradation, chemical degradation, and degradation by soil microorganisms, resulting in reduced exposure of rhizobia to the herbicidal compounds, which in turn has little to no effect on rhizobial growth (Bollich, 1985).

Observation of the effect of herbicides under both *in vivo* and *in vitro* conditions is necessary to separate the direct effect of the herbicide on rhizobia from the altered behavior of the herbicide under different environmental conditions as well as an effect on the host plant. Recognition of the influence of herbicides on symbiotic nitrogen fixation is extremely important in order to identify, in the case of a negative effect, the need to apply herbicides in a way that least threatens the normal function of the rhizobia or to develop herbicide-resistant strains.

Review of previous research on the effects of herbicides on rhizobia

The effects of herbicides on rhizobia may vary among species or be strain-specific. The positive and negative effects of herbicides on the symbiosis of legumes and rhizobia have been studied under *in vitro* and *in vivo* conditions. Table 1 provides an overview of previous research on the effects of herbicides on the symbiosis between rhizobia and legume host plants.

Table 1. Effects of herbicides on the establishment, growth and symbiosis of legumes and rhizobia

Herbicide	Host plant	Rhizobia	Conditions	Effect	Reference
2,4-D		*Rhizobium trifolii*	*In vitro*	Decreased growth and development.	Fletcher, 1956
	Trifolium subteraneum		*In vivo*	Decreased root dry weight, shoot dry weight and nodulation.	Eberbach and Douglas, 1989
	Medicago sativa	*Sinorhizobium meliloti*	*In vivo*	Inhibition of Nod expression.	Fox et al., 2001
	Cicer arietinum	*Mesorhizobium ciceri*	*In vivo*	Decreased root length, shoot length, chlorophyll content, seed production, nodulation and yield.	Khan et al., 2004
	Vigna radiata	*Bradyrhizobium sp. (vigna)*	*In vivo*	Decreased root growth, shoot growth, chlorophyll content, N content and grain protein.	Zaidi et al., 2005
Alachlor		*Rhizobium japonicum*	*In vitro*	Decreased growth of strains.	Malik and Tesfai, 1983
Amitrole	*Trifolium subteraneum*	*Rhizobium trifolii*	*In vivo*	Decreased plant growth, nodulation and nitrogenase activity.	Eberbach and Douglas, 1989
	Pisum sativum	*Rhizobium leguminosarum*	*In vivo*	Increased N accumulation, nitrogenase activity and nodule biomass.	Taylor, 2008
Atrazine	*Vigna radiata*	*Bradyrhizobium sp. (vigna)*	*In vivo*	Decreased root growth, shoot growth, nodule number, chlorophyll content and seed yield.	Khan et al., 2006b
		Mesorhizobium sp.	*In vitro*	Decreased growth of strains.	Drouin et al., 2010
Bentazone	*Trifolium repens*	*Rhizobium trifolii*	*In vivo*	Decreased plant growth and nodulation.	Clark and Mahanty, 1991
	Trifolium pratense	*Rhizobium leguminosarum b. v. trifolii*	*In vivo*	Root hair deformation, decreased nodule formation and plant dry weight.	Mårtensson, 1992
	Pisum sativum	*Rhizobium leguminosarum*	*In vivo*	Decreased nodulation, N content, nitrogenase activity.	Sing and Wright, 1999
	Cicer arietinum	*Mesorhizobium ciceri*	*In vivo*	Decreased root length, shoot length, plant biomass, chlorophyll content and yield.	Khan et al., 2004
Chlorsulfuron	*Medicago sativa*	*Rhizobium meliloti*	*In vivo*	Decreased N_2 fixation and plant dry weight.	Mårtensson and Nilsson, 1989
	Trifolium pratense	*Rhizobium leguminosarum b. v. trifolii*	*In vivo*	Root hair deformation, decreased nodule formation and plant dry weight.	Mårtensson, 1992
	Cicer arietinum	*Mesorhizobium ciceri*	*In vivo*	Decreased shoot biomass, root biomass, nodulation and N content.	Anderson et al., 2004
Cyanazine	*Lupinus albus*	*Bradyrhizobium sp. (lupinus)*	*In vivo*	Increased N_2 fixation and grain yield.	Pozuelo et al., 1989
Diquat	*Trifolium subteraneum*	*Rhizobium trifolii*	*In vivo*	Decreased root weight, shoot weight, nodulation and nitrogenase activity.	Eberbach and Douglas, 1989
Fluazifop	*Trifolium repens*	*Rhizobium trifolii*	*In vivo*	Decreased plant growth and nodulation.	Clark and Mahanty, 1991

Table 1. Continued

Herbicide	Host plant	Rhizobia	Conditions	Effect	Reference
Glyphosate	*Trifolium pratense*	*Rhizobium leguminosarum b. v. trifolii*	*In vivo*	Decreased nodule formation and plant dry weight.	Mårtensson, 1992
	Vigna radiata	*Bradyrhizobium sp. (vigna)*	*In vivo*	Decreased root growth, shoot growth and chlorophyll content.	Zaidi et al., 2005
	Pisum sativum	*Rhizobium leguminosarum*	*In vivo*	Increased N accumulation and specific nitrogenase activity.	Taylor, 2008
		Mesorhizobium sp.	*In vitro*	Decreased growth of strains.	Drouin et al., 2010
	Vicia faba	*Rhizobium leguminosarum b. v. viceae*	*In vivo*	Unaffected nodulation, plant growth and N_2 fixation.	Aynalem and Assefa, 2017
Isoproturon	*Cicer arietnium*	*Mesorhizobium ciceri*	*In vivo*	Decreased shoot length, plant biomass, chlorophyll content, nodule number and seed production.	Khan et al., 2004
	Vigna radiata	*Bradyrhizobium sp. (vigna)*	*In vivo*	Decreased root growth, nodule number, dry weight, chlorophyll content, seed yield and protein.	Khan et al., 2006b
Isoxaflutol	*Cicer arietinum*	*Mesorhizobium ciceri*	*In vivo*	Increased shoot growth and N accumulation.	Taylor, 2008
Linuron	*Glycine max*	*Bradyrhizobium japonicum*	*In vivo*	Decreased nodule number, nodule and plant dry weight.	Bollich, 1985
	Vicia faba	*Rhizobium leguminosarum*	*In vivo*	Decreased nodulation and N_2 fixation.	Haider et al., 1991
	Lens culinaris	*Rhizobium leguminosarum*	*In vivo*	Decreased nodulation and N_2 fixation.	Sandhu et al., 1991
	Cicer arietinum	*Mesorhizobium ciceri*	*In vivo*	Decreased chlorophyll and carotenoid content, improved nodulation and nodule dry weight.	Khan et al., 2004
		Bradyrhizobium sp.	*In vitro*	Decreased growth of strains.	Drouin et al., 2010
		Sinorhizobium sp.			
MCPA		*Rhizobium trifolii*	*In vitro*	Poor growth and development of rhizobia.	Fletcher, 1956
	Trifolium pratense	*Rhizobium leguminosarum b. v. trifolii*	*In vivo*	Root hair deformation, decreased nodule number and plant dry weight.	Mårtensson, 1992
	Pisum sativum	*Rhizobium leguminosarum*	*In vivo*	Increased N accumulation and nitrogenase activity.	Taylor, 2008
Metolachlor	*Medicago sativa*	*Sinorhizobium meliloti*	*In vivo*	Inhibition of Nod expression.	Fox et al., 2001
Metribuzin	*Lens culinaris*	*Rhizobium leguminosarum*	*In vivo*	Decreased nodulation and nitrogenase activity.	Sandhu et al., 1991
				Decreased nodulation and N_2 fixation.	Sprout et al., 1992
	Vigna radiata	*Bradyrhizobium sp. (vigna)*	*In vivo*	Decreased root growth, shoot growth, chlorophyll content, nodule number and seed yield.	Zaidi et al., 2005
				Decreased root growth, shoot growth, nodule number, chlorophyll content and seed production.	Khan et al., 2006b

Table 1. Continued

Herbicide	Host plant	Rhizobia	Conditions	Effect	Reference
Metribuzin	*Cicer arietinum*	*Mesorhizobium ciceri*	*In vivo*	Decreased shoot growth, N accumulation, nitrogenase activity and nodule biomass.	Taylor, 2008
	Pisum sativum	*Rhizobium leguminosarum*		Increased shoot growth and N accumulation.	
Oxadiazon	*Lens culinaris*	*Rhizobium leguminosarum*	*In vivo*	Decreased nodulation and N_2 fixation.	Sandhu et al., 1991
Oxyfluorfen					
Paraquat	*Trifolium repens*	*Rhizobium trifolii*	*In vivo*	Absence of growth and nodulation.	Clark and Mahanty, 1991
		Bradyrhizobium sp.	*In vitro*	Decreased growth of strains.	Drouin et al., 2010
Pendimethalin	*Glycine max*	*Bradyrhizobium japonicum*	*In vivo*	Decreased nodule dry weight, plant growth and N_2 fixation.	Bollich, 1985
		Rhizobium japonicum	*In vitro*	Decreased of rhizobial growth.	Moorman, 1986
	Lens culinaris	*Rhizobium leguminosarum*	*In vivo*	Increased nodulation and grain yield.	Yadav et al., 1990
	Cicer arietinum	*Mesorhizobium ciceri*	*In vivo*	Increased leghemoglobin content and N_2 fixation.	Pahwa and Prakash, 1992
	Medicago sativa	*Sinorhizobium meliloti*	*In vivo*	Inhibition of Nod expression.	Fox et al., 2001
Prometryne	*Cicer arietinum*	*Mesorhizobium ciceri*	*In vivo*	Increased N_2 fixation.	Kumar et al., 1981
	Pisum sativum	*Rhizobium leguminosarum*	*In vivo*	Decreased nodule size, leghaemoglobin content and nitrogenase activity.	Paromenskaya and Lebskii, 1985
				Decreased shoot growth, root growth, nodulation, photosynthesis and N content.	Singh and Wright, 1999
		Rhizobium leguminosarum	*In vitro*	Destruction of rhizobia cytoplasm and nucleous of bacteroids.	Paromenskaya and Lebskii, 1985
				Decreased rhizobial growth.	Singh and Wright, 2002
Simazine	*Cicer arietinum*	*Mesorhzizobium ciceri*	*In vivo*	Decreased nodule number and nodule fresh weight.	Kumar et al., 1981
	Lupinus albus	*Bradyrhizobium sp. (lupinus)*	*In vivo*	Decreased nitrogenase activity, nodule size, photosynthesis and grain yield.	De Felipe et al., 1987
				Increased N_2 fixation and grain yield.	Pozuelo et al., 1989
Simazine		*Bradyrhizobium sp. (lupinus)*	*In vitro*	Deformed nodule cells and decreased number of bacteroids.	De Felipe et al., 1987
Sulfentrazone	*Cicer arietinum*	*Mesorhizobium ciceri*	*In vivo*	Increased shoot growth and N accumulation.	Taylor, 2008
Sulfosulfuron	*Vigna radiata*	*Bradyrhizobium sp. (vigna)*	*In vivo*	Increased root growth, shoot growth, nodule number, chlorophyll content, seed yield and protein.	Khan et al., 2006b

Table 1. Continued

Herbicide	Host plant	Rhizobia	Conditions	Effect	Reference
Terbutryn	*Cicer arietinum*	*Mesorhizobium ciceri*	*In vivo*	Increased root growth, shoot growth, and grain yield.	Khan et al., 2004
Trifluralin	*Pisum sativum*	*Rhizobium leguminosarum*	*In vivo*	Decreased root growth, shoot growth nodulation, and N content.	Afifi and Dowidar, 1978
				Decreased nodulation and N_2 fixation.	Paromenskaya and Lebskii, 1985
		Rhizobium japonicum	*In vitro*	Decreased growth of strains.	Malik and Tesfai, 1983
	Glycine max	*Bradyrhizobium japonicum*	*In vivo*	Decreased nodulation, plant growth and N_2 fixation.	Bollich, 1985
	Trifolium subteraneum	*Rhizobium trifolii*	*In vivo*	Decreased number of nodules and nitrogenase activity.	Eberbach and Douglas, 1989
	Lupinus albus	*Bradyrhizobium sp. (lupinus)*	*In vivo*	Increased N_2 fixation.	Pozuelo et al., 1989
	Medicago sativa	*Sinorhizobium meliloti*	*In vivo*	Inhibition of Nod expression.	Fox et al., 2001

CONCLUSION

Symbiotic nitrogen fixation is one of the segments that sustainable agriculture should focus on. Through this process, plants benefit from the use of an environmentally friendly nitrogen source that also enriches the soil. However, it should be noted that any stress, whether triggered by chemical inputs or natural causes such as drought, flooding, temperature extremes, heavy metals, salinity, and nutrient deficiency stress, can have negative impact on symbiotic nitrogen fixation by limiting the host plant's ability to support a healthy relationship with the rhizobia or the rhizobia themselves. Of all the potential negative factors for symbiotic nitrogen fixation, herbicide application has the largest contribution. Therefore, when controlling weeds and applying herbicides, it is necessary to consider the effects they may have on the rhizobia and/or the host plant, now or in future years if the herbicide used is known to be persistent. It is necessary to avoid herbicides mechanism of action that may damage the susceptible host plant or rhizobia, as well as persistent herbicides that may remain in the soil for years and then damage the symbiotic relationship. Finally, the development of herbicide-resistant strains of rhizobia may be considered.

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