The Potential of Cold Plasma-Based Seed Treatments in Legume–Rhizobia Symbiotic Nitrogen Fixation: A Review

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Abstract: The use of cold plasma (CP) seed treatment is an emerging agricultural technology that exhibits the potential to enhance nodulation and symbiotic nitrogen fixation (SNF) in legumes. CP is composed of a diverse mixture of excited atoms, molecules, ions, and radicals that have the potential to affect the physical properties of the seed and influence gene expressions that could have a lasting impact on the nodulation, SNF, growth, and yield of legumes. The direct participation of the CP in the nodulation process and its correlation with the escalation of nodules and SNF is still not fully understood. This review discussed four areas in the nodulation and SNF process that can directly or indirectly affect CP seed treatments: root–rhizobia signal exchange pathways, root/shoot growth and development, phytohormone production, and the nitrogen fixation process. We also discuss the potential challenges and future research requirements associated with plasma technology to enhance SNF in legumes.

Keywords: biological nitrogen fixation; cold plasma; legumes; root nodulation; seed treatment; sustainable agriculture

1. Introduction

Legumes play a crucial role in soil fertility enhancement within diverse ecosystems, owing to their ability to establish a mutualistic relationship with nitrogen-fixing rhizobia bacteria, enabling them to utilize atmospheric nitrogen (N_2) for growth. Symbiotic nitrogen fixation (SNF) is the process through which rhizobia converts N_2 to ammonia (NH_3), contributing to the nitrogen (N) supply of legume hosts [1,2]. The significance of this process extends beyond legumes, benefiting non-legumes through interplant N transfer from legumes to non-legumes or from mineralized residues of legume crops [3,4]. While biological N fixation typically accounts for 11.3–34 kg ha^{-1} yr^{-1} in natural terrestrial ecosystems [5], the rates in agricultural ecosystems vary based on factors like legume species, soil properties, weather, and cropping systems. In 2018, the global total N fixation in grain legumes was around 35.5 Tg; of this amount, soybeans, pulses, and groundnuts fixed around 25 Tg, 7.2 Tg, and 3.3 Tg of N, respectively [6].

The essential role of N in plant life processes, such as amino acid and chlorophyll synthesis, underscores the critical need for an adequate N supply for optimal plant growth, development, and reproduction [7–9]. However, the use of synthetic N fertilizers, despite being widely adopted in crop production, poses significant challenges to environmental sustainability. The Haber–Bosch process, employed in producing commercial N fertilizers, is known for its high energy demand, accounting for 1–2% of the world's total energy

supply and emitting approximately 300 million tons of CO_2 annually [10,11]. In agroecosystems, only about 50% of the applied N is utilized by targeted crops, while the remainder undergoes various transformations, contributing to environmental pollution and soil degradation. These transformations include (i) nitrate leaching into groundwater, (ii) N runoff and the contamination of surface water bodies, leading to eutrophication, (iii) conversion to nitrous oxide (N_2O), a potent greenhouse gas, through nitrification and denitrification processes, or iv) the alteration of the soil chemical (e.g., soil pH) and biological properties (e.g., soil microbial diversity) [12,13]. Hence, using N fertilizers has many negative impacts on the environment, including a reduction in soil, water, and air quality, depletion of the ozone layer, and various contributions to climate change.

The escalating concerns regarding N-related environmental problems and soaring fertilizer prices emphasize the urgency to transition toward environmentally friendly and economically viable N sources. Integrating legumes into agricultural systems to leverage the benefits of SNF is recognized as a key strategy in sustainable agriculture [14]. However, addressing the N demand in intensive agroecosystems requires innovative approaches. Significant efforts have been made to develop innovative biological, agronomical, and management strategies to maximize the full potential of SNF. These include (i) conventional or marker-assisted plant breeding to enhance legume root development, nodulation, and overall SNF performance [15,16]; (ii) the use of more efficient rhizobial strains [17]; (iii) improved inoculants and inoculation methods [18]; (iv) the maintenance of soil moisture, pH, and macro- and micro-nutrients at optimum levels to maintain the inoculation success [19]; and (v) the development of superior, genetically engineered rhizobia strains and N_2-fixing cereals [20,21]. In addition, researchers are now exploring cold plasma (CP) seed treatment as a new approach to improve nodulation and SNF in legumes [22–24].

Plasma, considered the fourth state of matter alongside solids, liquids, and gases, consists of a diverse mixture of highly reactive oxygen species (ROS) and reactive nitrogen species (RNS) [25,26]. It is eco-friendly and chemical-free, making it studied widely for improving (i) seed germination [27]; (ii) plant growth, development, and reproduction [28,29]; (iii) insect and disease control [30]; (iv) SNF [22–24]; and (v) the shelf life of perishables and non-perishables [31]. ROS and RNS are naturally generated in plants under different environmental stresses. Plants have developed an array of antioxidants, oxidative enzymes, and other small molecules to neutralize reactive species safely [32]. When there is an imbalance between ROS production and their detoxification through enzymatic and non-enzymatic reactions, it leads to oxidative stress [32]. This imbalance results in increased net ROS formation, causing photooxidative damage to DNA, proteins, and lipids, ultimately leading to cell death [32]. However, at low concentrations, these reactive species have the ability to act as signaling molecules comparable to plant hormones and regulate plant growth and development, metabolism, and response to biotic and abiotic stresses [33,34]. Recent studies indicate that CP seed treatments activate biochemical and molecular processes within seeds beyond their surface impact on seeds. These include the regulation of oxidative and reductive pathways, abscisic acid catabolism, plant hormone biosynthesis, and isoflavone production, exerting a significant influence on germination and subsequent developmental stages [35–37]. The review by Priatama et al. [36] and Starič et al. [37] comprehensively discussed the effects of CP seed treatments on gene expression, enzymatic activity, morphological and chemical changes during seed germination, and seedling growth in plants/crops.

Previous studies indicate that the distinctive properties of CP position it as a promising and innovative method to boost plant growth, development, and yield in legumes. In the review by Šerá et al. [38], the impact of CP on the growth of legumes, including seed germination, seedling growth, and seed yield, was explored. This paper reviews the intricate details surrounding the potential of CP to influence nodule formation and SNF in legumes. Our review encompasses a comprehensive examination of how CP exerts its impact, closely analyzing both its direct effects on the distinct stages of the N fixation

process and its indirect influence achieved through the enhancement of plant growth and development.

2. Fundamentals of Cold Plasma Technology

Plasma is commonly recognized as the fourth state of matter that exhibits a higher energy state than solid, liquid, or gas. It comprises excited atomic, molecular, ionic, and radical species, along with various constituents like electrons, positively and negatively charged ions, free radicals, gas molecules, and atoms in both ground and excited states [39]. Additionally, electromagnetic radiation quanta, including ultraviolet photons and visible light, are integral components of plasma [39]. The presence of free electric charges, such as electrons and ions, renders plasma electrically conductive and highly responsive to electromagnetic fields [40].

Plasma is categorized as thermal (hot) or non-thermal (cold) depending on the thermal equilibrium between electrons and heavier particles. CP has an electron temperature (T_e) that is significantly higher than the macroscopic gas temperature (T_g) ($T_e \gg T_g$), resulting in a lack of local thermodynamic equilibrium [41]. This non-equilibrium state results from the asymmetrical momentum transfer during collisions influenced by an electrical potential difference [42]. In contrast, thermal plasma exhibits consistent average temperatures among electrons, ions, and neutrals. The temperature of any plasma is commonly defined by the mean energies of neutral and charged plasma species along with their corresponding degrees of freedom.

Historically, plasma discharges were generated under low-pressure conditions, effectively decreasing the number density of gas molecules (i.e., the number of gas molecules per unit volume) and enabling ionization at lower electric potential differences. However, with technological advancements, CP can now be initiated and sustained under ambient atmospheric conditions. This atmospheric pressure plasma generation system is simpler and more user-friendly compared to the low-pressure plasma generation system.

Plasma properties are governed by its chemistry, involving numerous reactions and a mix of positively and negatively charged particles, depending on the initial gas mixture's composition. Van Gaens et al. [43] developed a kinetic model with 1880 reactions and 84 reactive species. Chemical reactions in the air are mainly initiated by electron collisions with oxygen and nitrogen. The power input, working gas, and mode of operation significantly influence the species formed and their degree of formation in a plasma system. Various gases, including Ar, He, N_2, O_2, CO_2, specific gas mixtures, and air, have been utilized for plasma treatment. Air is the most suitable due to its abundance and low cost. Reactive oxygen and nitrogen species (RONS) are typically formed due to the collision between electrons that drift in the electric field and diffuse through the medium of neutral atoms, resulting in ions and excited atoms. Common RONS formed in plasmas include ROS, such as hydrogen peroxide (H_2O_2), ozone (O_3), superoxide anion ($O_2^{\bullet-}$), hydroperoxyl ($HO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), and carbonate anion radical ($CO_3^{\bullet-}$), and RNS, such as nitric oxide ($NO^{\bullet}$), nitrogen dioxide radical ($^{\bullet}NO_2$), peroxynitrite ($ONOO^-$), peroxynitrous acid ($OONOH$), and alkyl peroxynitrite ($ROONO$) [44]. Table 1 provides a comprehensive account of the physical processes that arise from electron collisions, which govern the reaction mechanisms leading to the formation of RONS.

Table 1. The reactions and processes during plasma generation.

Process	Reaction [‡]
Ionization	$e + M \rightarrow M^+ + 2e$
Dissociative ionization	$e + AB \rightarrow A^+ + B + 2e$
Electron attachment	$e + M \rightarrow M^-$
Dissociative electron attachment	$e + AB \rightarrow A^- + B$
Excitation (electronic, vibrational or rotational)	$e + M \rightarrow M * + e$
Dissociation (both products can be electronically excited)	$e + AB \rightarrow A + B$

[‡] Details were obtained from Misra et al. [45]. * shows excited molecule or atom.

These constituents induce surface oxidation, erosion, corrugation (i.e., change of shape), and hydrophilization of seed coats, thereby improving seed quality [46]. However, the efficacy of CP treatment varies depending on seed coat properties, moisture content, and hormesis—a phenomenon where a small amount of typically harmful substances (e.g., ROS) stimulates beneficial effects on living organisms [47]. Literature has highlighted diverse mechanisms contributing to the increased penetration of plasma-generated RONS in legume seeds. H_2O_2 interacts with the seed surface and can cross the plasma membrane, while superoxide, carrying a charge, necessitates voltage-dependent anion channels (porins) for passage through the plasma membrane [48]. Despite its charge, superoxide can decompose into hydroxyl and singlet oxygen, which can more easily traverse the plasma membrane. Occasionally, RONS can bypass the plasma membrane through aquaporins, membrane proteins facilitating the diffusion of water and small molecular weight solutes, including ROS and RNS [49,50]. It is important to understand the attributes of plasma constituents, particularly reactive species, due to their extended lifespan and complex reactions. Below, we present a brief overview of selected reactive species and their impacts.

UV radiation can have negative and positive effects on the various physiological processes related to seed growth [50]. It can facilitate the entry of RONS into the cell membrane by causing surface-etching damage to the seed coat [36].

Hydrogen peroxide is a long-lived ROS with a half-life from 8 h to 20 days [51]. It serves as a signaling molecule [52] and is highly interconnected with hormones, metabolism, and gene transcription [48]. H_2O_2 diffuses through anion channels and is converted to superoxide and hydroxyl radicals [36].

Hydroxyl radical is a short-lived ROS with a half-life of 10^{-9} s. It is an extremely potent ROS and can lead to the oxidative breakdown of cell wall polysaccharides, resulting in cell wall loosening [53].

3. Plasma Generation Devices

Plasma can be generated at room temperature by applying a strong electric field across a gas gap or exposing the gas to intense ultraviolet light. However, the latter method is rarely used in agricultural research. Common plasma sources for treating food and biological materials include corona discharges, microwave plasmas, dielectric barrier discharges, and plasma jets.

Corona discharges: Corona plasma is created through a high-voltage electrical discharge that happens between two electrodes with different radii of curvature, such as a sharp pin plate or a thin wire plate. These sharp edges create strong, localized electric fields that reduce the voltage needed to initiate the discharge. This results in a more stable discharge over the surface of the low potential or ground electrode [42]. It can be produced using various types of power sources including alternating current (AC), direct current (DC), and pulse voltages [54]. It has a simple design and low installation cost [55].

The main limitations of corona discharge are that it has an asymmetric electrode design and results in the formation of a local, unstable, and non-equilibrium discharge. Furthermore, the generation of the high electric field near the high voltage electrode results in the formation of streamers, thus resulting in a weakly ionized gas [54].

Microwave plasma: Microwave-powered discharges are commonly utilized for producing volumetric non-equilibrium plasma. Due to the significant variation in the mobilities of electrons and ions at microwave frequencies, the electromagnetic fields are able to selectively transfer energy to electrons, resulting in the creation of a CP with non-equilibrium properties at atmospheric pressure [56]. It is possible to manipulate the temperature and velocity of a microwave plasma jet by regulating the output power and gas flow rate. Microwave torch systems (Figure 1A) are commonly cooled using external water and/or air. To bring down the temperature of the plasma jet from several thousands of Kelvin to ambient temperatures, it can be passed through a heat exchanger before it is used for food treatment, as described in Schnabel et al. [57]. Microwave plasma sources offer several key benefits, including electrode-free operation, the ability to operate in atmospheric conditions,

efficient conversion of microwave energy into plasma, availability of low-cost sources at industrially approved microwave frequencies of 0.915 GHz (with wavelength λ ~32 cm) and 2.45 GHz (λ ~12 cm), and minimal maintenance requirements [58].
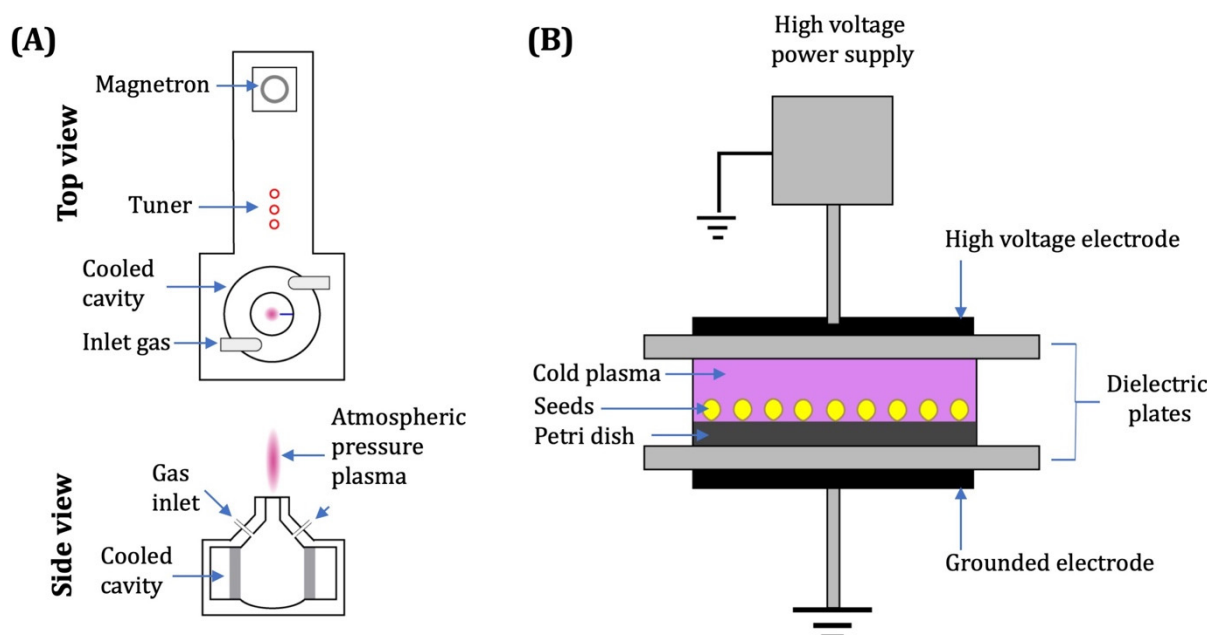

Figure 1. Graphical representation of plasma generation devices. (**A**) A microwave plasma torch, modified from Foltin et al. [58], (**B**) a dielectric barrier discharge system, modified from Shelar et al. [59].

Dielectric Barrier Discharge (DBD): Dielectric Barrier Discharge (DBD) is a barrier discharge involving two electrodes at different potentials separated by a dielectric material. The dielectric barrier limits current flow, thereby facilitating gas ionization within the inter-electrode space while preventing an electric arc. In the in-package DBD plasma concept, high voltages (~10^1–10^2 kV) are applied across a plastic package containing food, which ionizes the gas within the confinement. The plastic package serves as a dielectric barrier in this scenario (Figure 1B, [59]). The DBDs are safe and easy to operate and have flexible geometries including coplanar DBD, surface discharge DBD, and volume DBD [54,60]. The DBD discharge can be ignited using AC or pulsed DC [54]. The presence of a dielectric material limits the discharge current, thus preventing arc formation. The high electric field buildup between the discharge gap results in the formation of micro discharges, thereby minimizing the damage to the electrode over its lifespan [54].

Plasma Jets: A plasma jet is created by positioning two concentric electrodes with an inter-annular region through which a gas or gas mixture can flow. The inner electrode is made of metal and maintained at a high potential of approximately 10^2 kV using a high-frequency power source that operates at around 13.56 MHz (i.e., radio frequency), leading to the ionization of the gas. The resulting plasma is then directed onto the sample to be treated through a nozzle. Multiple plasma jets can be arranged to treat larger surfaces instead of using a single jet that only treats a localized area [61]. Plasma jets are safe and easy to operate and handle. They produce stable and homogenous plasma species without forming streamers or arcing. The electrical discharge can be ignited using AC, pulsed DC, radiofrequency, and microwave power [60].

4. Effect of Cold Plasma on Nodulation and Biological Nitrogen Fixation in Legumes

Legume nodules can be categorized into two morphological types: indeterminate (e.g., pea [*Pisum sativum* L.], alfalfa [*Medicago sativa* L.], red clover [*Trifolium pratense* L.]) and determinate (e.g., soybean [*Glycine max* L.], peanut [*Arachis hypogaea* L.], common bean [*Phaseolus vulgaris* L.]). Indeterminate nodules feature persistent meristems, initiating in the

root pericycle and progressing to the inner cortical cells, forming a cylindrical shape with a developmental gradient from apex to base, encompassing the nodule meristem, rhizobial infection zone, and N fixation zone [62,63]. In contrast, determinate nodules, with a spherical shape, have a shorter lifespan and originate from cell divisions in the middle/outer root cortex, where meristematic activity terminates early in nodule development [64]. Recent studies indicate that CP seed treatments enhance nodule number and SNF in legumes, irrespective of nodule type [23,24]. Although experimental evidence on the effects of CP on nodulation and SNF is limited, these studies propose that reactive plasma species may penetrate natural seed coats, interact with the plant's internal cellular machinery, and induce alterations in hormone signaling, enzyme activity, and gene expression that persist throughout the plant's life cycle [23,24].

In a recent investigation conducted by Mildaziene et al. [22], it was observed that red clover seedlings aged five weeks, derived from CP-treated seeds, exhibited an early onset of nodule formation. The CP treatment was administered using a planar geometry reactor at 200 Pa for a duration of five to seven minutes. The duration of treatment was identified as a factor influencing nodule formation and quantity, with the seven-minute CP treatment resulting in a 2.4-fold increase in nodule number compared to the untreated control [22]. Intriguingly, Ivankov et al. [24] noted that seed color influenced the performance variations in CP treatments. Red clover seeds of yellow, purple, and brown colors were subjected to five or seven-minute CP treatments, revealing the highest nodule number in the five-minute CP-treated brown seeds [24]. These findings underscore the significance of timing and seed color/type in CP treatments. Another study demonstrated the efficacy of high-frequency plasma (5.28 MHz) at atmospheric pressure (200 Pa) for five minutes in enhancing lateral root and nodule numbers (Table 2, [65]). These experiments suggest that CP treatments can impact root development and trigger the formation of lateral roots or nodules in legumes that form indeterminate nodules.

Table 2. Effect of cold plasma on symbiotic nitrogen fixation related traits in different legume crops.

Symbiotic Nitrogen Fixation Related Traits	References
Early formation of nodules	[24]
Alter nodule number and dry weight	[22–24,65]
Increase nitrogenase activity in nodules	[23]
Increase nodule leghemoglobin content	[23]
More flavonoid/isoflavonoid production	[22,65]
Increase root length	[22–24,65–68]
Increase lateral root number	[22,65]
Increase root biomass	[23,24,65,67,68]
Increase *GmEXP1* gene expression	[23,69]
Alter phytohormone levels (cytokinin and auxin)	[23]

Legumes with determinate nodules also exhibit enhanced nodule formation, nitrogenase activity, leghemoglobin content, and N content in nodules following the CP seed treatment (Table 2). Forty-day-old soybean plants derived from CP-treated seeds displayed a 23% higher average number of nodules on primary roots and a 73% higher total fresh nodule biomass per plant (Table 2, [23]). Furthermore, individual nodule dry and fresh weights were 48% and 72% higher, respectively, accompanied by 1.4-to-1.6-fold higher nitrogenase activity, 2-fold higher leghemoglobin content, and 2.1-to-3-fold higher N content in nodules compared to the untreated control (Table 2, [23]). These findings indicate that CP seed treatments have the potential to enhance nodulation and SNF in legumes, irrespective of nodule morphology.

5. Effect of Cold Plasma in Rhizobia Legume Roots Invasion

Legume roots encounter diverse microorganisms in the rhizosphere [70]. To safeguard against potential invasion by these microorganisms, host plants deploy both chemical and mechanical barriers [71]. Although rhizobia can partially suppress or overcome the plant's

defense responses, the host plant strives to maintain a balance, encouraging and impeding rhizobia colonization in its tissues [72,73].

The suberization and lignification process within cell walls play a crucial role in defending against the elongation of infection threads and the further advancement of rhizobia [74]. Although there is no evidence for more mature roots, recent research has demonstrated that three-day-old pea seedling roots treated with CP (generated with air, N_2, and O_2 administered for 40 s) exhibit enhanced lignification in sclerenchyma cells [75]. Additionally, five-day-old pea seedling roots from CP-treated seeds displayed notably increased endodermal cell development and the early progression of a secondary cell wall. This effect is potentially attributed to elevated lignin production induced by increased peroxidase enzyme biosynthesis due to the CP treatment [75]. Consequently, it seems that CP treatment conditions limit rhizobia root invasion, infection thread formation, and progression. This prompts the question of how CP can simultaneously promote nodulation and SNF while restricting the micro-symbiont. Several potential factors may contribute to the observed increase in nodulation and SNF after CP seed treatment, including (i) modifications to root–rhizobia signal exchange pathways, (ii) increase in plant/root growth and development, (iii) alterations to phytohormone production regulating nodule formation, and (iv) enhanced leghemoglobin production facilitating N fixation.

In legumes, the nodule development unfolds in three primary phases: pre-infection cross-talk, root invasion and nodule initiation, and the differentiation of mature N_2-fixing organs [76–78]. The initiation of root invasion involves signal exchange between legume roots and rhizobia [78]. Legume roots release flavonoid/isoflavonoid compounds that bind to bacterial NodD proteins [79,80]. This interaction activates the transcription of *nod* genes in rhizobia, resulting in the production of lipo-chitooligosaccharides, known as nodulation (Nod) factors [81]. These Nod factors initiate signaling cascades in host legumes, assisting in the recognition of rhizobia. Therefore, flavonoids/isoflavonoids play an important role in inducing *nod* genes at nanomolar to low micromolar concentrations [82]. It has been proposed that a mixture of flavonoids/isoflavonoids exerts a more substantial effect in inducing *nod* gene expression than a single compound [83]. Additionally, these compounds act as determinants of legume host specificity between the two partners [82].

Interestingly, research indicates that CP seed treatment has the potential to alter the release of flavonoids/isoflavonoids from legume roots [22,65]. Five-week-old seedlings cultivated from red clover seeds treated with air-generated CP for five and seven minutes exhibited higher levels of root exudate flavonoids/isoflavonoids compared to untreated controls (Table 2; [22]). The impact of CP on total flavonoids/isoflavonoids production was most pronounced in the seven-minute treatment, doubling the concentration compared to the untreated control. This suggests that the CP-induced production of these compounds is dependent on the duration of exposure [22]. The seven-minute CP treatment resulted in a two- to three-fold increase in the quantity of five out of nine measured flavonoids/isoflavonoids, including hesperetin, quercetin, kaempferol, daidzein, and 7,4'-dihydroxyflavone [22]. Nedved et al. [65] conducted assessments on one to two trifoliate leaves of red clover over two years and found similar results, with plants obtained from CP-treated seeds showing a 2-fold higher daidzein and a 1.4-fold higher formononetin content during the second production year. However, no difference was observed in genistein content compared to the untreated control [65]. The study suggested that the higher nodule number in CP-treated plants might be attributed to increased isoflavonoid production and enhanced lateral root development [65].

In contrast, Pérez-Pizá et al. [23] observed a 1.5–1.8-fold reduction in certain isoflavonoids (daidzein, genistein, and daidzin) in 15-day-old red clover seedlings derived from CP-treated seeds compared to the untreated control. However, no difference was observed for genistin production [23]. Nonetheless, CP-treated plants exhibited higher nodule number, nodule dry weight, leghaemoglobin, N content, and nitrogenase activity in nodules [23].

Overall, these findings suggest that treating legume seeds with CP can enhance flavonoids/isoflavonoids production either directly or as an indirect effect of stimulating

plant growth (Figure 2). The latter could potentially provide positive feedback on the nodulation phenotype. However, conclusive results are hindered by insufficient data, emphasizing the need for further research to gain deeper insights into this subject.
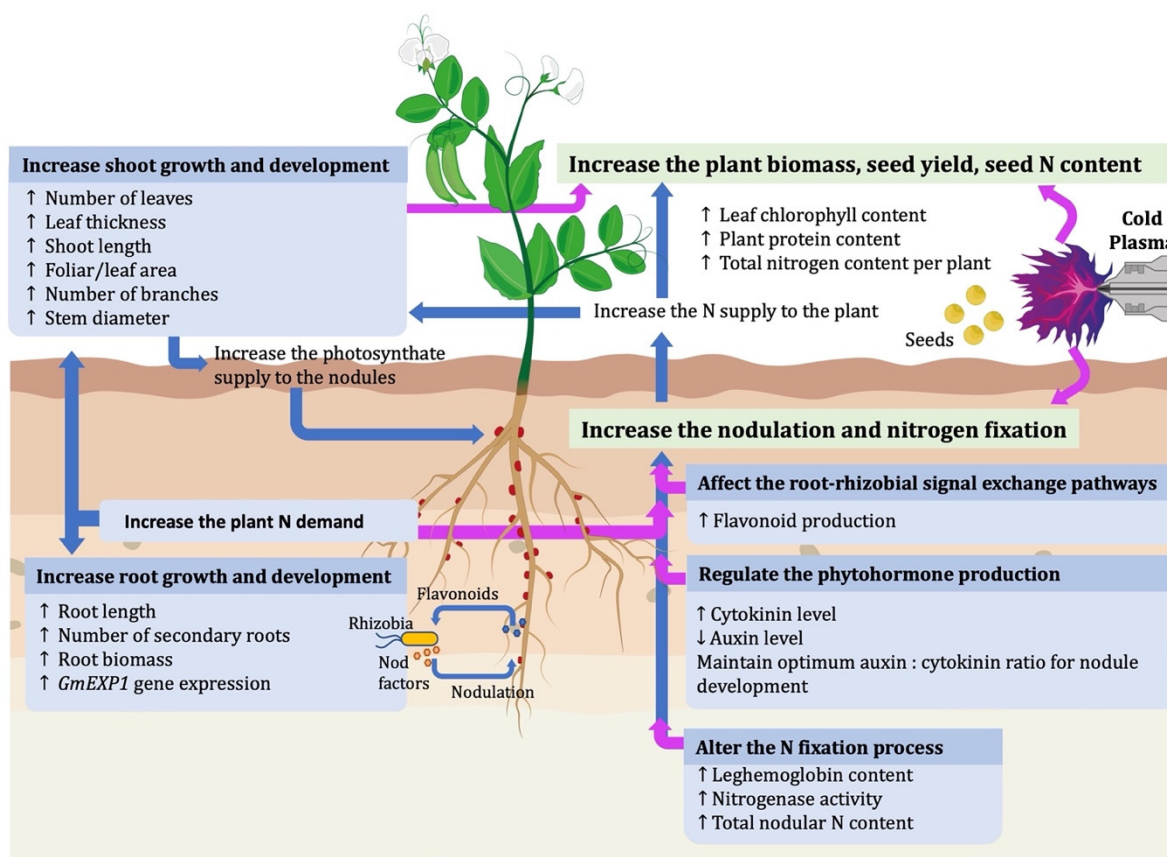

Figure 2. An illustration showing the impact of cold plasma seed treatment on the performance of a legume plant, including symbiotic N_2 fixation. Cold plasma has the ability to increase plant shoot and root growth, affect the composition of root exudates, regulate phytohormone levels, and influence the N fixation process.

6. CP Induces Root/Shoot Growth

During periods of rapid growth in legumes, there is an elevated demand for N. The observed increase in the number of nodules in plants derived from CP-treated seeds may stem from CP's direct impact on specific nodulation stages or from its stimulation of root and shoot growth. This growth stimulation, in turn, intensifies the demand for N, fostering enhanced nodulation [84]. In the upcoming section, we will discuss how CP seed treatment significantly influences plant growth, encompassing seed germination, root and shoot development, and its impact on SNF.

6.1. The Impact of CP on Seed Germination

Improved seed germination creates a conducive environment for the early and faster initiation and proliferation of roots and better seedling establishment, especially under field conditions when resources are limited. Enhanced root and seedling development are intricately linked to increased nodule development, elucidating the interconnectedness between early seedling establishment and subsequent SNF. CP seed treatment induces transformative changes in legume seed impacting its surface structural properties [36–38]. The seed coat of legumes, composed of epidermis, hypodermis, and parenchymatic cells, serves multifunctional roles in mechanical protection and various physiological functions [85]. The outer layer of the palisade cells is covered with a rough cuticle layer that serves as a pro-

tective structure for water and gas permeation [85]. The surface characteristics, thickness, and composition of the waxy cuticle affect the seed water uptake [86].

The literature emphasizes that plasma discharge over seeds removes organic contaminants and induces surface modifications such as oxidation, erosion, corrugation, and hydrophilization [35–37,87]. These alterations impact the seed coat's water contact angle, roughness, and hydrophilicity, enhancing water uptake during germination [49,87].

Seed surface roughness affects wetting, and it affects the seed imbibition. Greater roughness enhances surface properties that attract or repel water. If $\theta < 90°$, water enters the cavities, improving wetting [87]. If $\theta > 90°$, water does not penetrate, reducing wetting [87]. Volkov et al. [87] found that longer plasma treatment time (up to 19 min) decreased the water droplet contact angle on bush bean seeds, as well as increased in the hydrophilic pore sizes on the seeds. Stolárik et al. [88] reported that increasing the surface barrier plasma discharge from one to ten min increased the surface-modifying electroporation of the outermost waxy layer of peas and resulted in a higher imbibition process. Billah et al. [49] also observed an increased erosion of the cuticle layer and the subsequent thinning of the seed coat of black gram (*Vigna mungo* L.) following DBD plasma treatment for three min. The surface lipid erosion and an enhanced water absorption rate due to plasma etching was also reported in peas [88], black gram [89], and mung beans (*Vigna radiata* L., [90]). Moreover, an increase in the water imbibition is also related with an increased interaction between RONS and micropyles located on the seed coat, thus resulting in a larger pore size [49].

Apart from inducing mechanical changes, ROS in CP can bring about chemical and molecular alterations in the internal seed factors. These modifications involve gene expression, oxidative processes, protein synthesis, and hormonal activities [36,37]. Priatama et al. [36] proposed two hypotheses regarding the role of ROS in seed germination based on existing studies. One hypothesis suggests that cells in seeds detect and perceive ROS, initiating signal transduction from the outer layers of the seeds. The second hypothesis proposes that during imbibition, water plays a pivotal role in the absorption of ROS into the cell layers of the seeds, boosting seed respiration. This, in turn, sets off a chain reaction of sugar oxidation, releasing metabolic energy in the form of ATP to facilitate seed germination and seedling growth. Both dormancy and seed germination are regulated by the balance of two endogenous plant hormones: abscisic acid (ABA) and gibberellic acid (GA_3) [91]. ABA is required to maintain seed dormancy, and GA_3 promotes the release from dormancy, plant cell growth, and stem elongation. Le et al. [91] observed increased secretion of GA_3 in mung bean under plasma seed treatment condition (10 min using plasma jet), resulting in enhanced radical growth. Reviews by Priatama et al. [36] and Starič et al. [37] have describe in more details a more in-depth understanding of the molecular mechanisms underlying effect of plasma treatment on seed germination and plant growth.

The potential of CP treatments to optimize seed performance in legumes, and its indirect link to plant and root growth and development, as well as nodulation, is highlighted by the interplay between CP-induced changes in seed coat properties, and subsequent germination process.

6.2. The Impact of CP on Root Growth

Recent investigations have highlighted the significant influence of CP treatments on the growth of legume roots. Specifically, five-week-old red clover seedlings subjected to five and seven-minute CP treatments exhibited a 27% and 37% increase in root length, along with an 82% and 77% increase in the number of lateral roots, respectively (Table S1, [22]). Notably, the impact of CP treatment on root branching surpassed its effect on root length [22]. These findings imply that the duration of CP exposure may play a crucial role in determining the extent of its effect on root growth. Although the exact reason for the accelerated development and increased nodule number in plants derived from CP-treated seeds remains uncertain in this experiment, it could be linked to a positive sink–source feedback response.

In the red clover experiment based on seed coat color dependency by Ivankov et al. [24], seedlings with yellow seed coats from five- and seven-minute CP-treated seeds exhibited a 39% and 42% increase in root length, respectively. However, those from seven-minute CP-treated seeds showed a 54% reduction in root dry weight compared to the untreated control, with no significant increase in nodule number in either treatment [24]. Conversely, dark purple seeds subjected to a five-minute CP treatment displayed a 31% increase in root length [24]. Seedlings from five- and seven-minute CP-treated dark purple seeds exhibited a three and two-fold increase in root dry weight, along with a 27% and 19% increase in nodule number, respectively, compared to the untreated control [24]. These findings suggest that there is a positive relationship between the greater root mass and the number of nodules. Supporting this, the study by Nedved et al. [65] observed significant increases in root length and weight in red clover seedlings from CP-treated seeds, with 25% and 2.5-fold increments, respectively, during the one to two trifoliate leaf stage of the first year of vegetation. Additionally, CP-treated seeds resulted in a 50% increase in lateral root and nodule number (Table 2, [65]). A positive correlation was noted between enhanced root size and nodule number per plant, highlighting the need for a higher number of nodules to meet the N supply demands during periods of increased root/plant growth [65]. In the second year of growth, there was a 13% increase in root length and a higher lateral root number in red clover plants derived from CP-treated seeds (Table 2, [65]). Furthermore, CP seed treatment showed a 38% increase in nodule number per plant compared to the untreated control (Table S1, [65]). Additionally, no significant differences were observed in shoot parameters (shoot length and fresh shoot weight) with CP treatments. However, the physiological conditions leading to increased lateral roots and root length following CP treatments may contribute to a higher number of nodules.

In 40 day old mature soybean plants, Pérez-Pizá et al. [23,66] observed that plasma treatment led to an enhancement in both root length and root dry weight compared to untreated plants. Furthermore, as previously mentioned, they noted improvements in nodule parameters and activity following plasma treatment, including average nodule number in primary roots, total nodule fresh weight, individual nodule fresh and dry weight, nitrogenase activity in nodules, leghemoglobin content, and N content in nodular tissues [23,66]. This suggests a positive correlation between the enhanced root growth and the parameters related to nodulation. Pérez-Pizá et al. [23] and Li et al. [67,68] also reported an increase in root length and dry weight in soybean and peanut seedlings subjected to plasma treatment (Table S1). These findings imply that CP treatment has the potential to enhance root parameters in legumes, irrespective of the crop type.

Soybean seedlings at the age of five days, originating from CP-treated seeds, exhibited increased expression of the *GmEXP1* gene (Table 2). *GmEXP1* encodes a root-specific expansin crucial for facilitating cell elongation in the root's elongation region [23,69]. The observed increase in root length at the five-day-old stage in soybean was positively associated with the expression level of the *GmEXP1* gene [23]. Conversely, a reduced expression of the *GmEXP1* gene was noted in 15-day-old plants compared to the control (Table S1, [23]). The authors suggested that plants subjected to CP treatment may undergo transitions in root development phases, requiring less expansin secretion when assessing the *GmEXP1* gene in 15 day old plants [23]. These findings provide a reasonable yet preliminary explanation for the induced increase in root elongation by CP (Figure 2).

Moreover, root growth and development are influenced by various factors, including the availability of carbohydrates from photosynthesis or stored reserves, plant hormone signaling, and nutrient uptake from the soil [92]. Root development initiates during embryogenesis, with all root layers continuously forming from stem cells at the tip, regulated by a constant auxin flow and cytokinin-induced differentiation [93]. While young shoot tissues are the primary source of auxin, crucial for regulating root development, Pérez-Pizá et al. [23] observed higher trans-zeatin riboside (tRZ) cytokinin levels in soybean roots from CP-treated seeds. A well-developed shoot system aids root development by facilitating increased assimilate production and partitioning back to the root system.

CP treatments have been shown to increase photosynthetic tissues and leaf chlorophyll content, indirectly indicating an enhancement in photosynthesis (further details are provided in the section titled 'The Impact of CP on Shoot Growth and Seed Yield') [22,23,67]. The intricate network of roots provides a conducive environment for the establishment and growth of nodules, where the symbiotic relationship between legumes and N-fixing rhizobia bacteria flourishes.

6.3. The Impact of CP on Shoot Growth and Seed Yield

SNF relies on the provision of photosynthates to root nodules, with sucrose acting as the primary source of reduced carbon for nodule metabolism [94,95]. The breakdown of sucrose is facilitated by sucrose synthases and is metabolized through the glycolytic pathway, generating adenosine triphosphate (ATP) in the cytoplasm within the infected zone of the root nodule [96].

Throughout the root infection process, ATP-dependent molecular motors play a role in cytoskeletal remodeling, cell wall modifications, and membrane trafficking [97–99]. Following release from the infection thread, rhizobia undergo a transformation into a specialized symbiotic form called bacteroids [100]. Efficient nutrient and signal exchange between the host plant and bacteroids is crucial for nodule maintenance [101,102]. Energy availability emerges as the primary limiting factor for N fixation in legumes [103]. The enzymatic activity of nitrogenase, responsible for breaking the strong triple bond in dinitrogen molecules, necessitates a substantial amount of energy in the form of ATP. Legumes direct photosynthates to nodules to meet the energy demand, enhancing sucrose transport via the phloem [104].

Previous studies have demonstrated that the utilization of CP seed treatments can enhance plant growth and development which indirectly enhances the photosynthate production [22–24]. Although there is no direct evidence confirming that the increased production of photosynthates specifically contributes to greater allocation towards the development of nodules, we can reasonably assume that such a correlation exists. Li et al. [67] illustrated that peanut plants derived from CP-treated seeds exhibited increased leaf area, leaf thickness, leaf N, and chlorophyll contents at the fruiting stage. Additionally, they observed increased plant height, stem diameter, and branch number per plant during the maturity stage (Table S1, [67]). The outcomes further revealed an enhanced yield, with a 13% increase in pod numbers per plant, a 9% increase in 100-pod weight, and a 10% increase in seed yield compared to the control [67].

Indeed, various studies have highlighted that plants grown from CP-treated seeds manifest significant improvements in both fresh and dry shoot weight, as well as shoot length. Specifically, red clover plants exhibited increased shoot length and weight during early development in seven-day-old seedlings [22], continuing into later stages, such as five-week-old seedlings [22], and even in field-grown plants throughout their first and second years of vegetation [65]. These results indicate that CP treatments can foster growth, potentially influencing the entire plant development. Moreover, the enhancement in seedling weight and height observed in seven-day-old seedlings [22]. Mildaziene et al., [22] suggests that CP-treated seeds can stimulate growth and development even before the operational stage of SNF. Studies conducted by Gao et al. [46] on 15 day old pea seedlings and Pérez-Pizá et al. [23] on 15 day old soybean seedlings suggest that CP-treated seeds lead to higher seedling dry weight, height, and chlorophyll content. Pérez-Pizá et al. [23] also noted a 12% increase in aerial length, a 25–30% increase in total foliar area, a 5–10% increase in leaf chlorophyll content, and a 25% increase in total N content per plant in 40-day-old soybean plants obtained from CP-treated seeds. These findings propose that CP seed treatment can result in more robust and productive plants, as reflected by increased nodulation and N fixation (Figure 2).

A study by Zhang et al. [105], observed enhanced seed germination and seedling growth in soybeans with argon plasma seed treatment using the DBD system for 12 s. This was attributed to increased soluble protein, antioxidant enzymes, ATP, the target

of rapamycin (TOR: coordinates transcriptional networks that establish connections between central metabolism and biosynthesis, facilitating energy and biomass production), and growth-regulating factor (GRF: plant-specific proteins that play important roles in regulating plant growth and development). Moreover, enhancements in plant growth and development by plasma treatment were also attributed to the upregulated expression of antioxidant genes, pathogen resistance genes, and the modulation of phytohormone synthesis [36,37]. A comprehensive discussion on the impact of CP treatments on the regulation of these factors can be found in the studies by Priatama et al. [36] and Starič et al. [37]. These experimental results provide reasonable explanations for the observed enhancements of growth and development of plants derived from CP treated seeds.

7. CP Alters the Phytohormone Production That Regulates Nodule Formation

The initiation and development of nodules in legumes significantly rely on phytohormones, particularly cytokinin and auxin [106]. The perception of Nod factor leads to the upregulation of several cytokinin biosynthesis genes, a process dependent on the nodule type and legume species [106,107]. This localized upregulation of cytokinin signaling initiates cell divisions in the root cortex and pericycle, initiating the formation of nodule primordia [107,108] and also regulating rhizobial infection [109]. A proper balance between cytokinin and auxin is crucial for the production of active nodules [106]. Elevated levels of auxin stimulate lateral root formation, whereas a local excess of cytokinin may lead to the development of pseudo-nodules [106,110,111].

Maintaining an optimal number of N-fixing nodules is crucial for balanced nutrition and preventing excessive energy depletion in the host plant. Legumes employ a negative feedback mechanism known as auto-regulation of nodulation (AON) to control nodule numbers [77,112], with shoot cytokinins proposed as one component of AON [113]. A study revealed that CP-treated plants exhibited higher cytokinin (tZR) and no difference for auxin levels in their roots compared to control plants (Table 2 and Table S1, [23]). The authors suggested that the variation in the root cytokinin/auxin ratio could potentially contribute to the development of enhanced nodulation phenotypes in plants derived from CP-treated seeds [23]. However, further research is needed to confirm this as one of the contributing factors.

8. CP Induces Leghemoglobin Production and Nitrogenase Activity

Within root nodules, the rhizobial nitrogenase enzyme converts atmospheric N_2 into NH_3 [114]. However, sustaining a high N fixation rate poses a significant challenge due to the sensitivity of bacterial nitrogenase enzymes to oxygen, which is simultaneously required for facilitating rapid bacterial respiration to produce ATP needed for reducing N_2 to NH_3 [115]. The optimal scenario involves a high oxygen flux to fuel ATP synthesis while maintaining a low-oxygen environment to prevent nitrogenase inactivation. Legume nodules address this challenge by producing high levels of leghemoglobin [116]. Leghemoglobin safeguards nitrogenase by binding free oxygen, creating a high concentration of leghemoglobin-bound oxygen that facilitates a substantial oxygen flux to bacteroids for their respiratory needs [116,117]. The oxygen bound to leghemoglobin does not harm nitrogenase but is accessible to bacteroids, ensuring adequate respiration for N fixation [116,117]. Thus, leghemoglobin plays a crucial role in the N fixation process [118].

Pérez-Pizá et al. [23] observed that the application of CP to soybean seeds can elevate leghemoglobin content and nitrogenase activity in the root nodules of 40 day old plants (Tables 2 and S1). Nodules from CP-treated seeds exhibited twice the leghemoglobin content compared to those from untreated plants. Using an acetylene reduction assay, they measured nitrogenase activity and found that nodulated roots from CP-treated seeds displayed 1.4 to 1.6 times more nitrogenase activity than those from untreated plants. The total N content in both plants and nodules from CP-treated seeds was also higher, indicating enhanced N fixation compared to the untreated control. These findings suggest that CP seed treatment can improve the symbiotic relationship between rhizobia and the

host plant, leading to increased N fixation. This enhancement could result from a direct impact of CP seed treatment on processes relevant to symbiosis, such as leghemoglobin production, or indirectly, through positive sink–source feedback, boosting N_2 fixation to meet the enhanced demand for N in thriving, CP-induced plants (Figure 2).

9. Potential Challenges of the Plasma Seed Treatments and Future Work

While there has been notable progress in CP seed treatment research in legumes, the existing data primarily offer descriptive and reflective insights of controlled environmental conditions (Table S1). Few studies have explored the impact of CP seed treatment on SNF under actual field conditions, especially concerning its effects on grain yield in legumes [65,67]. It is imperative to conduct such studies before advocating CP seed treatment as a viable agronomic approach to enhance SNF. Additionally, there is a need for comprehensive research to investigate the long-term and multigenerational effects of plasma on seeds and plant growth.

It is essential to understand the durability of CP seed treatment effects. Ahmed et al. [119] demonstrated a significant enhancement in germination and growth rates persisting up to day 30 across three different plant species with CP treatment. Whether this duration can be extended by modifying treatment conditions remains to be investigated. Additionally, the exploration of CP treatments at the molecular level has been limited, with only a few researchers delving into this aspect. A study in *Arabidopsis* observed that the growth enhancement effects had not been passed on to the next generation, and there was no significant change in gene expression in second-generation seeds by the plasma treatment, indicating that the observed improvement in plant growth is not due to mutations but rather to epigenetic factors [120]. Hence, thorough explorations at the epigenetic, transcriptomic, and proteomic levels should provide better insights into the mechanisms underlying the enhanced crop growth, development, and SNF in legumes.

Exploring the role and impact of rhizobia in tandem with CP seed treatments is also important, especially considering the current lack of available information on this topic. Conducting a thorough examination of how plasma seed treatments affect rhizobia populations and activity is essential for gaining a deeper understanding of the factors contributing to the observed enhancement in nodulation, offering valuable insights to optimize agricultural practices and foster sustainable improvements in legume crop yields.

Interestingly, CP treatment has been shown to boost the vitality and activity of plant growth-promoting bacteria. Changes in the plant-associated microbiome have been observed in certain plant species (sunflowers, rice, etc., [121]) and experiments indicate that enhanced plant growth following CP seed treatment may be attributed to an increased abundance of some beneficial microbes. On the other hand, the application of RONS and UV irradiation in CP has been recognized for its efficacy in deactivating some microorganisms and their spores within seed microflora [121]. However, the effects of CP on microorganisms and spores depend on the dose and composition of the generated reactive species [121]. These contrasting findings suggest that CP treatment triggered a multifaceted response from seed microflora. Therefore, it is crucial to investigate microbiome alterations in plant parts and the rhizosphere in legumes as well to understand the ecological concerns and mechanisms behind enhanced crop growth and yield following CP seed treatments.

Recent research highlights the positive influence of plasma treatment in enhancing seed vitality and facilitating seedling establishment by mitigating the detrimental effects of environmental stressors like drought, salinity, heavy metal, and pathogen infections [122,123]. The CP seed treatment enhances stress tolerance by fortifying the antioxidant defense system, elevating the production of phenolic compounds, boosting antioxidant enzyme activities, and influencing stress-related gene expression [122]. It becomes imperative to delve into the effects of CP seed treatment on reducing both biotic and abiotic stresses in legumes, considering various crop growth stages. A comprehensive exploration of short-term and long-term stressors is essential to understand whether CP can effectively

ameliorate their negative effects. This is particularly relevant in the context of ongoing climate changes.

CP seed treatments to enhance SNF and crop production in legumes are still at the experimental stage. The regulation and validation of CP seed treatment are critical for safety and to ensure accurate outcome predictions. The commercialization of CP seed treatments faces challenges attributed to observed variations in the composition and concentration of plasma constituents. These variations depend on factors such as gas composition, gas flow rate, power level, frequency, treatment time, plasma chamber type, and the diverse responses of different plant species to CP. The regulatory approval for CP seed treatment has been limited due to these inconsistencies across experiments, emphasizing the need for comprehensive data demonstrating efficacy, safety, and reproducibility, with particular attention to potential health concerns. Work on securing approvals from regulatory agencies, including the Environmental Protection Agency (EPA), Food and Drug Administration (FDA), and the United States Department of Agriculture (USDA) in the United States, or with relevant regulatory agencies in specific jurisdictions, is imperative for the commercialization of this seed treatment as an agronomic approach to enhance nodulation, SNF, and crop growth and yield in legumes.

Despite the practicality of CP across a wide range of crop species, its advantages extend to ensuring the safety of food products for human consumption by avoiding hazardous agrochemicals and fertilizers to boost crop yields. This safety aspect is crucial for consumer acceptance and the widespread adoption of CP technology in the agricultural sector. However, it is essential to note that the impact of CP seed treatments on food quality can vary depending on the specific crop and treatment approach. Studies have indicated that fine-tuning treatment approaches, such as employing milder or intermittent treatments, can mitigate potential detrimental effects on quality attributes. Hence, extensive research is needed to investigate the safety of food or feed derived from CP seed-treated plants for human or animal consumption.

Finally, careful consideration must be given to the economic advantages when assessing the incorporation of CP as an agronomic practice. This thoughtful evaluation is crucial for ensuring the seamless integration of CP into the agricultural industry, promoting its successful adoption and implementation.

10. Conclusions

The necessity for sustainable agriculture and the demand for increased food production have redirected research initiatives towards exploring options to improve plant productivity while minimizing the use of N fertilizers. Notably, CP seed treatments have demonstrated promising outcomes as an agronomic approach, effectively enhancing plant growth, SNF, and seed yield in legumes.

CP seed treatments influence root exudation, modifying flavonoid production, potentially impacting communication between the host plant and rhizobia. These treatments also alter root phytohormone levels, including cytokinin (tZR) and cytokinin/auxin ratio, which are crucial for overall plant development and active nodule formation. CP treatments alter leghemoglobin production, facilitating nitrogen fixation process. The increased leaf area, thickness, leaf N, and chlorophyll content, along with parameters like plant height, stem diameter, branch number, and shoot weight, serve as evidence of CP's role in enhancing photosynthesis. The improvements in plant growth and development associated with plasma treatment are also indirectly evident through the upregulated expression of antioxidants, pathogen resistance, and photosynthesis-related genes, and enhanced levels of ATP, TOR, and GRF. This enhancement is thought to contribute to improved nodulation by providing ample photosynthates to developing root nodules and supporting the N fixation process. Notably, CP seed treatment affects root system architecture, promoting increased root length, augmented lateral root production, and overall root biomass. Increases in photoassimilate allocation to roots and the upregulation of the *GmEXP1* gene expression are

identified as factors behind enhanced root growth in plants treated with CP. In summary, CP seed treatments significantly enhance plant growth, development, and SNF (Figure 2).

Adjusting the configuration of the gas used, gas flow rate, power levels, frequency, treatment time, and plasma chamber type allows for the modification of the composition and concentration of various constituents of CP. The reviewed studies have employed different approaches, yet a commonality among these diverse CP seed treatments is their favorable influence on various legume species. To enhance the technology's commercial viability and make it more accessible to farmers, there is a need for the extensive validation of its effects. This involves assessing the reliability of these effects in real field production under various environments. Additionally, thorough evaluations of regulatory aspects, health implications, economic considerations, and ecological concerns are crucial steps in ensuring the successful commercialization of this technology.

In general, there is ample evidence indicating that CP technology has the potential to enhance plant performance, influencing both SNF and seed yield. Despite these positive outcomes, the precise mechanisms at play are not yet fully understood. Conducting additional studies at various levels, such as epigenetic, transcriptomic, and proteomic, can offer valuable insights. These comprehensive investigations will contribute to a more thorough understanding, enabling informed decisions regarding the utilization of CP as an agronomic application.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/crops4010008/s1, Table S1: Effect of cold plasma on symbiotic nitrogen fixation and plant growth related traits in different legume crops.

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