

Use of 2,4-dichlorophenoxyacetic acid (2,4-D) to facilitate bacterial infection of roots

F. Azam

Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan
E-mail: asim6006@fsd.comsats.net.pk

Abstract

2,4-dichlorophenoxy acetate (2,4-D) is a commonly used herbicide for the control of broad-leafed weeds in wheat, maize and rice. Its auxin-like action has been extensively exploited for induction of roots in plant tissue cultures. Over the past few years, however, 2,4-D has become focus of attention because of its potential to modify plant roots vis-à-vis bacterial infection. This role of 2,4-D has immense significance in understanding the mechanism of legume-*Rhizobium* symbiosis and extending this phenomenon to cereal crops. The need to make the plant roots harbour bacteria (particularly those capable of fixing atmospheric nitrogen) and the role of 2,4-D in facilitating the process has been reviewed.

Introduction

Nitrogen (N) is one of the most widely distributed elements in nature, with atmosphere as the main reservoir containing 99.96% of the total found in nature. Of the remaining 0.04%, biosphere contains only 0.005%. Present essentially in an inert form (legumes being the major beneficiaries through biological fixation), only a fraction of the atmospheric N is potentially available to plants. Limitations of N for crop plants have been met mainly through the application of chemical fertilizers a variety of which are now available for use in agriculture. According to statistics by FAO, about 42 million tons of fertilizer N is being used annually on a global scale for the production of 3 major cereal crops i.e., wheat, rice, and maize (17, 9 and 16 million tons, respectively). However, crop plants are able to use only 50% of the applied N, while 25% is lost from the soil-plant system through leaching (NO_3), denitrification and NH_3 volatilization; 25% being retained in soil in different organic forms. The N lost from the agroecosystems poses a serious threat to the environment and thus to biota, economic losses notwithstanding (Azam 2001, 2002). In view of the economic/environmental concerns and the fact that some plants (particularly legumes) meet their N demands through biological fixation, induction of nodulation in cereals has been a long-standing ambition of scientists. This consideration seems logical because the N_2 fixing plant systems do not require fossil fuel for their N supply and the fixed N is not as susceptible to losses as the fertilizer N.

On a global level, annual contribution of biological nitrogen fixation is estimated at 139 million tons.

The objective of inducing nodulation in cereals has met with success only to the extent that the root modifications can now be induced in cereals like wheat, rice, and maize (Ridge *et al* 1993, Kennedy and Tchan 1992, Tchan and Kennedy 1989, Kennedy and Islam *et al* 2001, Azam and Lodhi 2001) followed by enhanced bacterial colonization of roots. The most common approach used in these studies has been to expose the germinating seed to small concentrations of 2,4-D. Apparently, 2,4-D helps overcome the resistance of host cells to the entry of bacteria. This paper reviews the available information on the use of 2,4-D in modifying plant roots leading to enhanced colonization/infection by native or inoculated microorganisms.

2,4-D

It is an active ingredient in several formulations of herbicides recommended for the control of broad-leafed weeds in cereal crops including wheat, maize, and rice. Its auxin-like action has been extensively exploited for induction of roots in plant tissue cultures. 2,4-D is a very stable compound that is not subject to breakdown by plant and is effective at very low concentration (Ridge *et al* 1993). In soil, however, 2,4-D has a relatively short half-life and is rather immobile and biodegradable, average half-life being a few days. Whether applied through foliage or soil, the herbicide accumulates in the meristematic tissues leading to changes in all sorts of metabolic processes causing death to target plants (mainly

due to the abnormal metabolism of nucleic acids) and malformation of plant parts in others.

2,4-D and nodulation in legumes

It is generally believed that formation of nodules in legumes requires an exquisite coordination of gene expression of two very distinct organisms, a prokaryote (*Rhizobium*) and a eukaryote (Hirsch and LaRue, 1997). However, despite genetical studies lasting more than a decade, only fragmentary evidence has been obtained to suggest that the legume-rhizobium symbiosis involves unique gene products restricted to plants that successfully nodulate and fix N₂. In addition, neither of the proposed steps in this symbiosis offers a uniqueness that would absolutely exclude different plant species allowing establishment of persistent symbioses with diazotrophs. It is now known that the Rhizobia may not necessarily be required for nodulation to occur (Truchet *et al.*, 1989). Sprent and de Faira (1989) emphasized that many of the widely accepted dogmas for “normal” symbioses, e.g., root hair infection and the necessity of the bacteria to be released from infection threads before they differentiate into N₂ fixing forms, are not universal. Infection through wounds is now well established as a normal part of nodule initiation and infection threads develop following damage to cells, while the damage could be caused in different ways. In fact, the entire terminology used in the literature to define/discuss the legume-bacterium association e.g., host, bacterial invasion, infection, infection thread, resistance to infection and legume defense mechanism etc. suggests a kind of host-parasite relationship. According to Sharifi (1984), the pre-infection events, the process of infection and nodulation in the colonization of the legumes by *Rhizobium* are similar to those of other parasitic associations. Similarly, the host responses to the *Rhizobium* entry, infection thread synthesis and bacteroid formation are comparable to those of other plants when they encounter phytopathogens. Broughton and Perret (1999) used the term invasion to describe bacterium legume interaction and successful invasion has to precede with host cell damage.

Indeed nodulation or nodule-like structures may not necessarily result from bacterial invasion alone. Plant growth hormones or phytohormones have indeed been found to play a role akin to *nod* factors in inducing root modifications that lead to the development of nodules (Hirsch and LaRue, 1997). Effective nodulation in otherwise non-

nodulating soybean following use of 2,4-D has also been reported (Akao *et al.*, 1991). This finding suggests that the non-nodulating type has acquired resistance against the damage by rhizobial attack, while 2,4-D effectively overcame the resistance. Apparently, 2,4-D removed one of the barriers to nodulation and facilitated the entry of the bacterium into the roots. An effect similar to that of 2,4-D, has been obtained by using cell wall degrading enzymes. Cellulase and pectolyase are reported to facilitate the entry of N₂ fixing microorganisms into the roots of many legumes and non-legumes including cereals (Cocking *et al.*, 1990). This would mean that a sufficiently damaging substance is required for malformation or so-called nodulation followed by bacterial entry into the wound, where it may or may not work depending upon the preferences and capabilities of the plants. Quite certainly, the plants that nodulate do not have the means to resist the entry of Rhizobia into their roots, while the ones which do not nodulate have such an ability. There is ample evidence to suggest that the most common mode of infection is through fissures from where lateral roots emerge and 2,4-D enhances such a probability by increasing the number of lateral roots. It will appear, therefore, that the Rhizobia become part of the nodule only after the plant has started reaction to some foreign agent e.g., exogenous chemicals like 2,4-D or biochemicals secreted by the microbes destined to become microsymbiont. In legumes, Iannetta *et al.* (1997) suggested that species of rhizobia have evolved towards symbiotic status by development of cell-wall degrading enzymes on 2 levels, firstly to stop or limit secretion and/or synthesis of wall-degrading enzymes capable of eliciting host plant defence reaction, and secondly to express high levels of enzymes stimulated by the presence of compatible host tissue polysaccharides or polysaccharide containing moieties. The cereals not only appear to have developed a stronger defense mechanism against such an invasion, but are able to disallow the bacterial invader from normal functioning including N₂ fixation.

The nodules or nodule-like structures (paranodules) formed after bacterial invasion or due to physical root damage may differ. In fact, different structures are formed as a result of differences in the agent e.g., types of rhizobia. Different rhizobia could secrete different types of chemicals similar to 2,4-D in function. These chemicals may be different for different organisms leading to variation in the degree of damage caused to the root system and plant responses

expressed in the form of variable types of nodular structures. Genetic diversity of the host is also an important factor in the maintenance of polymorphism within the symbiont population. The nodules formed on 2,4-D treated roots in the presence of bacteria were structurally different than those formed without inoculum (Glagoleva *et al.*, 1997). Thus nodules or nodule-like structures can be formed by chemical treatment of roots, while presence of bacterium only extends this occurrence to a sort of effective nodulation commensurate with N₂ fixation. Physical wounding and rhizobial inoculation results in the triggering of similar genes e.g., chalcone synthase gene which codes for the first enzyme of the flavanoid pathway (Rolfe *et al.*, 1997). Differences in cell wall chemistry of non-nodulating and nodulating legumes may have an important bearing on the subsequent nodulation. The bacteria that do well once in the nodule but are unable to cause nodulation will be lacking cell wall degrading chemicals. Bacteria with a 2,4-D like effect must succeed in causing nodulation. Some of these diazotrophs have indeed been used in an attempt to establish N₂ fixing association with non-legumes treated with 2,4-D (Kennedy and Tchan, 1992, Kennedy *et al.*, 1997).

Nodulation in cereals and 2,4-D

In non-leguminous dicots and cereals, significant progress has been made over the past two decades towards inducing root modifications through the use of auxins including 2,4-D. When treated with low concentrations of various auxins, particularly 2,4-D, graminaceous crops such as wheat, rice, and maize etc. develop tumorous structures (paranodules) along primary and secondary roots. These structures are modified lateral roots that resemble a legume nodule at least morphologically (Francisco and 1993, Kennedy and Tchan 1992). Ridge *et al.*, (1993) suggested that 2,4-D disturbs the orientation of cell division causing abnormal root growth. Histologically, auxin induced tumors or paranodules in cereals appear as cancerous grown out root meristems and are thus comparable to stem nodules of *Sesbania rostrata*. Nie *et al.* (1992) have described in detail the structure of 2,4-D induced paranodules in wheat. They showed a central vascular tissue surrounded with several layers of cortical cells and connected to the root stele. These structures are also reported to develop a central vacuolar system similar to that in roots (Christiansen-Wengier, 1997). This is in contrast to the organization of vascular tissue in legume nodules in which the

stele surrounds the infected cells. However, in tissue organization, paranodules of wheat resemble those of *Parasponia*, a non-legume (Trinick, 1988). The structures formed on roots of rice seedlings following exposure to 2,4-D have smooth epidermis, distinct zoning of tissue, internal development of proto-vascular elements typical of a vascular bundle and a close resemblance to the structures induced in *Parasponia* by *Bradyrhizobium* (Ridge *et al.* 1993). The nodules formed on 2,4-D treated roots in the presence of bacteria are structurally different than those formed without inoculum. Significance of the structures induced by 2,4-D treatment in root-microbe interactions was recognized relatively recently by Nie and co-workers but without any acceptable evidence of N₂ fixation (Nie *et al.*, 1992).

The bacteria colonize intercellularly usually in the basal zone of the paranodules (Kennedy *et al.*, 1997) and there is no evidence of intracellular infection (Nie *et al.*, 1992) as is the case in legumes. The bacteria infect through the cracks created by lateral root emergence. Kennedy and Tchan (1992) suggested that crack entry or the entry through damaged root portions (e.g., due to 2,4-D) is the main route for endophytes. Similar mode of entry into wheat roots treated with 2,4-D has been proposed for *Nostoc* (Gantar and Elhai, 1999). Gough *et al.* (1997) reported reproducible crack entry of *Azorhizobium caulinodans* into roots of *Arabidopsis thaliana* where they occur in high frequency. Interestingly, this mode of entry is shared by many legumes that grow under aquatic conditions (De Bruijn, 1995). Attachment of rhizobia to rice roots (Terouchi and Syono, 1990), deformation of root hairs and formation of nodule like structures/hypertrophies (Al-Mallah *et al.*, 1989, De Bruijn *et al.*, 1995, Rolfe and Bender, 1990) or thick short laterals (Cocking *et al.*, 1993) are reported.

In wheat, Kennedy *et al.* (1997) did not find intracellular infections with bacteroids, while significant infections were found only in 10-20% cases with most bacteria being located in pockets away from the central vascular system. Inoculation with rhizobia did not result in appreciable N₂ fixation. In their review, Tchan and Kennedy (1989) also did not report any evidence of N₂ fixation for these nodules in the presence of rhizobia. However, when *Azospirillum* instead of *Rhizobium* was used as the microbial partner, substantial amounts of N₂ were fixed in wheat nodules obtained through 2,4-D treatment (Zeman *et al.*, 1992). Kennedy *et al.* (1991) found an

effective colonization of paranodes by *Azospirillum* although a direct evidence of N₂ fixation could not be obtained. Compared to this, studies by Christiansen-Weniger (1998) have reported NH₄ excreting mutant of *A. brasilense* to survive inside these nodules in large numbers in maize and rice; measurable quantities of N being fixed at the expense of C supplied by the host were also found.

Colonization of wheat paranodes by several other bacteria has also been reported (Kennedy *et al.*, 1997). Using ¹⁵N methodology, Kennedy and Tchan (1992) obtained concrete evidence of N₂ fixation in paranodulated wheat. Kennedy and Islam (2001) proposed that 2,4-D has an effect akin to bacteria that produce cellulases and polygalacturanases. According to them, these enzymes are involved in loosening as well as breaking the intercellular bonds. The cells thus released will add to the amount of rhizodeposition with a consequent increase in bacterial colonization of roots. Hurek *et al.* (1994) found that apical region of the root behind the meristem was the most intensively colonized by bacteria. Indeed, the release of root border cells or root cap cells is a common feature in most crops including wheat and serve as substrate/bate for bacteria/pathogens (Hawes *et al.* 1991 and the references therein). Presence of 2,4-D in the rooting medium may facilitate the release of such cells. Francisco *et al.* (1993) observed 2,4-D to cause sloughing off of the epidermis as the cells proliferated from the actively dividing apical and adjacent lateral meristems. Schlöter and Hartmann (1998) attributed effective colonization of root tip cells by different bacteria to the root cap cells or root border cells which served as the carbon source.

Christiansen-Weniger (1992) showed that auxin-affected portions of rice nursery are attractive infection sites for the bacteria, with enhanced N₂ fixation in 2,4-D treated wheat seedlings. Yanni *et al.* (1997) found good colonization of rice root interior by diverse types of rhizobia which could effectively nodulate clover (*Trifolium alexandrinum*) and fix significant amounts of N. Certain rhizobial inoculants significantly improved different growth parameters of rice under both laboratory and field conditions. Since no attempt was made to determine contribution through N₂ fixation, they hypothesized that the positive effects may be more through hormonal means. Gough *et al.* (1997) used *Azorhizobium* in such studies and found high intercellular endophytic colonization of both rice

and wheat roots. Reasonable N₂ fixation (using acetylene reduction assay, ARA) was detected and the inoculated plants showed better growth. Surface colonization by rhizobia (Chabot *et al.*, 1996) of cereal roots and their entry through cracks (Cocking *et al.*, 1992, De Bruijn *et al.*, 1995) has been reported. Christiansen-Weniger and Vanderleyden (1994) have described the colonization of maize roots by *Azospirillum*. Treatment with 2,4-D is reported to correlate positively with increased internal bacterial colonization in wheat roots (Kennedy and Tchan 1992). The 2,4-D treated plants were shown to carry more associated azospirilla than untreated plants (Katupitiya *et al.*, 1995), a dense layer of bacteria was observed covering the surface of young parts of 2,4-treated roots and nitrogenase activity was high especially when inoculum was also given.

Using ¹⁴C, Elencheyhian and Panwar (1997) found higher translocation of photosynthates to the rhizosphere due to the 2,4-D treatments. Hence, one of the mechanisms whereby 2,4-D enhances bacterial colonization of roots may be through an increase in rhizodeposition by the plants probably due to cells becoming leaky. Zeman *et al.* (1992) demonstrated that energy source required for N₂ fixation by colonizers of wheat roots treated with 2,4-D was supplied by the plant. Nitrogen fixation associated with 2,4-D treated and inoculated seedlings was demonstrated using ¹⁵N as well as nitrogenase activity (Yu and Kennedy, 1995). Some of our unpublished work shows rapid proliferation of bacteria following transfer of wheat seedlings in the medium containing 2,4-D. Sabry *et al.* (1997) showed that wheat grown in pots and inoculated repeatedly with *A. caulinodans* showed colonization at the point of emergence of lateral roots and appeared to obtain significant amounts of nitrogen through fixation. By inducing many lateral root initials simultaneously, 2,4-D may increase the entry of microbes into the root interior and enhance N₂ fixation.

In rice, progress in achieving nodulation has been more significant than that in wheat. There is a fair degree of optimism that legume-rhizobium association could be extended to rice, as the latter is also able to enter into associations with mycorrhizal fungi (Secilia and Bagyaraj, 1992) and genetic links between the processes involved in nodulation and arbuscular mycorrhizae have been found in legumes (Gianinazzi-Pearson, 1996). Thus rice may possess part of the genetic machinery necessary for entering into mutually beneficial, endosymbiotic associations with other

soil microorganisms. Rolfe and Bender (1990) used genetically modified rhizobia to induce nodulation in rice. The structures thus formed were fairly similar to legume nodules with a good proportion of cells containing bacteroids. However, no significant nitrogenase activity was detectable. In another study, Ridge *et al.* (1993) induced structures on rice roots with 2.5 to 100 μM 2,4-D but no internal infection or colonization by *Rhizobium* or *Azorhizobium* was found.

The above account suggests substantial bacterial colonization of cereal roots, both surface and endophytic. The endophytes have the advantage of avoiding competition with rhizospheric population for rhizodeposits. Instead they are sitting at the site of exudates export and may thus benefit immediately from the C materials diverted to the roots. In order to obtain results of choice, the plants can be modified in a way that they support desirable microbial diversity. Indeed, a remarkable diversity of bacteria is found associated naturally with roots of field-rice (Yanni, 1991). Transgenic plants are reported to have a different effect on rhizospheric microbial diversity (Di-Giovanni *et al.*, 1999). In fact it is the plants control in the rhizosphere that some genotypes are resistant to soil-borne plant pathogens while others are not. Same will be true for N_2 fixing plant types. Indeed, the so-called plant-microbe specificity will depend on specific compounds released into the rhizosphere and the resultant threshold level of the particular microbial population to achieve effective plant-microbe association. Co-inoculation of plant growth promoting rhizobacteria (PGPR) with effective rhizobia in chickpea resulted in significant increase in nodule weight and other plant characteristics.

In summary, endophytic root colonization could be achieved to the extent that measurable N_2 fixation could take place. This mode of N_2 fixation could be induced more conveniently in crops like rice, which grow under higher moisture conditions. Success in this direction has already been witnessed in sugarcane, which provides enough carbonaceous materials to support reasonable N_2 fixation activity. Use of 2,4-D could indeed help achieve the objective of enhanced microbial colonization of cereal roots. Optimism seems to prevail therefore that some sort of N_2 fixing symbiosis could be achieved in cereals, particularly in rice. This will enable this important cereal fulfil part of its N requirements through bacterial fixation leading to savings in fertilizer inputs and consequently the environment.

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