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Endophytes eradication technology in the production of symbiotically modified grasses

Technologia eradykacji endofitów w produkcji traw modyfikowanych symbiotycznie

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1. Introduction

Fungal endophytes that live inside plant tissues without causing any apparent symptoms in the host plant, are important components of plant micro-ecosystems (Grabka et al., 2022). Cool-season grasses (C3) establish symbioses with fungal endophytes of the genus *Epichloë.* These endophytes form a group, commonly known as Clavicipitaceous endophytes (Hypocreales, Ascomycota) (Leuchtmann et al., 2014), and are characterized by intercellular colonization of leaves, culms and meristematic tissue with transmission of the fungus via the seeds (White et al., 2016). The mutualistic symbiosis of the *Epichloë* and the grass confers a number of benefits for the host plant such as pathogens, insects and nematodes resistance, drought tolerance (Saikkonen et al., 2016) and manage vital mechanisms such as photosynthesis, osmotic regulation, antioxidants, and the activity of essential enzymes of host physiology processes under abiotic stressors, thereby promoting the health, growth, persistence, seed production and seed survival of host grasses and improve the competition with other plant species (Li et al., 2017; Wang et al., 2020). However, grasses inhabited by wild endophytes can pose a threat to livestock due to the production of toxic compounds (Schardl et al., 2013). Safe associations are those with desirable metabolites profile, the so-called novel endophytes (Kaur et al., 2015). Nevertheless, the introduction of such endophytes to the plant requires prior removal of toxic, endosymbionts naturally occurring in the cultivar. The purification process is challenging and time-consuming activity. It is usually carried out by breeding, with the use of fungicides or high temperature (Cheplick & Faeth, 2009).

The development of cold atmospheric plasma (CAP) technology has offered a promising and environmentally friendly solution for addressing global food security problems. Besides many positive effects, such as promoting seed germination, plant growth, and development, CAP can also serve as sterilizing agent. It can be considered as a method for decontamination of microorganisms alternative to the traditional use of fungicides (Florescu et al., 2023). It also seems to be promising method of eradication endophytes from grasses.

2. Literature Review

2.1. Food production challenges

The UN report 2024 revealed that the global population will grow from 8.2 billion in 2024 to approximately 10.3 billion by the mid-2080s, increasing an urgent concern of the need for food security (Lay, 2024). The efficient agriculture that is preferably sustainable is required, because during the last half century, increases in food production maximised with the growth in global consumption of chemical pesticides, significantly increased the adverse consequences on the environment and human health (Tudi et al., 2021).

For thousands of years, one of the main factors affecting food supply and the evolution of human society has been plant diseases (Palmgren et al., 2015). According to estimates, the diseases cause 10–15% of the world's major crops to be lost each year, with direct economic losses of up to hundreds of billions of dollars. For instance, yield loss estimates for wheat (10.1–28.1%), rice (24.6–40.9%), potatoes (8.1–21.0%), soybeans (11.0–32.4%), and maize (19.5–41.1%) range globally and by hotspot if the diseases are not properly controlled (Savary et al., 2019). Globalisation facilitates the faster spread of viruses, as evidenced by the increasing occurrence of quarantine diseases. While the number of available insecticides is declining, the number of resistant infections is steadily rising (Bebber et al., 2014). A higher rate of disease incidence will emerge from these facts, as well as climate change and the creation of more conducive conditions for infections. This could lead to manufacturers utilising more chemicals, which would raise the proportion of pesticide residues in food products (https://ec.europa.eu/food/horizontal-topics/farm-forkstrategy_ en).

Frequent droughts and uneven rainfall distribution have made the climate issue worse, and the past ten years have been the warmest on record (Spinoni et al., 2015). The production of plants and the management of diseases are made more challenging and complex by the changing global climate. Temperature and CO_2 concentration increases may alter plant's vulnerability to infection by particular pathogens, which could result in the formation of new issues with diseases (Elad & Pertot, 2014).

Currently, a large portion of crop production relies on synthetic fungicides to prevent losses from fungal diseases. This widespread use of these chemicals has increased adverse health and environmental effects and developed fungicide-resistant pathogens (Fisher et al.,

2024). The urgent need to make food production systems more sustainable, preserving both quantity and quality while reducing the negative impact on the environment and health, has led scientists to focus on the use of fungi as biological control agents for the control of plant diseases (Jiang et al., 2019; Palmieri et al., 2022)

Furthermore, European's concern for the condition of our natural environment is evidence of the growth of environmental awareness in recent decades. They are also interested in more sustainable intensive agricultural production systems. The importance of grassland systems in Europe will therefore be increasing even faster. Recent studies have demonstrated that boosting the biodiversity of grassland agroecosystems may lead to livestock feed of a calibre equivalent to that of intensively fertilised grass, which could mitigate the adverse effects on the environment since nitrogen fertiliser use would be drastically reduced (Huyghe et al., 2014)

2.2. Grasses and their importance

Grassland ecosystems are important to the economy and to human survival. Since permanent grasslands make up around 25% of the world's land area, they are a part of our agricultural environment. Combined with rough grazing areas, meadows and pastures make up almost 30% of the agricultural land in Europe, accounting for more than 20% of the total area under cultivation. Europe has gradually seen a decline in grassland areas in recent decades. Grassland fragmentation, land-use abandonment, grazing population reduction, and the growing use of concentrates in animal feedstuffs are some of the causes of this(Huyghe et al., 2014) Grasslands are therefore crucial in mitigating the adverse environmental effects that economic expansion may have. They have a significant impact on the standard of living in both urban and rural communities. They have high biocenotic value, which boosts biodiversity, in addition to their indisputable aesthetic value (Gunnarsson et al., 2017), according to their research, people who live in urbanised areas recognise and value the issue of biodiversity.

The European Community has long been concerned about issues like pollution, climate change, biodiversity loss, and the destruction of the natural environment. As a result, the European Union developed the European Green Deal, which was unveiled on December 11, 2019. It includes the most recent growth strategy and action plan to address the issues

raised, together with the initiatives that were introduced on May 20, 2020: According to the Farm to Fork Strategy, by 2030, 50% less chemical plant protection and hazardous pesticides will be applied, 50% of the nutrients will be lost while maintaining soil fertility, at least 20% less mineral fertiliser will be applied, and organic farming will be supported to occupy 25% of all arable land in the EU(https://food.ec.europa.eu/horizontal-topics/farm-fork-strategy_en). These goals are extremely challenging and demanding. But they also provide the European Community a chance to grow dynamically and sustainably and become more competitive on the world market. The extended phrase "From Farm to Fork" now reads, "Our food, our health, our planet, our future," and it sums up the importance of our long-term objectives well (https://food.ec.europa.eu/horizontal-topics/farm-fork-strategy_en). Successfully achieving the objectives can only be ensured by contemporary, creative solutions that offer an alternative to the ones already in use. The need for more sustainable, efficient, and non-chemical-based, safer technologies for intensive agricultural production is growing steadily. To ensure the success of the European Green Deal policies and satisfy consumer expectations, creative and contemporary solutions that are alternatives to those now in use are required. Consequently, the pursuit of novel non-chemical methods to enhance plant growth and development, speed up seed germination, and increase resilience to biotic and abiotic stressors has recently gained significant attention (Pańka et al., 2021).

2.3. Perennial ryegrass and its benefits in farming

Perennial ryegrass (*Lolium perenne* L.), originated from Europe, Asia, and Northern Africa, is a cool-season perennial grass with a breeding history of more than 100 years that is produced all over the world (Sun et al., 2020). This temperate perennial forage grass is distinguished by its quick establishment, outstanding quality, and great productivity during the cool season. It has dark green, glossy, smooth, glabrous, and noticeably ridged leaf blades that are folded in the bud. Small, claw-like auricles and a thin-membranous, obtuse ligule measuring 0.5 to 2.5 mm are features of the tight collar. The base of leaf sheaths is frequently crimson. The spike-like inflorescence of flowering culms, which range in length from 50 to 100 cm, with 5 to 40 sessile, awnless spikelets arranged edgewise to the rachis. Perennial ryegrass thrives in irrigated areas and warm, humid climates. It can tolerate soils with a pH between 5.0 and 8.3 that have poor drainage. The ideal temperature range for growth is 20 to 25°C

(Hannaway et al., 1997). In temperate pastoral farming systems, which consist of grazing livestock, perennial ryegrass is mainly cultivated due to its quick establishment and longer growing season as well as its grazing quality relative to other perennial grasses (Richardson et al., 2023). Perennial ryegrass has the potential to be used as a substitute protein source in animal diets, particularly in monogastric feeds like those for pigs. This is a major benefit. According to a study, ryegrass might be processed using a green biorefinery to produce protein concentrates that could take the place of conventional protein sources like soybean meal, offering livestock nutrition that is more environmentally friendly (Ravindran et al., 2021). Additionally, ryegrass's high digestibility makes it a vital part of animal feed in places like New Zealand, where it is frequently combined with white clover to improve pasture quality. This makes it a great source of fodder for ruminants (Cranston, 2017). Due to this high digestibility factor, which leads to efficient energy conversion in animals, making it perfect for dairy, sheep, and meat sectors. It lowers production costs and eliminates the need for repeated replanting by producing strong yields over several year(https://alfagenseeds.com.au/seed-category/pasture-grasses/). Ryegrass is essential for improving soil quality and reducing erosion. Because of its robust root system, the soil is stabilized and erosion on slopes and other susceptible regions is avoided. Compacted soil is broken up by the roots, which improves water infiltration and aeration. By fixing nitrogen, the plant raises the nutrient content of the soil for subsequent plantings. Because of this characteristic, it is useful for farmlands and gardens where preserving soil fertility is essential. Ryegrass helps ensure long-term soil stability and resistance to severe weather by establishing a strong root system (https://compassgm.co.uk/pros-and-cons-of-ryegrass/). As outstanding cover crops are ryegrasses, including annual and perennial varieties. They increase the amount of organic matter in the soil, reduce weeds, and improve soil structure. Their extensive root systems are great for enhancing soil health and halting soil erosion. In crop rotation systems, where preserving the health of the soil is crucial, these cover crops are extremely advantageous(https://www.farmraise.com/blog/a-comprehensive-guide-to-ryegrass-farming). Compared to other perennial grasses, it can be cultivated at a comparatively low price. It spreads quickly and flourishes with little help, particularly in areas with enough rainfall. Ryegrass uses fertilizers more efficiently than many other grasses, even though it needs nitrogen, therefore fertilizer expenditures are moderate. Even if it has a high nutritional requirement, yet it reacts well to organic or synthetic nitrogenous fertilizers with

moderate levels of potassium and phosphorus, maximizing input costs without sacrificing output. Sustained development and excellent yields are supported by applying nitrogen fertilizers based on soil tests and dividing treatments over the growing season. By fixing atmospheric nitrogen, legumes like white clover can be incorporated into ryegrass swards to reduce the demand for fertilizer and further cut input costs (https://pasture.io/ryegrass/perennial-spring-growth).

2.3.1. Use of perennial ryegrass for stunning lawn and forage production

For homeowners looking to create lush lawns, perennial ryegrass is a favourite cool-season grass because of its vivid green colour and quick growth. In comparison to other cool-season grasses, it has a respectable degree of drought tolerance and good resistance to lawn diseases, which enable it to stay vibrant even during times of lower rainfall (Cool et al., 2004). Since perennial ryegrass seeds usually sprout in 5–14 days, damaged areas can be repaired, or a lawn can be established quickly. In a comparatively short amount of time, this quick growth guarantees a dense, rich lawn. It generates a lawn that is brilliant, deep green, and has fine to medium-sized leaf blades that give it a pleasing, consistent look. Because of its aesthetic appearance, it is quite popular for decorative lawns. It is perfect for high-use places like parks, sports fields, and family gardens because of its remarkable resistance to wear and foot traffic. Even with heavy use, its durability helps keep a tidy, healthy lawn. After it has established, it needs moderate watering and frequent mowing. Due of its rapid recovery from damage, less frequent overseeding is required. Additionally, modern cultivars exhibit better resistance to common lawn diseases, improving the beauty and health of lawns (https://www.grassseedonline.co.uk/collections/lawn-landscaping).

Even though perennial ryegrass already has several advantageous qualities, breeders have been able to enhance it for years. Consequently, more than 100 cultivars of *L. perenne* have been created, each with somewhat different characteristics from the original species. Additionally, these are combined to create a healthy, well-suited grass, some cultivars such as Adagio, Barolympic, Ceretec Centurion, Corsica, Esquire, Eventus, New Orleans, Shorty (https://plantura.garden/uk/lawn/varieties/perennial-ryegrass). With considerable breeding efforts worldwide, perennial ryegrass is one of the most popular grass species for establishing

grasslands in temperate regions. It is particularly popular in Europe, where it accounts for up to 50% of all grass seeds sold. This green, highly tillering grass can generate large amounts of herbage over several years that are suitable for ruminant animal's diets. Because it can tolerate animal treading and the somewhat confined defoliation brought on by grazing better than other temperate grass species, it is especially well suited to grazing. When it comes to perennial ryegrass, the spike emergence date varies greatly, spanning over 7 weeks. Breeders have created late cultivars that are suited to a lengthy spring grazing season, as well as early and intermediate cultivars that are more practical for ensiling the initial cut and grazing or cutting the regrowth as green fodder. European National Lists typically divide perennial ryegrass varieties into multiple earliness groups based on spike emergence date for convenience (Sampoux et al., 2011).

It is consistently among the highest for crude protein (CP; 200 g kg^{-1}), equine digestible energy (DE; 2.35 Mcal kg^{-1}), and bovine metabolizable energy (ME; 2.6 Mcal kg^{-1}), moderate for non-structural carbohydrates (NSC; 136 g kg^{-1}), and among the lowest for neutral detergent fiber (NDF; 518 g kg^{-1}) and additionally, horses and cattle prefer perennial rye grass (Catalano et al., 2020). By using cool-season perennial grasses, stocker cattle farmers may see an improvement in returns and a reduction in annual establishment costs (Islam et al., 2011). Breeders of perennial ryegrass are motivated by market needs to produce cultivars with varying maturity, high, consistent yield, and good dry matter quality that are resistant to severe weather conditions like frost and drought. It states that the original breeding material needs to be diverse, containing a variety of genotypes from either wild populations or cultivars (Sokolović et al., 2010).

2.3.2. The use of perennial ryegrass for wildlife and soil conservation

A dense, tufted grass cover produced by perennial ryegrass offers cover and nesting places for a variety of fauna, including small mammals and ground-nesting birds. By providing cover and protection, its blades promote biodiversity in places like waste ground, harsh pastures, and roadside verges. Beneficial insects, which are essential for pollination and preserving ecological balance, are drawn to and shelter in the grass.

This relationship promotes the larger food web and enhances the local biodiversity (https://www.wildlifetrusts.org/wildlife-explorer/grasses-sedges-and-rushes/perennial-rye-grass).

Wildlife can also benefit from perennial ryegrass as feed. Elk, deer, rabbits, wild turkeys, geese, coots, widgeons, and other ducks graze this feed. Seeds are the food source for Quailand songbirds such the Savannah sparrow, golden-crowned sparrow, white-crowned sparrow, and brown towhee, as well as pocket mice. Soil conservation applications are a good fit for perennial ryegrass. It effectively reduces soil erosion because of its wide, shallow, fibrous root structure. It is advised to be used either by itself or as a fast-starting ingredient in combinations, where it quickly creates cover and promotes the establishment of more resilient or long-lived species (https://greatbasinseeds.com/customer-favorite-oro-verde-perennial-ryegrass/). In delicate environments, its fibrous, deep root structure efficiently stabilizes soil, minimizing flow and erosion. Because this soil stability preserves habitat quality and stops ecosystem degradation, it benefits wildlife at the same time. The ability of perennial ryegrass to use a lot of nitrogen from biosolids and manure contributes to the recycling of nutrients in the environment. In order to maintain wildlife figures, this capacity promotes healthy soil and plant communities (Hannaway et al., 1999).

2.3.3. Production of perennial ryegrass in Europe

Perennial ryegrass is the leading grass species in Europe, which yields around 90,000 metric tons of grass annually, or almost 50% of all grass production. It is widely used for turf and fodder, and production volumes for both uses are currently about equal. Due to its excellent production and quality in oceanic conditions with mild winters and adequate precipitation, the species is preferred in Northern and Western Europe. Because perennial ryegrass is less productive at temperatures above 25°C, its usage is considerably restricted in Southern Europe by warm, dry summers, where more drought-resistant species are preferred (https://www.seedworld.com/europe/2016/05/03/breeding-targets-ryegrass-europe/). It is the most widely used species of fodder grass in Europe due to its high nutritional value for ruminant animals, rapid establishment, strong capacity for renewal, and tolerance to frequent cutting and grazing. But unlike other cool-season forage grass species, perennial ryegrass does not fare well in adverse environmental conditions.

As a result, climate change makes it extremely difficult to grow perennial ryegrass in the Baltic/Nordic region (https://eeagrants.org/archive/2014-2021/projects/LV-RESEARCH-0008). It was first tested for the UK National List (NL) in Ireland in the 1970s. The Republic of Ireland (ROI) used five sites, while Northern Ireland (NI) used one as part of the UK NL network. The two locations used different testing methods. Early variety lists prioritized persistency and yield, which improved output, especially in the spring. Sales of grass seeds have been dominated by these lists, which have had a significant impact. However, constraints are indicated by decreased reseeding levels and usage of late-maturing types. Digestibility and other quality parameter testing have increased because of farmer's increased demand for higher nutritional value. Improvements in perennial ryegrass varieties have been shown to boost milk and meat production, according to scientific research; nevertheless, more specific data is required to satisfy the needs of local farmers (Grogan & Gilliland, 2011).

2.4. Challenges in the production of perennial ryegrass

2.4.1. Effects of drought and heat stress

The growth and developmental stages of perennial ryegrass are negatively impacted by heat stress and drought, which results in notable output reductions. Perennial ryegrass demonstrates significant phenotypic adaptations to cope with various environmental stresses. Lack of soil moisture during drought stress has a direct impact on plant development and growth. Drought stress impairs the ability of perennial ryegrass roots to absorb water, upsetting the equilibrium between root water uptake and canopy transpiration. Parameters include leaf relative water content, dark respiration rate, wilting coefficient, tiller number, and biomass of both aboveground and root systems exhibit declining patterns as drought stress increases (Patel et al., 2015). In addition, the plant wilting results from the significant inhibition of normal physiological activities in perennial ryegrass seedlings caused by the combined effects of heat stress and drought (Rahman et al., 2022).

Generally, proper soil conditions are essential for seed development and seedling growth. These parameters vary based on the type of plant and include variables like temperature and humidity (Reed et al., 2022). Seed germination is frequently negatively

impacted with drought and higher soil temperatures, particularly influence intricate physiological metabolic reactions including enzyme activity and seed cell division (Guo et al., 2024). The appropriate amount of water is necessary for seed germination in perennial ryegrass since it facilitates enzyme absorption and activation when in its absence slows germination, which decreases the effectiveness rate of the total germination process (Wang et al., 2024). Elevated soil temperatures accelerate the transpiration process which affect seed germination by decreasing the efficiency of seed hydration. Additionally, heat stress can lower enzyme activities and disrupt metabolic functions linked to seed germination(Wang et al., 2024; Zou et al., 2023). Perennial ryegrass grows well at temperatures between 15 and 24°C; above this range, heat stress is produced, which restricts its use in temperate climates. Perennial ryegrass seedling traits, including relative germination rate, germination index, vitality index, and root and seedling length, typically exhibit a declining trend when exposed to heat stress. The germination rate drastically drops at temperatures above 35°C, and it becomes steadily more obvious how heat stress inhibits the formation of roots and stems (Javaid et al., 2022). According to the research, the drought stress can minimize ryegrass biomass by up to 79%, cut the frequency of annual harvests from four to three, and reduce the production of fresh grass and hay by 45 and 28%, respectively, when there is extreme water scarcity (Kemesyte et al., 2017; Shariatipour et al., 2022). In reaction to drought stress, plants usually increase below ground biomass while limiting aboveground biomass to minimize excessive demand for water. By strategically restructuring biomass, roots become more capable to absorb water, increasing plant resistance to drought stress(Yang et al., 2014). As the first organ to sense and react to water deficiencies, the root system is essential for development and biomass buildup as well as for absorbing water and nutrients and using soil resources (Wasaya et al., 2018).

Perennial ryegrass adapts to drought by growing thicker, narrower leaves and increasing the surface area of its roots to absorb more water. Although root malformations and decreased numbers may occur, this plant increases in root length and area during severe drought circumstances and this strategy shows unique adaptive methods used by plants in arid regions (Wang et al., 2024). When perennial ryegrass plants experience heat stress, at temperatures exceeding 35°C can cause direct plant death; the leave’s original emerald green colour changes to a faded yellow, which eventually turns brown (Chen et al., 2023). The

Damage at the junction of the penultimate and antepenultimate leaf sheaths is the first indicator of heat stress, which can seriously impair ryegrass plant's growth status. At the same time, some leaves start to wilt and progressively become yellow from the base to the tip. There are also signs of damage on lodging and aging leaves. Reduced forage covering is the ultimate result of these injuries becoming irreversible if stress continues (Lei & Huang, 2022). The effective number of spikes per unit area can be considerably decreased by heat stress during the development period. It severely impairs reproductive growth by preventing ryegrass's vegetative growth and material transfer, which results in sharp drops in pollen grain weight and protein content. This lowers the number of grains per ear, which impacts the plumpness and thousand-grain weight of the grain and ultimately lowers yield (Jung et al., 2021). In response to heat stress, plants undergo several intricate morphological changes to adapt to high temperatures. Certain thermomorphogenic characteristics, which improve photosynthetic and reproductive capacity under heat stress, can be seen in plants under heat stress. These characteristics include early flowering, expansion of hypocotyl leaves, and elongation of hypocotyls and petioles (Kan et al., 2023). Through adaptive modifications like decreasing plant height, reducing leaf width, decreasing leaf length, and decreasing leaf area, perennial ryegrass improves its heat tolerance (Yang et al., 2024).

2.4.2. Effect of cold stress, disease and pest pressures

Globally, temperate climates cultivate perennial ryegrass, a type of perennial gramineous grass. Therefore, cold stress in the winter and early spring of each year is an inevitable occurrence for perennial ryegrass. Improving perennial ryegrass's capacity to withstand cold stress and recover from it, is essential to enhancing grassland performance. Low temperature reprogrammes gene expression and directly inhibits metabolic enzymes, which are the sources of cold stress's effects on plant metabolism (Chinnusamy et al., 2006). While freezing stress occurs at temperatures below 0 ◦C, cold or chilling stress occurs at temperatures chilly enough (0 to 15 ◦C) to cause the damage without ice crystals forming in plant tissues. Various degrees of frost tolerance are thought to be exhibited by plants from temperate climates (Sanghera et al., 2011). Additionally, low temperature stress suppresses several metabolic processes, changing phenotypic traits. Reprogramming metabolism and gene expression, as well as remodelling cells and tissues, are all part of cold acclimatization

(Förster et al., 2018). During the winter, photosynthesis in temperate grasslands is limited by short days and limited solar radiation, leading to source restrictions. Growth can be limited by mechanisms that prevent cell division and expansion at low temperatures once light conditions improve. Growth becomes sink-limited in these circumstances (Wingler, 2015). Research indicates that there is minimal growth variance between perennial ryegrass accessions under cold stress, indicating a strict physiological regulation of growth at low temperatures. However, the disparities in growth responses between genotypes become more noticeable in more favourable environments. Furthermore, a measure of cell membrane damage called electrolyte leakage can be increased by cold stress and is associated with decreased plant vigour and yield (Förster et al., 2018). Reduced biomass buildup and chlorophyll content are two examples of changed phenotypic features caused by cold stress, which also slows photosynthesis and other metabolic activities. During colder months, these impacts lead to a decrease in forage yield (Miao et al., 2022). As the temperature drops further below zero, the damage gets more severe, and the thresholds of injury for roots and leaves vary (for example, root damage occurs at -10 °C, whereas leaf tissue freezes between -20 and -25 °C) (Iwaya-Inoue et al., 2004).

The production and use of perennial ryegrass are significantly restricted by fungal infections (Altpeter, 2007). Biotrophic infections such as crown rust (*Puccinia coronata* f.sp. *lolii*) and stem rust (*Puccinia graminis* subsp. *graminicola*) are common diseases that impact ryegrass(Jo et al., 2008), and vascular wilt disease (*Fusarium oxysporum*), necrotrophic pathogens disease dollar spot (*Sclerotinia homoeocarpa* F.T. *Bennett*), powdery mildew (*Blumeria graminis* DC.), and leaf spot (*Bipolaris sorokiniana*) (Boutaj et al., 2022). The fungal infection *Pyricularia grisea*, which causes gray leaf spot, kills the leaf blades of all the turf it infests, causing significant damage. When leaf infections spread to the crown region, individual plants may die. Thin, discoloured turf clusters are the outcome of moderate gray leaf spot disease. However, severe outbreaks will swiftly destroy large sections of turf, harm and degrade extensive areas if left untreated. In warm, humid weather (usually between 26 and 32 °C), Gray leaf spot can appear rapidly and damage turf in within days. Stands of immature or recently planted perennial ryegrass are particularly susceptible, and significant harm frequently happens within 48 hours when there are favourable conditions for this disease (https://www.lebanonturf.com/education-center/diseases/gray-leaf-spot).

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Numerous plants are harmed by the fungus *B. sorokiniana*, which can cause necrotic lesions, wilting, or even death (Smiley et al., 2005). In countries including India, Nepal, Canada, Scotland, and Brazil, *B. sorokiniana*-caused diseases have been shown to significantly affect the crop yield (Iftikhar et al., 2009). Another important limiting factor that affects the growth of perennial ryegrass is insect infestations, especially those linked to drought and overgrazing, which also negatively affect the ability of perennial ryegrass to endure (Hewitt et al., 2024). For instance, when the population of African black beetles (*Heteronychus arator*) reaches 20 per square meter, the instar larvae cause significant harm by feeding on grass roots close to the crown ryegrass seedling (Bell et al., 2011; Ferguson et al., 2019). Furthermore, the year-round presence of the root aphid (*Aploneura lentisci*) in New Zealand can drastically lower ryegrass production (Müller, 2019). Grass blight is caused by fungi of the genus Fusarium, which can kill grass at various stages of growth. Both stains in established turf and seedling deaths during lawn establishment are caused by them. Extremely wet conditions (such heavy rain or intensive watering) may precede high temperatures, which might exacerbate the severity of the sickness. Brown grass with black roots and stolons is considered damaged. Symptoms on leaves could be rings or stripes with healthy plants in the middle (Wiewióra et al., 2015).

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2.5. Applications of endophytes in perennial ryegrass

Fungi known as *Epichloë* endophytes are asymptomatic and inhabit the intercellular spaces of grasses' leaf sheaths and shoot meristems. Their vertical transmission through seeds is one of these fungi's defining characteristics (Moon et al., 2004; Selosse & Schardl, 2007). The mycelium grows into kernel primordia in this process. The genotype of the endophyte in seeds from infected plants is identical to that of the mother.

The genus *Epichloë* contains symbiotic endophytic fungus that may help the grass they colonise grow and develop. *Epichloë* spp. may have a wide range of beneficial effects on the plant, such as enhanced resilience, a greater quantity of dry matter and green matter, and a higher yield of the seeds (Clay & Schardl, 2002). In addition, these plants exhibit enhanced resistance to the adverse environmental circumstances, such as better tolerance to water scarcity (Schardl et al., 2004) , pathogen infection and pest damage. The primary cause of these effects is the endophyte's potential influence on plant metabolic processes, as well as the colonised plants generation of hormones and other metabolites (Bastias et al., 2017).

By using symbiotic organisms to improve grass biologically, less chemical plant protection products and synthetic fertilisers would be applied, benefiting the environment (Ayilara et al., 2023; Ons et al., 2020; Pirttilä et al., 2021).It would also contribute to more economical use of water resources in the locations where grass and endophytes associate. With the use of endophytes, many varieties of grass grown for feed and seeds or to shape landscapes would be cultivated in a more sustainable manner, low costs of care, low maintenance, and less harmful to the environment. Such a solution would present an opportunity to use local species and varieties of grass (Kauppinen et al., 2016). Mycorrhizal, arbuscular fungus (such as *Rhizophagus* etc.), and fungal species of the genus *Epichloë* (Clavicipitaceae, Ascomycetes), formerly *Neotyphodium* (the asexual form of many *Epichloë* species), are among the diverse and broad symbiotic relationships that grasses establish with microorganisms (Rodriguez et al., 2009; Selosse & Schardl, 2007; Żurek et al., 2012). The relationships between *E. festucae var. lolii* (formerly *N. lolii*) infecting perennial ryegrass, *E. coenophiala* (formerly *N. coenophialum*) infecting tall fescue (*Lolium arundinaceum*), and *E. uncinata* (formerly *N. uncinatum*) infecting meadow fescue (*Festuca pratensis*) are the most thoroughly researched (Leuchtmann et al., 2014) these listed grass species are crucial for forage

production and turf purposes. Additionally, endophyte infections of wild Triticum and Aegilops (a Triticum-related plant) were discovered (Marshall et al., 1999). Nonetheless, this proves there is a great diversity of *Epichloë* endophytes in nature, which could provide a plentiful source of strains that might be applied in breeding programs to create new commercial crop grass cultivars that are more resilient to stressful conditions (Card et al., 2014; Simpson et al., 2014; von Cräutlein et al., 2021). Even though there are now no practical applications yet, their potential is drawing increased attention from researchers worldwide, and they might get important soon.

2.6. Disadvantages of endophyte presence in grasses

Grass infected with endophytes can produce a variety of alkaloids (Faeth & Saari, 2012). They can reduce the quality of fodder by protecting the host grass from grazing vertebrates like horses, sheep, and cattle, while others increase the association's resistance to pests and diseases (Qin et al., 2016). In the natural environment, endophyte-infected grasses are therefore more competitive and persistent than their endophyte-free, more often grazed counterparts due to those anti-quality characteristics. Egopeptines (ergovaline, ergonovine, and ergotamine), clavines, lysergic acid, and its amides, as well as diterpene alkaloids (lolitrems), are the most toxic substances that affect animals (Malinowski & Belesky, 2019). Ryegrass staggers in livestock, a neuromuscular condition that primarily manifests in late spring, summer and autumn, are caused by endophyte-infected perennial ryegrass that produces lolitrems (Di Menna et al., 2012). Ergot alkaloids have the potential to cause toxicoses in grazing animals, such as bovine fat necrosis, fescue foot, and fescue toxicosis (summer syndrome) (Arnold et al., 2014). The harmful effects of diterpene or ergot alkaloids on grazing animals in Europe are not well documented in the literature (Di Menna et al., 2012; Philippe, 2016). Numerous factors influence the kind and number of alkaloids present in plant-endophyte relationships. The most crucial ones are the endophyte and grass species and genotypes. Alkaloid synthesis, however, may also be significantly impacted by environmental factors, plant age, or the degree of endophyte colonisation present (Żurek et al., 2017). There is a lot of promises for lawn turf, sports facilities, and landscaping applications from endophyte strains that produce alkaloids that are somewhat harmful to cattle as well as other defensive compounds in the host grass. Degraded ecosystems in Europe may also be

successfully restored using these kinds of grass-endophyte relationships, which are long-lasting and require minimum maintenance (Pańka et al., 2021).

2.7. Endophytes eradication methods

- Endophytes eradication by physical methods

To drive out endophytes in seeds, physical techniques have been studied as a substitute to chemical and biological procedures. Particularly, heat treatment has attracted a lot of attention. According to preliminary studies, endophytes within seeds can be successfully eradicated by applying controlled heat without compromising their viability.

In order to drastically lower endophyte viability (Rolston et al., 1986), developed a hot water treatment procedure in which seeds were immersed at temperatures between 58 and 65°C for 10 to 30 minutes. In Australia and New Zealand, this method has since been improved and integrated into commercial seed washing procedures. Another non-aqueous option that has been considered is dry heat treatment.

According to (Kirkby et al., 2010) endophyte infection rates were considerably decreased when endophyte-infected ryegrass seeds were incubated at 60°C for a few hours. However, as high temperatures or prolonged exposure might hinder germination or seedling vigour, both dry and wet heat treatments need to be precisely calibrated.

The sterilizing potential of radiation-based techniques, such as gamma irradiation and ultraviolet (UV-C) light exposure, has been also investigated in endophyte eradication techniques. Although these methods have demonstrated some promise in lowering fungal pollutants on the surface, their effectiveness against systemic endophytes is limited because of their limited ability to penetrate tissue (Borzouei et al., 2010). In addition, the practical application of high-dose irradiation is limited since, it frequently results in irreparable seed damage. Furth more, physical eradication techniques, especially heat treatments, provide a practical way to control endophyte contamination in seed batches. Although these techniques work well in controlled settings, they need to be optimized to strike a compromise between endophyte suppression and seed viability.

- Endophytes eradication by chemical methods

In breeding and commercial seed production, chemical treatments to eliminate endophytes especially *Epichloë* species have been a typical method to guarantee forage safety and seed purity. According to (Cheplick et al., 2009), endophytes can offer host plants substantial advantages like pest and drought resistance, but they can also create poisonous alkaloids that are bad for cattle, such ergovaline and lolitrem B. The goal of chemical eradication techniques is to eradicate these endophytes while preserving seed viability and reducing detrimental impacts on plant performance. The effectiveness of systemic fungicides, such triazoles and benzimidazoles, has varied based on the active component, concentration, and seed penetration. For example, benomyl used as a seed dressing decreased endophyte transmission rates in *Lolium perenne*, although total eradication was rarely attained, as shown by(Latch & and Christensen, 1982). Similarly, studies have shown that diethofencarb and flutriafol can reduce endophyte levels in tall fescue with varying degrees of efficacy (Bacon & White, 1994).

However, the placement of endophyte hyphae, which are entrenched within the seed's inner tissues (embryo and scutellum) and are challenging to reach by external application, frequently limits the efficacy of fungicides (Latch, 1993).

Furthermore, in recent years, there has been less enthusiasm for their use due to fungicide residues, environmental concerns, and possible phytotoxicity. Chemical methods have involved injecting chemotherapeutic drugs or systemic fungicides into plants or seedlings. Compounds like carbendazim and thiabendazole sprayed to young seedlings were explored in early research (Gorfu & Sangchote, 2003). Although a certain amount of fungal colonization was reduced, these treatments frequently had phytotoxic side effects, especially when given to sensitive genotypes or at greater dosages.

Another strategy to facilitate in the elimination of endophytes is the chemical soaking of seeds in solvents (such as ethanol, acetone, or aqueous surfactants). Seeds treated in diluted ethanol-based solutions showed partial endophyte eradication, according to (Miles et al., 1998). The reasoning behind this is because organic solvents can inactivate hyphae by rupturing fungal membranes or penetrating seed tissues.

Although this approach frequently produced inconsistent results, it also raised questions about seed viability and regulatory permission for solvent residues. Another widely

used technique for lowering surface microbial burdens is seed disinfection with sodium hypochlorite (NaOCl) or home bleach. It works well against epiphytic fungi, but it is not very efficient against endophytes that live inside seeds (Gilbert et al., 2023). However, to increase overall efficacy, it is sometimes combined with physical techniques (such as heat or scarification). Through host-induced resistance or competitive exclusion, some chemical elicitors and growth regulators, such as analogues of salicylic acid and jasmonic acid, have been investigated to displace or reduce endophytic colonization (Faeth & Sullivan, 2003). But rather than being direct eradicants, these techniques usually work as modulators of host endophyte interaction. Although endophyte treatment has historically relied heavily on chemical methods, interest in alternate approaches has grown due to these methods' shortcomings, which include incomplete eradication, environmental and phytotoxic concerns, and uneven performance.

Physical or biological control methods have gradually replaced chemical treatments in the seed industry, however chemical treatments may still be useful in some situations, such as quarantine programs or pre-breeding.

- Endophytes eradication by biological and genetic manipulation methods

The use of biological techniques to eradicate endophytes is a new and environmentally friendly strategy for controlling undesirable fungal endophytes in grasses. In contrast to physical or chemical techniques, biological strategies aim to suppress or displace endophytic colonization without sacrificing seed viability or environmental safety by taking use of natural antagonistic interactions, plant-microbe dynamics, or ecological niches (Clay & Schardl, 2002). For instance, it has been demonstrated that *Pseudomonas fluorescens* and *Bacillus* species create antifungal compounds, which in turn decrease seed-borne fungal infections (Compant et al., 2005). Preliminary research indicates that co-inoculation with antagonistic rhizobacteria may hinder endophyte establishment in grass seedlings, despite the lack of direct proof against *Epichloë* endophytes (Rojas et al., 2014). The antifungal capabilities of *Trichoderma* spp. have also been investigated for their potential to inhibit endophytic colonization in tall fescue and ryegrass. According to (Mastouri et al., 2010) *Trichoderma harzianum* may prevent *Neotyphodium coenophialum* from developing its hyphal stage in vitro, which may indicate that it could be used as a seed treatment or in soil.

A different biological approach is to choose or breed host plants that are resistant to reinfection or that do not naturally colonize systems with endophytes. Studies have revealed that the sensitivity of grass genotypes to endophyte colonization varies significantly (Mwangi et al., 2024). Breeding strategies can indirectly eradicate endophytes over multiple generations without requiring chemical or physical treatments by choosing for endophyte-free or low-colonization lines. To replace toxic endophyte strains in current plant populations, competitive displacement by benign or non-toxic *Epichloë* strains has been suggested. For example, seedlings inoculated with a specific strain that does not produce ergot alkaloids can prevent colonization by pathogenic strains (Johnson et al., 2013). This approach maintains the mutualistic advantages of endophyte colonization, such as pest and drought resistance, while simultaneously addressing toxicity issues. Endophyte eradication via biological techniques provides a viable and possibly long-term way to control harmful endophyte strains in fodder grasses. While still in the early stage considering to chemical and physical treatments, strategies including host selection, microbiome engineering, and microbial antagonism offer a lot of potential for future.

2.8. Novel endophytes in grasses

The term "novel endophytes" refers to the introduction of new endophytes into commercial grass varieties. Marginal or nil production of alkaloids, which are hazardous to grazing livestock, must be a crucial component of the new association. Novel endophytes can therefore be added to breeding programs, improved, and developed into cultivars. Breeding material can be used to inoculate plants with the symbiont, and infected plants are subsequently multiplied to provide farmers with an adequate supply of E+ seeds. The endophytes are vertically transmitted to the following plant generations through seeds because of their high level of specialisation (Hartley & Gange, 2009; Hunt & Newman, 2005; Kaur et al., 2015) there is no need of a constant application.

Because of its developmental specialisation, the endophyte can multiply inside host seeds and grow into a new plant after being sown. Gundel et al. (2013) introduced the term "symbiotically modified organisms" (SMOs) to describe these naturally occurring, non-genetic grass-novel endophyte relationships. However, the ultimate positive result of the plant-symbiont interaction depends on the genetic compatibility of those association components

as well as the impact of environmental factors. To improve forage and turf grass cultivars, new endophytes are being used, and the technology is continually being developed. Important forage grasses have improved productivity, persistence, pest resistance, and adaptation to marginal environments through the use of endophyte-enhanced grass cultivars that do not contain poisonous alkaloids (Ferguson et al., 2021). Certain cultivars for sports and turf grasses have been enhanced with endophyte strains, which improve their durability and disease and insect resistance (Funk et al., 1993; Rochefort et al., 2007).

2.9. Cold Atmospheric Plasma and its applications

The basic classification of plasma in technological applications is into high-temperature, "hot" plasma and low-temperature, non-thermal plasma, commonly called "cold" plasma. Hot plasma reaches temperatures of thousands or even millions of degrees Celsius. In cold plasma, only electrons reach high temperatures, while atoms and ions remain at room temperature or only slightly elevated. Plasmas with temperatures not exceeding several dozen degrees Celsius are of particular interest to biologists because they do not cause thermal damage to living matter (Zhou et al., 2024). Numerous studies have demonstrated that the application of cold plasma is a novel, effective, and environmentally beneficial way to reduce infections without resorting to pesticides, which are hazardous for the environment and consumers (Ahmad et al., 2022; Hoppanová et al., 2020; Jo et al., 2014).

Plasma is made up of a variety of species, including electrons, free radicals, positive and negative ions, gas atoms and molecules in their fundamental or excited states, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as electromagnetic radiation (UV photons and visible light) (Dharini et al., 2023). It is regarded as a "quasi-neutral" medium since its net electrical charge is zero. But because plasma has characteristics that set it apart from neutral gases and liquids, including free charge carriers, it is electrically conductive (Gibbon, 2016; Tendero et al., 2006). Electric current, electromagnetic radiation, or thermal energy can all be used to power the process (Conrads & Schmidt, 2000). Cold plasma is often generated by an electrical discharge using direct current (DC) or alternating current (AC). The temperature of this "cold" plasma depends on the type of discharge and can be slightly higher than the temperature of the surrounding gas (Zhou et al., 2024). However,

a microwave discharge (beyond 100 MHz) or a radio frequency discharge (between 100 kHz and 100 MHz) can also be used to produce it (Fridman et al., 2004; Misra et al., 2016; Tendero et al., 2006; Varilla et al., 2020).

A dielectric barrier discharge, a plasma jet, a plasma torch, microplasma arrays, a gliding arc, and high voltage nanosecond pulsed plasma are the most often used cold plasma generators (Chukhlantsev, 2018; Ji et al., 2016; Zhou et al., 2016). A dielectric barrier discharge (DBD), which consists of two electrodes separated by a dielectric barrier, is well-known kind of AC discharge (Bryjak et al., 2010). The key benefit of DBD is its ability to function in both ambient air and normal atmospheric pressure. Cold plasma technologies have recently been used in agriculture to decontaminate seeds or crops that are ready to be sown or stored, increase seed germination and growth, decrease pathogen contamination, produce nitrogen-based fertilisers, remediate soil, disinfect processing surfaces and equipment, and remove ethylene from the air to slow down the ageing process (Hayashi et al., 2015; Ito et al., 2018; Ohta, 2016).

Pathogen-infected seeds are the main cause of plant disease in a variety of food crops, according to scientific evidence(Kumar & Gupta, 2020). Numerous fungi, including *Alternaria*, *Aspergillus*, *Bipolaris*, *Botrytis*, *Colletotrichum*, *Fusarium*, and the *Sclerotinia* species, spread when seeds are sowed and sprout (Sbai et al., 2024). Due to poor quality of seeds and sowing conditions, germs restrict the germination and growth of both seedlings and plants, which has an impact on crop establishment, crop health, and eventually yield in both direct and indirect ways (Lamichhane et al., 2018). Fungicides have long been used extensively to combat seed-borne diseases (Addrah et al., 2020; Saranya et al., 2017). In numerous investigations, cold plasma has shown promise as a substitute method for effectively and safely controlling plant pathogenic microorganisms by deactivating phytopathogenic bacterial and fungal cells (Adhikari et al., 2020).

Since the seed coat is where plant diseases are mostly found, the surface structure of seeds is altered by cold plasma, which also inhibits the growth of pathogens (Binyam, 2015; Rusu et al., 2018). Therefore, it is a practical instrument for sterilising the seed surface. Cold plasma application satisfies the requirements for the improved seed treatment method since it is simple to use, effective against a variety of diseases, and non-toxic to both humans and animals (Jo et al., 2014; Sayahi et al., 2024). A combination of ions, electrons, free radicals,

stable reaction products, reactive oxygen species and molecules, or ultraviolet (UV) radiation produced by cold plasma has been shown to have fungicidal effects. The sterilizing process is still not entirely understood. Cell permeability and external cell disruption are generally caused by plasma. The effectiveness of cold plasma is also influenced by the device model and operational parameters, including gas composition, flow rate, temperature, humidity, voltage, and frequency (Bourke et al., 2017; Dharini et al., 2023; Kolb et al., 2012; Thirumdas et al., 2018).

Furthermore, oxidative stress, or severe biological cell damage, can result from biological material coming into contact with reactive oxygen species (ROS) and reactive nitrogen species (RNS). The oxidation of membrane proteins and lipids impairs and ultimately destroys the cell membrane's ability to function properly (Afshari & Hosseini, 2014; Imlay, 2013). Studies on microorganisms, particularly filamentous fungi, have shown that UV radiation has a negligible impact on inactivation (Misra et al., 2011). ROS are far more efficient as seen by inactivation of *Penicillium digitatum* spores (Hashizume et al., 2013; Ito et al., 2012). Ozone is a potent oxidant among reactive oxygen species, ranking second only to the hydroxyl radical (Segat et al., 2014). *Fusarium* spp. is frequently found on seeds and are thought to be among the most sensitive fungi to cold plasma. For instance, when common buckwheat seeds were treated with oxygen in a low-pressure radiofrequency (RF) system, the growth of fungi, including *Fusarium* species, was reduced (Mravlje et al., 2021). For rice-pathogenic *Gibberella fujikuroi* (*Fusarium fujikuroi*), high antibacterial activity of non-thermal plasma produced by an air dielectric barrier discharge (DBD) was also observed, where treatments at atmospheric pressure successfully decreased the overall number of colonies on the seed surface(Jo et al., 2014).

Researchers observed that the number of *F. culmorum* on wheat grains was not significantly affected by prolonged plasma treatment (Kordas et al., 2015). Furthermore, it is noted that the surfaces of wheat and barley seeds may be decontaminated from this pathogen, demonstrating that plasma is a useful technique for successfully treating cereal seeds against illness (Hoppanová et al., 2020). It has also been demonstrated that cold plasma effectively decontaminates and improves the quality of pepper (*Capsicum annuum* L.) seeds by preventing the growth of *F. culmorum* on the seed surface. inhibition of *Colletotrichum gloeosporioides*, one of the most harmful plant diseases, in terms of mycelium growth and

spore germination was revealed (Ahmad et al., 2022; Rampersad & Teelucksingh, 2012). It has been demonstrated that pepper seeds can effectively eliminate *Didymella licopersici* spores using cold plasma. According to (Nishioka et al., 2014), inactivating Rhizoctonia solani on brassicaceous seeds decreased the fungal survival rate from 100% and 83%, respectively, to less than 2% after a 10-minute treatment with cold atmospheric plasma at low pressure. On ginseng seeds, plasma also had antifungal action.

When compared to the untreated controls, the number of fungal colonies from the genera *Coniochaeta, Pyrenochaeta*, and *Fusarium* was much reduced in seeds treated with argon-generated plasma (argon utilized as input gas for the DBD plasma generator) (Lee et al., 2021). Several studies demonstrate that cold plasma inhibits the growth of Aspergillus species. On maize seeds, a decrease in *A. flavus* was observed (Zahoranová et al., 2018). Coffee treated with cold plasma with helium at 1.5 L min^{-1} flow successfully eliminated *A. niger*, A. *westerdijikiae*, A. *steynii*, and *A. versicolor*, lowering the amount of ochratoxin A in roasted coffee (Casas-Junco et al., 2019). However, melanin, which has been linked to the dark-pigmented *Aspergillus* species' resilience to environmental stress, may restrict the efficiency of cold plasma. Melanin was found to boost the conidia's resistance to ROS in *Aspergillus fumigatus* (Perez-Cuesta et al., 2020; Tsai et al., 1998). The presence of melanin may have caused a lower plasma impact on A*. flavus* than on *Fusarium* species (Zahoranová et al., 2016). Moreover, the main cell walls and septa of *Alternaria* species multicellular conidia contain melanin (Carzaniga et al., 2002), It could account for its resistance to cold plasma action as previously described.

According to the research findings, cold plasma is efficient against microorganisms that live on the seed surface. This environmentally benign approach, however, might be effective in preventing seed-borne pathogenic fungus from growing inside the seeds. For instance, the *Diaporthe*/*Phomopsis* complex species reduction on soybean seeds is a typical example of this preventing inside seeds diseases (Pizá et al., 2018).

3. Research hypothesis, purpose and scope of research

Due to the presence of the *Epichloë* endophytes, grasses are usually more resistant to numerous stress factors, both abiotic and biotic. Selected isolates of endophytic fungi of the genus *Epichloë*/*Neotyphodium*, the so-called "novel endophytes" can be used for symbiotic modification of grasses (SMG) to provide plants with greater resistance to stress factors. However, the introduction of these isolates into the plant requires the prior removal of wild endophytes from the variety to be symbiotically modified. The eradication process is very difficult and time-consuming. It is usually carried out using fungicides or high temperature and the efficiency of those methods is very low.

Therefore, the main purpose of the conducted research was to determine the suitability of low-temperature plasma for the eradication of wild endophytes from plants in order to develop a proposal for an innovative, effective, and environmentally friendly technology for removing undesirable endophytes from grasses and then using them in the process of symbiotic modification of new varieties. Based on literature data and previous research, a working hypothesis was formulated that due to antibacterial and antifungal activity of low-temperature plasma growth of *Epichloë festucae* endophyte can be significantly inhibited after treatment by Dielectric Barrier Discharge plasma and Gliding Arch plasma.

To achieve the main objective, the following specific objectives were formulated:

- To collect grass genotypes and isolates of endophytic fungi inhabiting different genotypes available commercially as well as ecotypes and to characterise the endophyte infected genotypes based on the chosen parameters.
- To identify the collected isolates of endophytes and to determine their genetic diversity.
- To determine the boundary and optimal operating parameters of the DBD plasma and GA plasma generator ensuring the highest level of endophyte eradication.
- To check the overall performance between DBD plasma and GA plasma.
- To determine the effect of Cold Atmospheric Plasma (DBD plasma and GA plasma) on seeds contamination by fungi.

- To determine the effect of Cold Atmospheric Plasma (DBD plasma and GA plasma) on the activity of chosen enzymes (Pathogenesis Related Proteins (PRP).
- To propose the guidelines for the prototype design of the endophytes' eradication technology using Cold Atmospheric Plasma.

4. Research Methodology

4.1. Research material

To collect research material in the form of seeds and plants, a field expedition was organized in the southern and northern parts of Poland. Grass ecotypes were obtained in the vicinity of Zakopane, Wisła, Bydgoszcz and Gdańsk. The locations were selected to increase the probability of obtaining plants colonized by endophytic fungi. In previous studies conducted at the Department of Microbiology and Plant Ecology, Faculty of Agriculture and Biotechnology (WRiB), Bydgoszcz University of Science and Technology Jan and Jędrzej Śniadecki (PBŚ), the above locations were characterized by the highest degree of colonization. Research material was also obtained from companies involved in the trade and production of grass seeds, including Hodowla Roślin Grunwald Sp. z o. o. – Grupa IHAR, Mielno 163, 14-107 (HR Grunwald). Collected plants were planted to the pots containing peat substrate at pH 6.2 (Profi Substrate, Gramoflor, Germany). Seeds were sown on plates, and the resulting seedlings were transplanted into multi-pots and later into larger pots. All pots were placed in the greenhouse for further analyses. Remaining seeds were stored in room temperature.

4.2. Detection of endophytes using Agrinostics, Ltd. Co. Phytoscreen immunoblot kit "*Neotyphodium* field tiller"

The endophytic status of each plant was checked at the laboratory by analysing the sampled tillers. The presence of endophytes of the *Epichloë* genus was confirmed by a serological method using the Phytoscreen Immunoblot Kit "*Neotyphodium* field tiller" test catalogue number ENDO797-3 (Agrinostics, Ltd. Co., USA, Watkinsville, GA 30677). The test for visualization was carried out according to (Hiatt III et al., 1999) as follows: a nitrocellulose membrane was placed on a sponge ensuring constant access to the buffer in a reaction vessel filled with previously prepared extraction buffer. For each plant, 1-2 mm cross-sections of a tiller taken from its base were placed on the membrane (Figure 1). The next stage was incubation at 4°C for 24 hours to bind the proteins of the endophyte overgrowing the cross-sections to the membrane. Then, the membrane was dried at 70ºC for 15 minutes and then placed back in the reaction vessel, and 20 ml of BWW solution (Blocking/Washing/Working reagent) was added to block the membrane. It was shaken using orbital shaker for 30 minutes.

After shaking, the BWW solution was poured off and 20 ml of MAB solution (Pooled monoclonal antibodies 4H2, 5C7 and 15D7) diluted with the BWW solution was added. After 1 hour of shaking and rinsing twice with BWW solution, 20 ml of RAM (Rabbit anti-mouse antibody) solution were added and placed on a shaker for 1 hour. After rinsing twice with BWW solution, 20 ml of PA solution (protein A coupled to alkaline phosphatase) were added. Nitrocellulose membrane was shaken for 30 minutes, then rinsed twice with BWW solution, and then 20 ml of staining solution (TRIS diluted with distilled water + Napthol + Fast Red Chromogen reagent) were added. Shaking in the dark was carried out for 20-30 minutes. The stained membrane was evaluated.

The Phytoscreen Immunoblot Kit "*Neotyphodium* field tiller" was also used for examination of seedlings treated with Cold Atmospheric Plasma (CAP) and tillers grown from seeds treated with CAP.

Figure 2. Detection of endophyte using Phytoscreen Immunoblot Kit "*Neotyphodium* field tiller" test – nitrocellulose membrane with cross-sections of tillers.

4.3. Detection of endophytes by Rose Bengal staining method

Obtained seeds were analysed for endophyte presence using Rose Bengal (RB) staining method according to Saha et al. 1988. Seeds were placed into falcon rounded bottom

tubes 50ml and first staining solution (100ml H_2O, 2g Na OH, 0.2g Rose Bengal) was added. After 15 to 18 hours seeds were rinsed by tap water and soaked in second staining solution (0.5g Rose Bengal, 5ml ethyl alcohol, 100ml H_2O) to complete the staining. After 3 hours seeds were ready to be examined (Figure 1) under microscope at 100-400x magnification. Seeds were peeled seed coat and the remaining (embryo and endosperm) crushed on microscope slide and covered with coverslips.

The Rose Bengal staining method was also used for examination of tillers in the collection and tillers grown from seeds or seedlings treated with CAP. Inner epidermis of leaf sheath was peeled off and a drop of second staining solution was applied on it. The pieces of epidermis were examined microscopically at 100-400x magnification after 1 min of staining.

RB staining of inner epidermis of the leaf sheaths was also used for assessment of the endophyte hyphae density according to the method described by Panka et al. (2013). Four tillers per plant were collected randomly. Basal part sections of the leaf sheath cut from the two the most matured leaves were examined. 0.5 cm long sections were stained, and hyphae were counted under the microscope at 400× magnification. Counts were carried out in duplicate. Hyphal density was calculated as the average number of hyphae per 1mm width of leaf sheath.

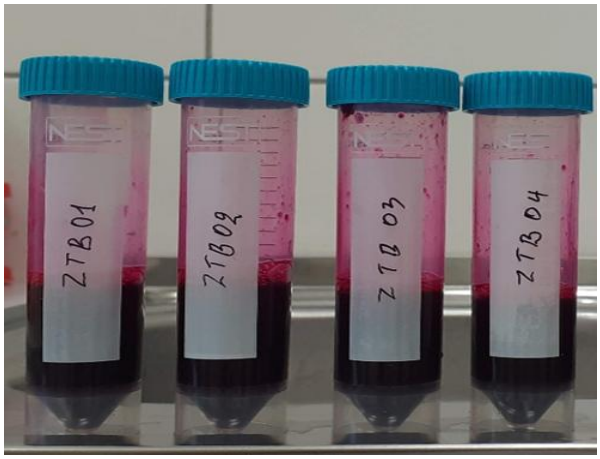

Figure 2. Seeds stained by Rose Bengal solution in Falcon tubes ready to be examined under microscope.

4.4. Molecular identification and determination of endophyte's genetic diversity

4.4.1. Molecular identification of endophyte by Real-Time PCR

- Isolation of total DNA

DNA in the tested samples was isolated from 40 mg of lyophilized and mechanically homogenized *L. perenne* tillers. DNA isolation was performed using a modified Doyle and Doyle (1990) protocol. The composition of extraction buffer used in this protocol was described in Table 1.

Table 1. Extraction buffer composition for Real-Time PCR analysis.

Reagents – initial concentration	Final concentration in buffer	Amount per one sample (600µl)
CTAB 5.0 %	2 %	240 µl
NaCl 5.0 M	1,4 M	168 µl
EDTA 0.5 M	20 mM	24 µl
Tris-HCl (pH 8.0) 1.0 M	100 mM	60 µl
PVP* (solid phase)	2 %	0,012 g
β-mercaptoetanol	0,2 %	1,2 µl
Sterile H_2O	-	106,8 µl

After adding 600 µl of extraction buffer and thoroughly mixing by vortexing for 30 seconds, the tube contents were incubated in a water bath at 65ºC for 50 minutes. Then, 600 µl of phenol, chloroform, and isoamyl alcohol mixture (25:24:1 v:v) in a was added to each tube, vortexed, and centrifuged at 12000 rpm for 10 minutes. The supernatant was transferred to 1.5 ml Eppendorf tubes, and 600 µl of a 24:1 volume ratio of chloroform and isoamyl alcohol mixture was added to each tube. The contents were mixed for four minutes by inversion and centrifuged at 12000 rpm for 10 minutes. The collected supernatant was transferred to a new set of 1.5 ml tubes after adding 50 µl of 5M NaCl and mixed by inverting. DNA was precipitated from the resulting mixture in each tube by adding 700 µl of frozen (- 20°C) 96% ethanol, vortexing vigorously for 1 minute, and then placed in a – 20°C in freezer for 20 minutes to maximize nucleic acid precipitation. The samples were then centrifuged at 4°C at 12000 rpm for 10 minutes to sediment the DNA pellet. The ethanol above the DNA was

removed and the material was washed with 400 µl of 70% ethanol by centrifugation at 4°C at 12000 rpm for 10 minutes. The alcohol was poured off, and the samples were left to dry for approximately 20-25 minutes. After this time, 50 µl of double-distilled, nuclease-free sterile water was added to each tube and left in the refrigerator for another 24-48 hours to dissolve the DNA pellet. The resulting material was stored in the freezer until molecular analysis.

- Identification of endophyte by Real-Time PCR

Analyses were performed using two pairs of primers flanking a fragment of the NRPS-1 (nonribosomal peptide synthetase) and ChitA (Chitinase A) genes. The primer sequences are presented in Table 2.

Table 2. Primer sequences used for specific identification of *Epichloë festucae*.

The primer sequences 5'-3'	Product length (bp)	Reference
ChitA-F: 5'-AAGTCCAGGCTCGAATTGTG-3' ChitA-R: 5'-TTGAGGTAGCGGTTGTTCTTC-3'	353	Rasmussen S., et al. 2007
NRPS-1-F: 5'-GTCCGATCATTCCAAGCTCGTT-3' NRPS-1-R: 5'-TGGTGGGAAGTTCCCTGCAC-3'	153	Rasmussen S., et al. 2007

- Preparation of DNA for Real-Time PCR analysis

DNA concentration in samples from individual experimental combinations was measured fluorometrically using a Quantus fluorometer (Promega, USA) and the QuantiFluor ONE dsDNA System reagent kit (Promega, USA). All samples were diluted in deionized, sterile water to a concentration of 10 ng·µl^{-1}.

Reactions were performed in a LightCycler 480II apparatus (Roche, Switzerland) using LightCycler 480 SYBR Green I Master reagent mix (Roche, Switzerland). To each sample, 0.25 µl of each pair of species-specific primers at a concentration of 10 pM·µl-1 was added. The resulting reaction premix was pipetted (5.5 µl each) into the wells of a 96-well PCR plate, and

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then 4.5 µl of DNA from each sample was added, yielding a final volume of 10 µl. Reaction wells containing sterile deionized water instead of DNA were negative controls.

- Thermal profile of Real-Time PCR analysis

The amplification program was preceded by a 10-minute pre-incubation of the samples at 95ºC. The thermal profile for both the NRPS-1 and ChitA gene fragment amplifications included 45 cycles, each consisting of:

- denaturation – 95ºC for 10 seconds,
- primer annealing – 62ºC for 20 seconds,
- primer elongation – 72ºC for 20 seconds.

Fluorescence readings were taken immediately after each cycle. The final step was melting curve analysis in the 65ºC–95ºC temperature range, allowing for the identification of those combinations that produced a species-specific fragment of the *Epichloë festucae* genome.

4.4.2. Molecular analysis of genetic diversity of endophyte isolates

- Isolation of endophyte mycelium from tillers

Isolation of viable endophytes was performed from perennial ryegrass samples, in which the presence of *E. festucae* was confirmed by Real-Time PCR. Isolation was performed according to a modified method by Pańka and Jeske (2009). Leaf sheath fragments were collected from the oldest part of the tiller, cleaned of contaminants, and cut under sterile conditions into approximately 5 mm long segments. These were then cut lengthwise to facilitate mycelial isolation. The obtained fragments were decontaminated using 75% ethyl alcohol for 1 minute and 10% sodium hypochlorite solution diluted 50:50 with water for 5 minutes. Next, the plant fragments were washed three times in sterile distilled water for 2 min, transferred to PDA (Difco) plates, and incubated for up to 4 weeks at 22°C in the dark. The grown colonies of endophyte were transferred to PDA medium for further use.

- Isolation and preparation of DNA for analysis

Three discs of 5-mm ø PDA medium overgrown with endophyte mycelium were excised and transferred to potato dextrose broth (PDB) (A&A Biotechnology, Poland) plates, protected with Parafilm M, and incubated in the dark for 5–7 days at 23 ◦C. DNA was extracted according to the modified Doyle and Doyle (1990) method from 30 mg of mycelium rinsed in sterile water, filtered, and initially drained with a Büchner funnel, blotting paper, and vacuum pump. Obtained samples were lyophilized in a CoolSafe lyophilizer (Scanvac, Denmark) and homogenized with quartz beads and sand in a MagnaLyser homogenizer (Roche, Switzerland). Extraction buffer (900 µL) consisting of 5.0% cetyltrimethylammonium bromide (CTAB), 0.5 M ethylenediaminetetraacetic acid (EDTA), 5.0 M NaCl, 1.0 M Tris-HCl (pH 8.0), β-mercaptoethanol, and 2.0% polyvinylpyrrolidone (PVP) was used. In the next steps, phenol, chloroform, isoamyl alcohol and ethyl alcohol were added. The concentration of DNA suspended in 100 µL ddH2O was measured fluorometrically (Quantus; Promega, USA).

- PCR assays

The internal transcribed spacers (ITS) regions were amplified with ITS1 and ITS4 primers (White et al., 1990) in an Eppendorf EP Mastercycler (Eppendorf, USA), in 37.5 µL of PCR Core Kit reagent (Qiagen, USA) reaction mixture. The final concentration of reagents was as follows: 1× buffer, 1× Q solution, 1.0 mM $MgCl_2$, 0.2 mM dNTP, 0.6 pM ITS1/ITS4, 0.5 U Taq polymerase, and 2 ng DNA template. The PCR analysis started with an initial denaturation step of 5 min at 95 ◦C, followed by 35 cycles of 95 ◦C for 1 min, an annealing temperature of 55 ◦C for 1 min and 72 ◦C for 2 min, followed by 5 min at 72 ◦C for a final extension. The presence of PCR products was verified by electrophoretic separation of 2 µL of the postreaction mixture in tris-borate-EDTA (TBE) buffer on a 1.2% agarose gel (Pronadisa, Spain) stained with SimplySafe (EURX, Poland).

- Sequencing of ITS Regions and Data Analysis

The obtained amplification products were purified and sequenced by Genomed (Poland). The sequencing of the ITS regions of forward and reverse strands of amplified fragments of each isolate was performed twice, as two separate replicates. A FinchTV 1.4 sequence alignment editor was used to analyse the obtained sequences. Multiple sequence alignment (ClustalW) analysis was performed in a MEGAX software (Kumar et al., 2018) to

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compare sequences. For endophyte identification, the E-value and percent identity parameters of the basic local alignment search tool (BLASTn) in NCBI were used. The Tamura 3-parameter model (Tamura, 1992) was used to generate maximum likelihood trees from ITS rDNA sequences in MEGAX to demonstrate the variation in the ITS regions. The dendrogram was prepared to visualize the differentiation between the tested *E. festucae* isolates, isolates of this species from GenBank NCBI and other isolates of the genus *Epichloë* and to graphically confirm the correctness of their identification.

4.5. Characteristics of the grass genotypes collection

Part of the research conducted was to assess the collected grasses to obtain information about their overall performance in pot and field conditions. The best plants can be used in the breeding process of the new varieties in HR Grunwald company. Based on the results obtained regarding the colonization of plants in the collection, perennial ryegrass was subjected to further research. Chosen parameters of the morphological characteristics based on UPOV (International Union for the Protection of New Varieties of Plants) and CPVO (Community Plant Variety Office) protocols and the breeders requirements (HR Grunwald) were assessed: the condition before winter (SPZ), overwintering (PZ), the energy of spring regrowth (ENOD), regrowth after mowing (ODR), beginning of inflorescence (PKŁ), length of longest stem (WYS), length of flag leaf (DLF), width of flag leaf (SLF), overall aspect (OA). Majority of the parameters was assessed based on 1 to 9 scale (1 – undesirable/poor trait/vigor and 9 – desirable/excellent trait/vigor) commonly used in this type of research. The following protocols were used: UPOV-TG/4/8 – Ryegrass (https://www.upov.int/edocs/tgdocs/en/tg004.pdf) and CPVO-TP/004/1 – Ryegrass (https://cpvo.europa.eu/sites/default/files/documents/TP/agr/TP_LOLIUM_004.pdf). These protocols of UPOV and CPVO are used during the registration process of the new *Lolium* spp. varieties providing guidelines for conduct of tests for Distinctiveness, Uniformity and Stability (DUS). The best plants were subjected to vegetative propagation to obtain more research material. In each year of the study, seeds were collected from the best plants.

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4.6. Treatment of seeds and plant material with cold atmospheric plasma

4.6.1. Cold Atmospheric Plasma generators

Studies on the effectiveness of cold atmospheric plasma on the *Epichloë* endophytes in plants and to determine the boundary parameters of the plasma generator operation were carried out using prototypes of Dielectric Barrier Discharge (DBD) and Gliding Arc (GA) plasma generators in the Department of Microbiology and Plant Ecology, Faculty of Agriculture and Biotechnology, Bydgoszcz University of Science and Technology. The design and control of power electronic generators for generating DBD and GA plasma were described in more detail by Mućko et al. 2020, Mućko 2025 and in the patent No. P.428969 (Mućko et al. 2022). These generators can be used both for the surface treatment of plastics and organic materials such as seeds or seedlings. The electrical diagrams of the DBD and GA generators, along with the equivalent reactor diagrams used for the research, are shown in Figure 3 (Mućko et al. 2020, patent No. P.428969 (Mućko et al. 2022) and Figure 4 (Mućko 2025).

The electrical diagrams of the generators shown in Figures 3 and 4 are similar. They differ in power, number of mains phases, and used components. The construction of the high-voltage, high-frequency transformer is also different. Both the discharge chambers for DBD plasma and for GA plasma (Plasma Jet) are electrically a combination of electrode capacitances, dielectric capacitances (if DBD discharge) and non-linear electrical discharge resistance. The generators' outputs are high-voltage transformers, which are inductive in nature. The transformer inductances and additional inductances, together with the capacitances of the discharge electrodes, create resonant circuits. The use of electrical resonance reduces switching losses and allows higher power supply efficiency and higher power density factor of the entire power supply system for plasma reactors. The generators used in the conducted research use resonance phenomena.

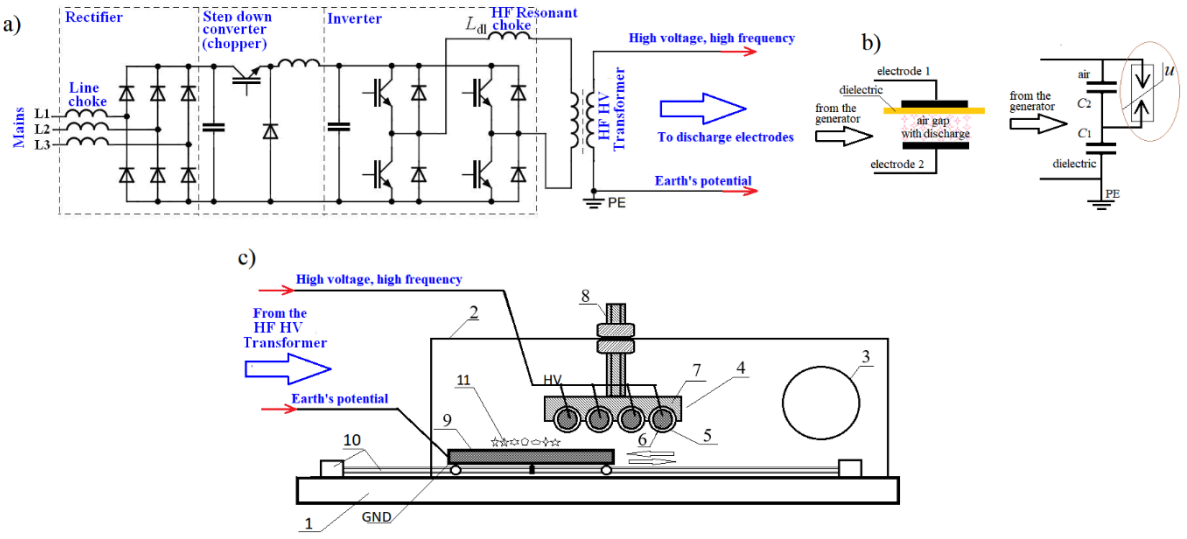


Figure 3. High frequency high voltage generator and DBD discharge chambers used for disinfection and/or sterilization of organic materials (based on Mućko et al. 2020, patent No. P.428969 (Mućko et al. 2022): a) generator, b) the idea of discharge chamber and its equivalent diagram, c) discharge chamber with a sliding electrode (which was used for research during the dissertation); base (1), discharge chamber (2), suction hole for ozone (3), electrode assembly (4), dielectric layers (5), HV electrodes (6), insulating support (7) with electrode gap adjustment knob (8), transport trolley (9), feed mechanism (10), processed material (11).

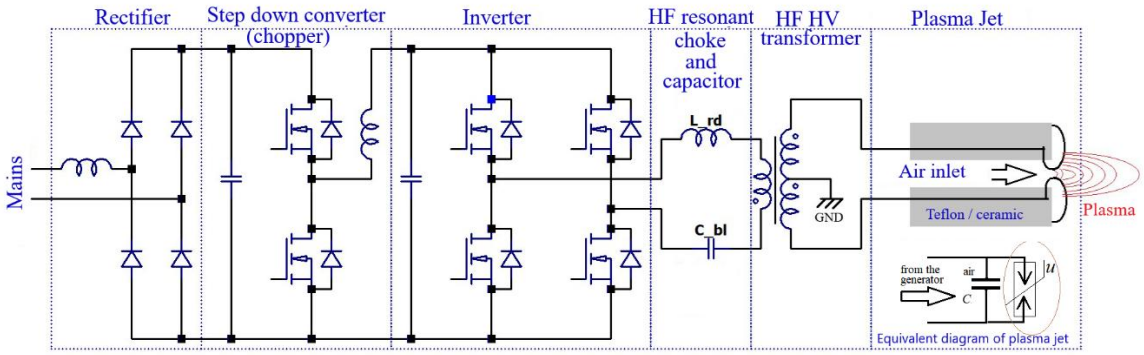


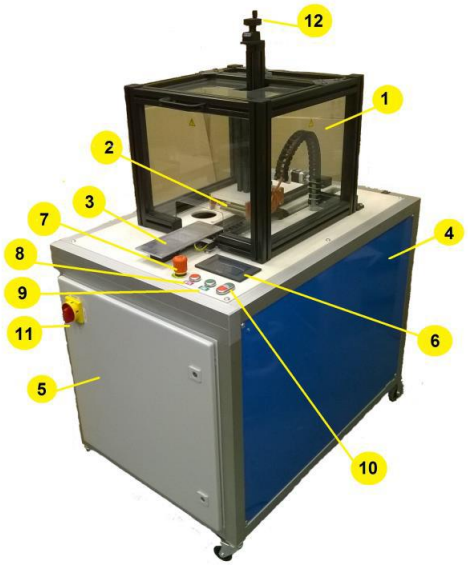
Figure 4. High frequency high voltage generator powering a plasma jet with a gliding arc discharge - simplified diagram (based on Mućko 2025).

The presented structure enables power control in plasma reactor by means of regulation of: - inverter supply voltage (amplitude modulation, AM), - inverter output voltage frequency (frequency modulation, FM), - pulse density modulation (PDM) or by a combination of the listed methods (Mućko 2008, Mućko et al. 2020, Mućko 2025 and in the patent No.

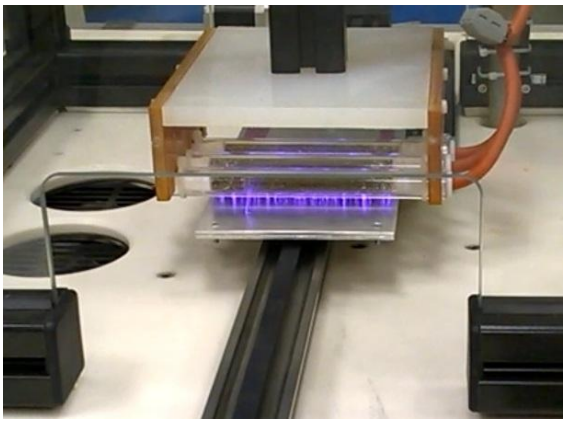
P.428969 (Mućko et al. 2022). The control of inverter supply voltage can be adjusted using the chopper. The control of output voltage frequency can be adjusted using the inverter. PDM modulation involves periodic changes in voltage or frequency. The systems shown in Figures 3 and 4 enabled power regulation in all the above-mentioned ways. The FM method was used in the DBD generation system (the unused chopper was not installed), while the AM method was used in the plasma jet system.

The prototype of DBD plasma generator used in the conducted research is shown in Figures 5a, 5b (scheme is presented in Figure 3). This device was developed at the Bydgoszcz University of Science and Technology (Mućko et al. 2022) as the result of the research project "Innovation Incubator +", No. 17/02/2018/UTP under the title "Investigation of decontamination technology of crushed dried plants using low-temperature plasma".

The operator touch panel (Figure 5c) is used to set and read parameters of the plasma treatment process. The panel buttons allow to set: the trolley speed, trolley start position, trolley position at which the generator starts and stops, number of trolley passes, set the power (200 – 1000 W) and modulation type (FM/PDM+FM). When working in PDM OFF mode and set power in the range from approx. 200 W to 300W, the discharge may be uneven. Switching to PDM ON mode increases the uniformity of discharges. The touch panel indicators show, among others: measured power and energy density, i.e. the amount of energy per cm^2 of the grounded electrode during one pass of the trolley as well as the total amount of energy per cm^2 for a given test. The device enabled precise selection of the energy dose needed to process biological material.



a)



b)

c)

Figure 5. The test station with discharge chamber with a sliding electrode: a) view of the stand (taken from the user manual), b) view of the DBD discharges in this device, c) touch panel; 1) discharge chamber, 2) high-voltage electrode, 3) grounded electrode on a transport trolley, 4) device body (inside: HV transformer and ozone exhaust fan), 5) electrical switchgear (inside: generator, PLC controller, safety relay with contactors), 6) operator touch panel, 7) safety switch button, 8), 9) off and on button, 10) start/stop button set for trolley and generator, 11) main switch, 12) electrode gap adjustment knob with indicator.

The GA plasma prototype used in the conducted research is presented in Figure 6 (developed by Mućko (2025), designed to power two plasma jet simultaneously, theoretical maximum power of approximately 400W).

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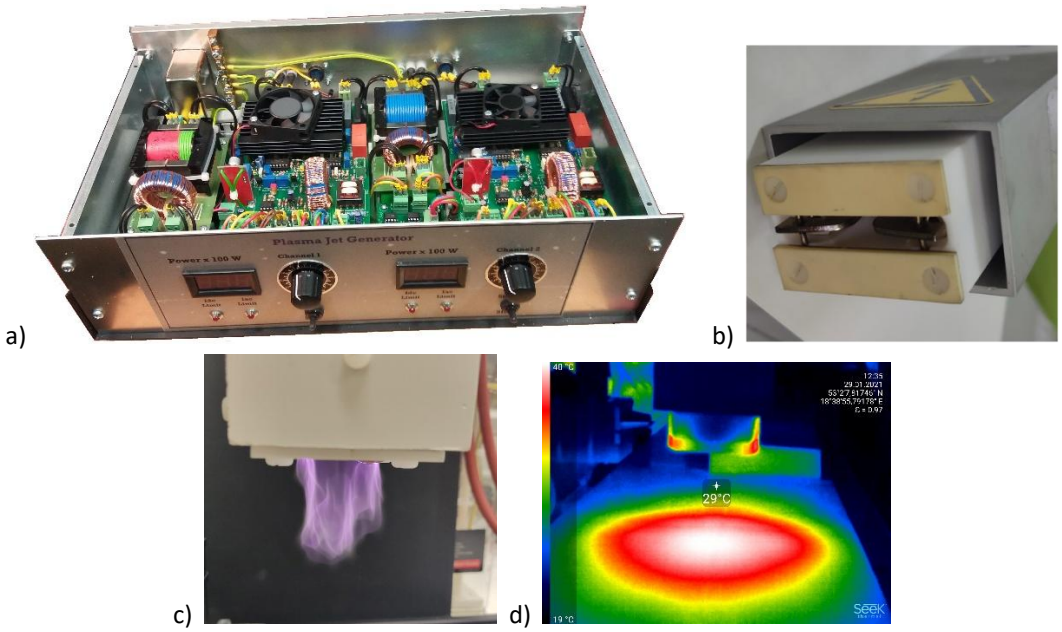

Figure 6. Gliding arc plasma generator powering the Plasma Jet reactor (a), a view of the plasma jet without (b) and with discharges (c), as well as a thermogram Plasma Jet temperature profile (d) (Mućko 2025).

4.6.2. Determination of CAP operating parameters for endophyte treatment

- Seed treatment

Perennial ryegrass seeds colonized by the endophyte were exposed to DBD plasma and GA plasma with varying generator parameters. Five power levels of the DBD plasma generator were tested: 200W, 300W, 400W, 500W, and 600W, as well as one level – 200W for GA plasma, due to technical limitations of the Plasma Jet device. Three exposure times were used: 10 s, 20 s, and 30 s. At least 100 seeds were treated in each combination (Figure 7). Table 3 presents approximate energy density values applied in individual experimental combinations in research using DBD and GA plasma generators. The treated area for DBD plasma was 103,5 cm^2, and for GA plasma – 19,6 cm^2 (5 cm-in-diameter plate). Most of the thermal energy generated in the process was absorbed by the airflow associated with the cart movement (DBD plasma generator) or the plate movement (GA plasma generator), which prevented excessive temperature rise. An example is the temperature distribution for the Plasma Jet shown in Figure 6d. The maximum temperature at the central point did not exceed approximately 40°C.

CAP-treated seeds were sown into pots filled with peat substrate (Profi Substrate, Gramoflor, Germany) and placed in a greenhouse. After 4 weeks of growth, the presence of

the endophyte was assessed by staining the leaf sheaths with Rose Bengal according to the methodology described in section 3.3. The plants were watered as needed during the growth period.

Figure 7. Treatment of seeds with DBD plasma generator.

- Seedlings treatment

Seeds were rinsed by tap water for 20 min and decontaminated using 75% ethyl alcohol for 1 minute and 10% sodium hypochlorite solution diluted 50:50 with water for 5 minutes. Next, the seeds were washed three times in sterile distilled water. Then, 100 seeds were transferred to the incubation container with moist blotting paper (120ml of sterile water added) and incubated for 3 days at 22°C in the dark, in INCU-line incubator (Germany) (Figure 8). Blotting paper was kept moist to allow the seeds to germinate. 3-days-old seedlings on blotting paper covered by dry gauze bandage to reduce the contact of seedlings and electrodes were put on a trolley of the DBD plasma generator and treated with plasma under 200W, 300W and 400W power for 10s, 20s and 30s (Figure 8). The 500W and 600W power used damaged the seedlings and inhibited their further growth. Table 3 presents approximate energy density values applied in individual experimental combinations. The treated area was 103,5 cm^2. Treated seedlings were kept in INCU-Line incubator (Germany) for 3 days again to recover their strength then planted in pots filled with peat substrate (Profi Substrate, Gramoflor, Germany) and placed in a greenhouse. After 4 weeks of growth the presence of the endophyte was assessed by staining the leaf sheaths with Rose Bengal according to the

methodology described in section 3.3. The plants were watered as needed during the growth period.

Table 3. Density of energy applied on seeds treated with DBD and GA plasma.

Power [W]	Exposure time [s]	Density of energy [J × cm^{-2}]
DBD plasma		
200	10	19.3
200	20	38.6
200	30	58,0
300	10	29.0
300	20	58.0
300	30	87.0
400	10	38.6
400	20	77.3
400	30	115.9
500	10	48.3
500	20	96.6
500	30	144.9
600	10	58.0
600	20	115.9
600	30	173.9
GA plasma		
200	10	102.0
200	20	204.1
200	30	306.1

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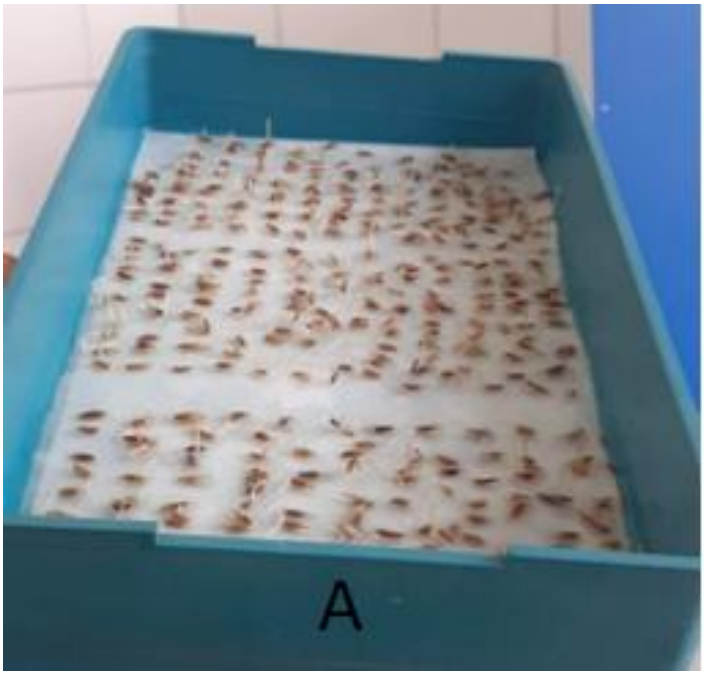

Figure 8. Perennial ryegrass seedlings grown on blotting paper.

4.7. Effect of Cold Atmospheric Plasma on seeds contamination by fungi

The effect of CAP plasma on perennial ryegrass seeds contamination by fungi was examined in laboratory conditions on Petri plates. Seeds from chosen genotype samples were treated by DBD plasma and then placed on Petri plates with Potato Dextrose Agar (PDA, Difco, USA) and incubated in the dark at room temperature (22°C). The number of growing fungal colonies was counted after 7 days of incubation. Non plasma treated seeds washed under tap water for 20 min were used as a control combination.

4.8. Effect of Cold Atmospheric Plasma on the activity of chosen Pathogenesis Related enzymes

Chosen perennial ryegrass seed samples (ZTB01-ZTB07) treated with DBD plasma have been sown into pots filled with peat substrate (Profi Substrate, Gramoflor, Germany) and kept in the glasshouse for 30 days. Harvested plant material was subjected to the extraction and analyses of Pathogenesis Related Proteins (PRP) β-1,3-glucanase (GLU), chitinase (CHI) and guaiacol peroxidase (GPOX) which are considered one of the main mechanisms of higher resistance of the plants against pathogens.

- Extraction of enzymes

Enzymes were extracted with use of 50 mM sodium phosphate buffer, pH 7.0 with 1 M NaCl, 1% PPVP, 1 mM EDTA and 1 mM sodium ascorbate. In total, 250 mg of freshly harvested plant tissue was homogenized in a chilled mortar and pestle with 2 mL of ice-cold extraction buffer with addition of sterile quartz sand. Homogenized plant material was transferred to 2 ml Eppendorf tube (Figure 9), centrifuged at 12000 rpm for 30 minutes and supernatant was collected in a new tube and the pellet was discarded.

- Analyses of β-1,3-glucanase and chitinase activity

The activity of chitinases and β-1,3-glucanases was determined using the modified method of Abeles (Abeles et al. 1971, Abeles and Forrence 1970). For glucanase analysis 100 mL of the enzyme fraction was mixed with 100 mL of a solution of colloidal laminarin (1mg/mL) and then suspended in acetate buffer at pH 4.5. For chitinase analysis, enzyme fraction was mixed with a solution of colloidal chitin (2mg/mL) and suspended in acetate buffer. The reaction was carried out at 37 ^{o}C for 60 min After incubation, the samples were centrifuged for 5 min at 5500 rpm. After that, 100 mL of DNS reagent was added to 100 mL of the supernatant and heated at 95 ^{o}C for 5 min, then cooled to 25 ^{o}C and measured at 550 nm. The amount of reducing sugars was read based on the standard curve prepared for the glucose solution. Chitinase and glucanase activity was determined based on the difference in reducing sugar content between the blank and the tested samples. Enzymes activity was determined in 1 nmol per 1 h and 1 g of fresh weight.

- Analyses of guaiacol peroxidase activity

The activity of guaiacol peroxidase was determined using the modified methods described by Zahir et al. (2021) and Chance and Maehly (1955) in a 96-well microplate format. The reaction mixture consisted of 10 mL of enzyme extract, 10 mL of guaiacol, and 250 mL of reaction buffer (phosphate buffer, 50 mM, pH 7.0) per well. The reaction began after the addition of 10 mL of H_2O_2 solution. The absorbance (470 nm) was measured immediately for 2 min 260 µL of phosphate buffer was used instead of enzyme extract for the blank. Guaiacol peroxidase activity was determined in 1 nmol per 1 min and 1 g of fresh weight.

Figure 9. Eppendorf tubes (2mL) containing the homogenized plant material prepared for enzyme analysis.

4.9. Statistical analyses

Data were analysed using JASP (https://jasp-stats.org/; JASP Team, University of Amsterdam). A three-way factorial analysis of variance (ANOVA) was performed (to analyse data treated by DBD plasma) with Grass genotype, Time of exposure, and Power as fixed factors, and the number of hyphae as the dependent variable, and a two-factorial analysis of variance (ANOVA) was performed to analyse data treated by Plasma Jet (GA plasma). The model tested for main effects of each factor as well as their two-way and three-way interactions. Effect sizes (ω^2) and 95% confidence intervals were calculated to estimate the magnitude of effects. When significant main effects were found, post-hoc pairwise comparisons with Tukey's HSD correction were carried out. The significance threshold was set at $\alpha = 0.05$.

5. Results

5.1. Collection of grass genotypes

The conducted research allowed to obtain a collection of plant material and seeds of perennial ryegrass and meadow fescue. A total of 47 perennial ryegrass and 27 meadow fescue samples were obtained (Tables 4, 5). Seed colonization by *Epichloë* endophytes (E+) was assessed microscopically after staining with Rose Bengal. To confirm the viability of the endophyte mycelium, the seeds were sown in pots, and the resulting plants were then examined serologically using Agrinostics kits. These kits were also used to examine plant samples collected during field expeditions. The presence of the viable endophyte was confirmed by the presence of pink stained spots on the nitrocellulose membrane (Figure 10). Due to the varying intensity of staining at the places where the cross-sections were attached to the membrane, indicating varying amounts of endophyte hyphae in the tiller, and in equivocal cases, the colonized samples were examined microscopically after staining the endophyte mycelium with RB. In the case of perennial ryegrass, viable endophyte mycelium was detected in 17 out of 47 samples collected, representing 36.2% (Table 4). In turn, the colonization of meadow fescue samples was 29.6% (8 E+ samples out of 27 collected) (Table 5).

Analysis of endophyte mycelium density in leaf sheaths revealed variation between genotypes (Table 6). The number of endophyte hyphae per 1 mm of leaf sheath width ranged from 4.8 (PR42) to 20.8 (PR34/ZTB07). The mean value for all genotypes was 11.8.

To economise the research, due to limited funds of the project only perennial ryegrass genotypes were chosen for further experiments. Both perennial ryegrass and medow fescue genotypes were planted in a field owned by HR Grunwald and maintained for breeding purposes. Based on the level of mycelium density in leaf sheaths, characteristics of the perennial ryegrass genotypes (Tables 6-8) and to economise the research 7 genotypes were chosen for tests with CAP plasma effect.

Table 4. List of the obtained and studied perennial ryegrass genotypes.

No.	Code of the genotype	Code of the studied genotype	Viable endophyte presence
1	PR1	-	+
2	PR2	-	-
3	PR3	-	-
4	PR4	-	-
5	PR5	ZTB01	+
6	PR6	-	-
7	PR7	-	-
8	PR8	-	+
9	PR9	-	-
10	PR10	-	-
11	PR11	-	+
12	PR12	-	+
13	PR13	-	+
14	PR14	ZTB02	+
15	PR15	ZTB03	+
16	PR16	-	+
17	PR17	-	-
18	PR18	-	-
19	PR19	-	-
20	PR20	-	-
21	PR21	ZTB04	+
22	PR22	ZTB05	+
23	PR23	-	-
24	PR24	-	-
25	PR25	-	-
26	PR26	-	-

Table 4. Continuation.

No.	Code of the genotype	Code of the genotype	Viable endophyte presence
27	PR27	ZTB06	+
28	PR28	-	+
29	PR29	-	-
30	PR30	-	-
31	PR31	-	-
32	PR32	-	-
33	PR33	-	-
34	PR34	ZTB07	+
35	PR35	-	+
36	PR36	-	+
37	PR37	-	-
38	PR38	-	-
39	PR39	-	-
40	PR40	-	-
41	PR41	-	-
42	PR42	-	+
43	PR43	-	-
44	PR44	-	-
45	PR45	-	-
46	PR46	-	-
47	PR47	-	-

Table 5. List of the obtained meadow fescue genotypes.

No.	Code of the genotype	Viable endophyte presence	No.	Code of the genotype	Viable endophyte presence
1	MF1	-	15	MF15	-
2	MF2	-	16	MF16	-
3	MF3	+	17	MF17	-
4	MF4	-	18	MF18	+
5	MF5	-	19	MF19	-
6	MF6	-	20	MF20	-
7	MF7	+	21	MF21	+
8	MF8	-	22	MF22	-
9	MF9	+	23	MF23	-
10	MF10	-	24	MF24	-
11	MF11	-	25	MF25	-
12	MF12	-	26	MF26	+
13	MF13	+	27	MF27	+
14	MF14	-			

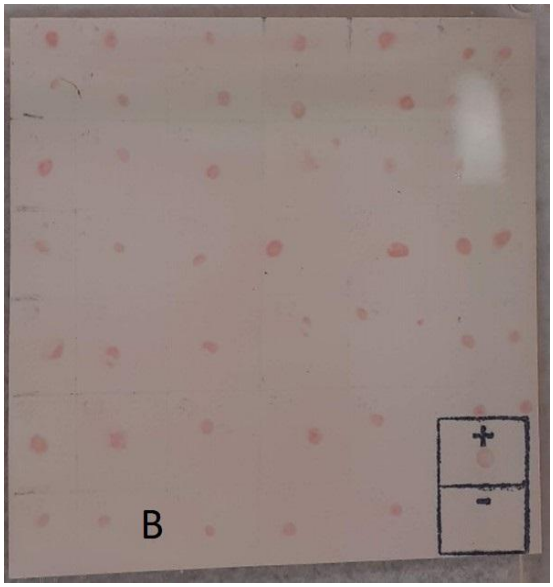

Figure 10. Nitrocellulose membrane with coloured pink spots indicating the presence of bounded endophyte proteins. Reference + means endophyte presence (E+) and – means no presence of endophyte (E-).

Table 6. Mycelium density in leaf sheaths of endophyte inhabited perennial ryegrass genotypes.

No.	Code of the genotype	Mycelium density [number of hyphae per 1mm of width of leaf sheath]
1	PR1	10,8
2	**PR5/ZTB01**	**15,0**
3	PR8	8,0
4	PR11	5,0
5	PR12	10,5
6	PR13	7,3
7	**PR14/ZTB02**	**17,5**
8	**PR15/ZTB03**	**13,8**
9	PR16	9,3
10	**PR21/ZTB04**	**17,0**
11	**PR22/ZTB05**	**16,0**
12	**PR27/ZTB06**	**19,5**
13	PR28	6,5
14	**PR34/ZTB07**	**20,8**
15	PR35	7,5
16	PR36	10,8
17	PR42	4,8

5.2. Molecular identification and determination of endophyte's genetic diversity

5.2.1. Identification of *Epichloë festucae* endophyte by Real-Time PCR

23 tested samples were analysed using Real-Time PCR. Analyses were performed using the nonspecific dye SYBR Green I, which intercalates into all double-stranded DNA fragments in the test sample, allowing it to detect nonspecific reaction products for both the NRPS-1 and ChitA gene fragments (Figures 11 and 12). To confirm the target product was amplified, a melting curve analysis was performed for the NRPS-1 and ChitA gene fragments. Obtained results showed that the expected reaction product was formed in 11 samples (10 samples + positive control) (Figure 13 and 14). Performed analyses confirmed the presence of *Epichloë festucae* var. *loli* in 10 out of 23 tested perennial ryegrass samples.

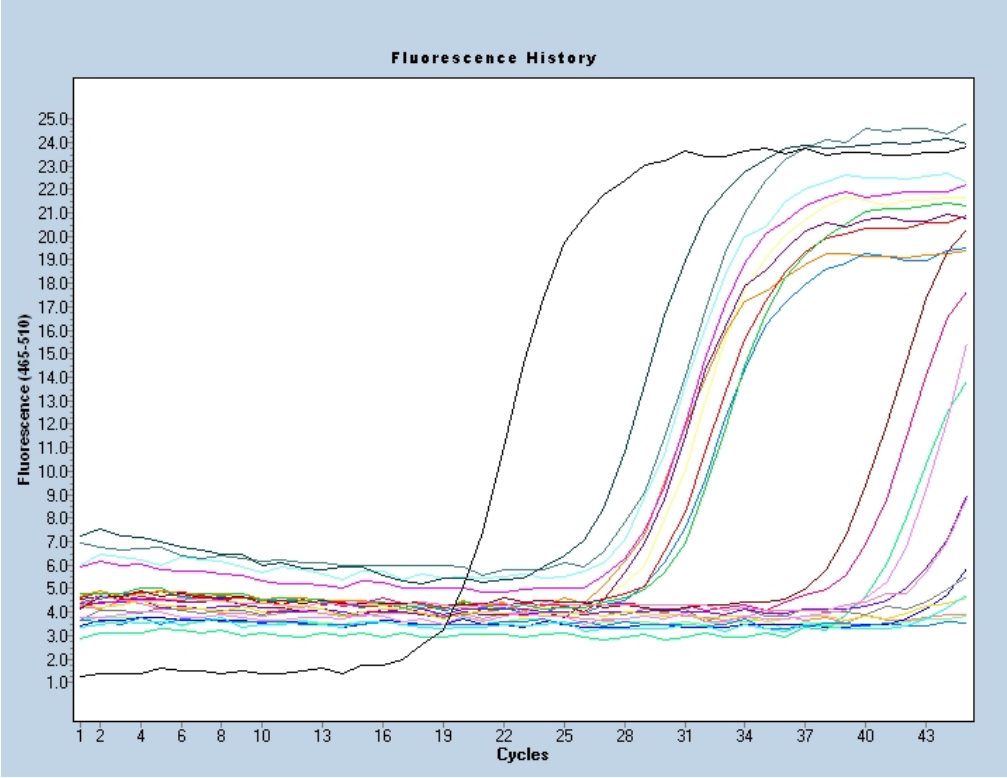

Figure 11. Result of the Real-Time PCR analysis of the NRPS-1 gene fragments.

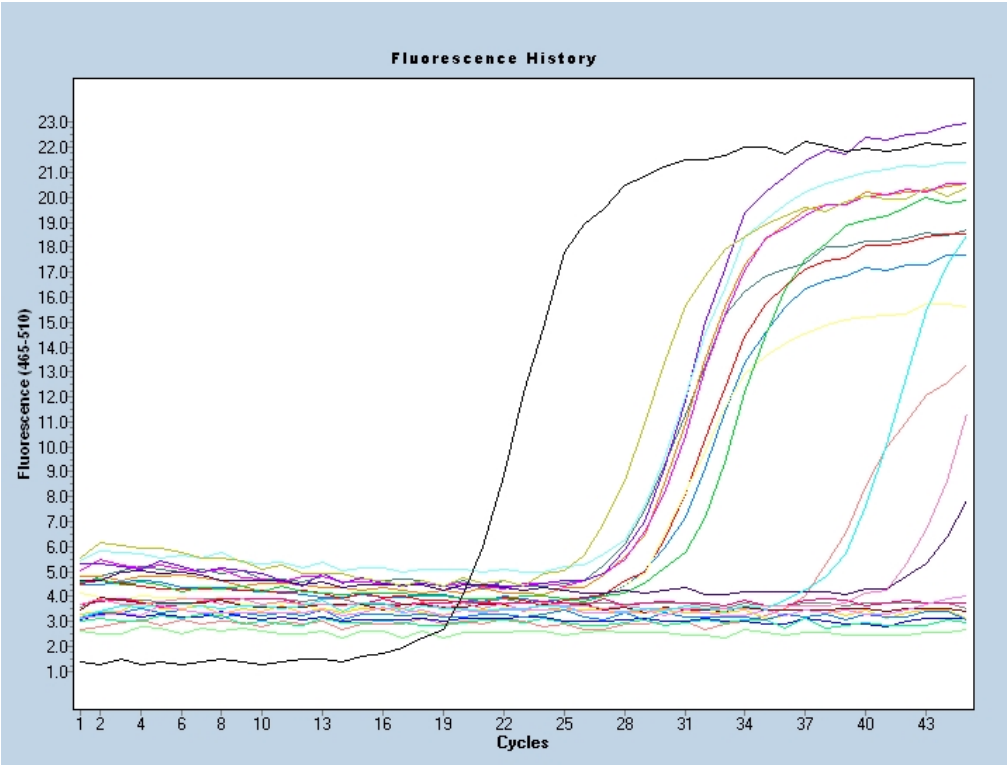

Figure 12. Result of the Real-Time PCR analysis of the ChitA gene fragments.

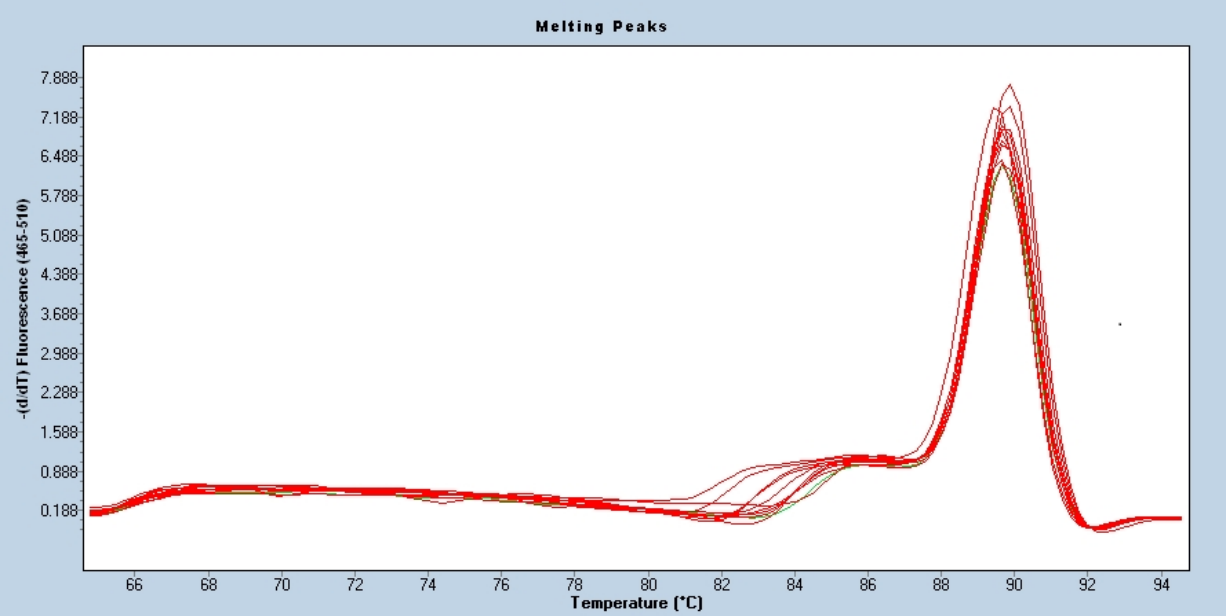

Figure 13. Result of the Real-Time PCR melting analysis of the NRPS-1 gene fragments.

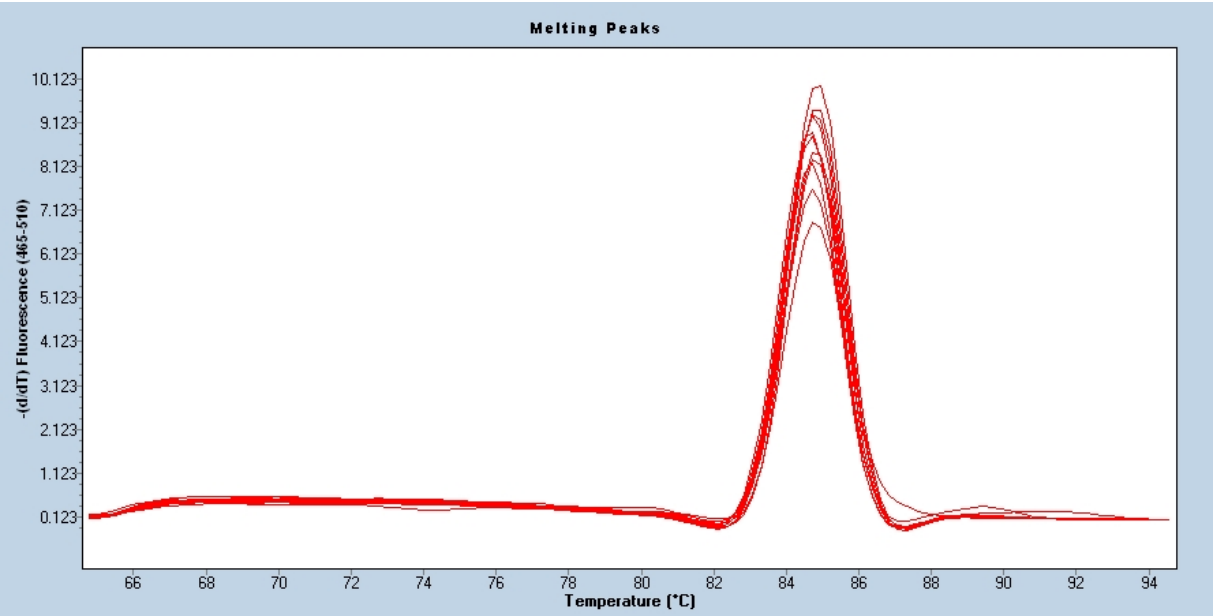

Figure 14. Result of the Real-Time PCR melting analysis of the ChitA gene fragments.

5.2.2. Genetic diversity analysis by Maximum Likelihood method

Viable endophyte mycelium was isolated from perennial ryegrass genotypes only in two cases. Based on molecular tests using ITS region sequence analysis, the tested *E. festucae* isolates showed no variation in the ITS sequence. They were identical with some sequences deposited in the NCBI Gen Bank, e.g. MK342195 or MK342203. With other sequences available in this database they showed similarity above 99%, i.e., at levels of 99.82% (MK342191), 99.61% (MK342022), 99.42% (AF059731), which was associated with 1-3 nucleotide differences, similarly to *E. typhina* – 99.63% (JN049832). With other *Epichloë* species, i.e., with *E. coenophiala* similarity was at the level of 99.12%, and with *E. baconii* – 97.86% (Figure 15).

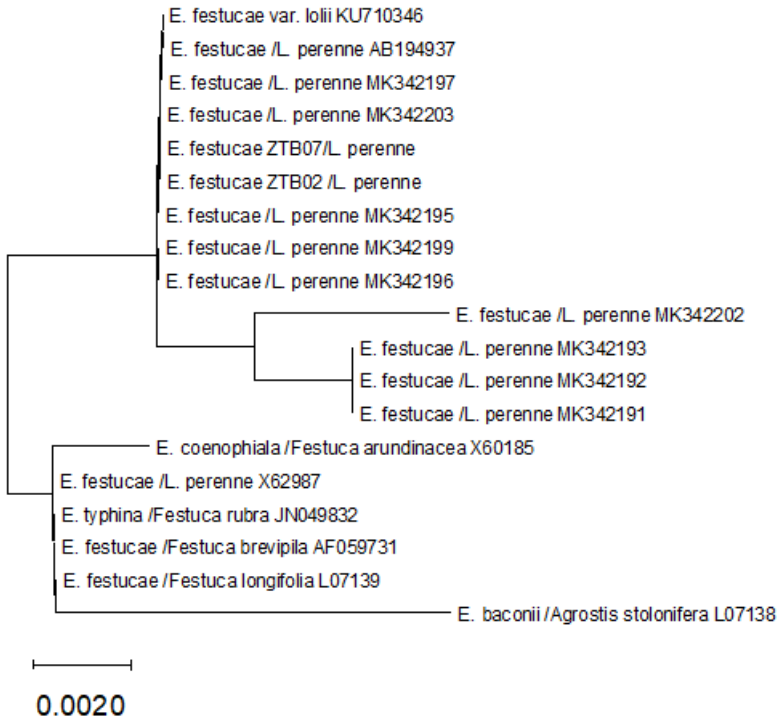

Figure 15. Dendrogram constructed based on the ITS rDNA sequences of tested *Epichloë festucae* isolates using the maximum likelihood method based on the Tamura 3-parameter model in MegaX.

5.3. Characteristics of the grass genotypes collection

The obtained genotypes were placed in a greenhouse to ensure proper growth and development before being transplanted into the field. After initial selection the most promising plants were propagated vegetatively to obtain more research material.

In the following year of study (2022), plants were planted into the field in spring (April 9, 2022). In the fall 2022, the condition of the plants before winter was assessed. A variability in plant vigour was observed ranging from 6 to 8. In the following years, all plants were fully assessed, analysing the parameters listed in Chapter 3.5. The results are presented in Tables 7, 8, 9.

In 2023, the variation within the analysed traits ranged as follows: PZ – 5-9, ENOD – 6-9, ODR – 5-9, SPZ – 6-8, and OA – 6-8 (Table 7). The mean values of the above-mentioned traits ranged from 6.0 to 8.4. Values above 7.0 were recorded for seven genotypes designated ZTB01 – ZTB07. The earliest date of beginning of inflorescence was characterized by genotype PR35 (24 May), and the latest by PR16 (31 May). The length of the longest stem ranged from 60 cm (PR16) to 75 cm (ZTB04). The length of the flag leaf ranged from 14 cm (PR12) to 21 cm (ZTB03), and the width from 0.4 cm (PR35) to 0.65 (PR13, ZTB02, ZTB04, ZTB05).

In 2024, the variation within the analysed traits ranged as follows: PZ – 5-9, ENOD – 6-9, ODR – 6-9, SPZ – 5-8, and OA – 6-9 (Table 8). The mean values of the above-mentioned traits ranged from 6.2 to 9.0. Values above or equal 7.0 were recorded for eight genotypes designated ZTB01 – ZTB07 and PR36. The earliest date of beginning of inflorescence was characterized by genotype ZTB02 (22 May), and the latest by ZTB04 (30 May). The length of the longest stem ranged from 60 cm (PR8) to 75 cm (ZTB02). The length of the flag leaf ranged from 13.5 cm (PR1) to 21 cm (PR13), and the width from 0.45 cm (PR42) to 0.65 (ZTB02, ZTB05, ZTB07).

In 2025, the variation within the analysed traits ranged as follows: PZ – 6-9, ENOD – 6-8, ODR – 5-9, and OA – 6-9 (Table 9). The mean values of the above-mentioned traits ranged from 6.0 to 8.5. Values above or equal 7.0 were recorded for nine genotypes designated ZTB01 – ZTB07, PR1, and PR13. The earliest date of beginning of inflorescence was characterized by genotype PR1 (26 May), and the latest by PR16 (2 June). The length of the longest stem ranged from 60 cm (PR42) to 75 cm (PR13, ZTB02, ZTB03, ZTB06, ZTB07). The length of the flag leaf

ranged from 15.0 cm (PR1, PR12) to 20.5 cm (ZTB06, ZTB07), and the width from 0.50 cm (PR28, PR35, PR36) to 0.75 (ZTB02).

In all years of research, genotypes marked as ZTB01-ZTB07 were characterized by the most favourable features. They showed the highest utility value and usefulness in the further breeding process of new varieties.

Table 7. Characteristics of the chosen traits of tested perennial ryegrass genotypes inhabited by endophytic fungus *Epichloë festucae* var. *loli* (season 2023).

No.	Gen. code	PZ	ENOD	ODR	PKŁ (May)	WYS [cm]	DLF [cm]	SLF [cm]	SPZ	OA	Mean (3,4,5,10,11)
1	2	3	4	5	6	7	8	9	10	11	12
1	PR1	6	7	7	25	70	14.5	0.60	7	7	6.8
2	**PR5/ ZTB01**	**8**	**8**	**8**	**25**	**70**	**19.0**	**0.60**	**7**	**8**	**7.8**
3	PR8	7	7	7	28	65	16.5	0.60	6	6	6.6
4	PR11	6	6	7	30	70	18.5	0.55	7	6	6.4
5	PR12	6	6	7	30	65	14.0	0.60	7	7	6.6
6	PR13	6	7	7	29	70	20.0	0.65	6	7	6.6
7	**PR14/ ZTB02**	**9**	**8**	**9**	**25**	**70**	**20.5**	**0.65**	**7**	**8**	**8.2**
8	**PR15/ ZTB03**	**9**	**9**	**9**	**30**	**70**	**21.0**	**0.60**	**7**	**7**	**8.2**
9	PR16	7	7	5	31	60	16.0	0.60	6	7	6.4
10	**PR21/ ZTB04**	**8**	**9**	**8**	**29**	**75**	**20.5**	**0.65**	**8**	**8**	**8.2**
11	**PR22/ ZTB05**	**8**	**7**	**9**	**25**	**65**	**19.5**	**0.65**	**8**	**8**	**8.0**
12	**PR27/ ZTB06**	**8**	**8**	**8**	**26**	**70**	**20.0**	**0.55**	**7**	**8**	**7.8**
13	PR28	5	6	7	25	70	18.0	0.45	6	7	6.2
14	**PR34/ ZTB07**	**9**	**8**	**9**	**30**	**70**	**19.0**	**0.55**	**8**	**8**	**8.4**
15	PR35	5	6	7	24	70	17.0	0.40	6	6	6.0
16	PR36	6	7	7	26	75	16.0	0.45	7	7	6.8
17	PR42	7	6	6	27	65	18.0	0.45	7	7	6.6

Assessed traits: overwintering (PZ), the energy of spring regrowth (ENOD), regrowth after mowing (ODR), beginning of inflorescence (PKŁ), length of longest stem (WYS), length of flag leaf (DLF), width of flag leaf (SLF), the condition before winter (SPZ), overall aspect (OA).

Table 8. Characteristics of the chosen traits of tested perennial ryegrass genotypes inhabited by endophytic fungus *Epichloë festucae* var. *loli* (season 2024).

No.	Gen. code	PZ	ENOD	ODR	PKŁ (May)	WYS [cm]	DLF [cm]	SLF [cm]	SPZ	OA	Mean (3,4,5,10,11)
1	2	3	4	5	6	7	8	9	10	11	12
1	PR1	6	6	6	24	65	13.5	0.55	7	7	6.4
2	**PR5/ ZTB01**	**7**	**8**	**7**	**23**	**70**	**18.0**	**0.60**	**7**	**7**	**7.2**
3	PR8	6	7	7	26	60	15.0	0.55	5	7	6.4
4	PR11	6	6	8	29	65	18.0	0.50	7	7	6.8
5	PR12	6	6	7	28	65	14.5	0.50	6	6	6.2
6	PR13	5	6	7	27	65	21.0	0.60	6	7	6.2
7	**PR14/ ZTB02**	**9**	**9**	**9**	**22**	**75**	**19.5**	**0.65**	**8**	**8**	**8.6**
8	**PR15/ ZTB03**	**8**	**9**	**8**	**28**	**65**	**20.0**	**0.60**	**8**	**7**	**8.0**
9	PR16	6	6	6	29	65	16.5	0.55	7	7	6.4
10	**PR21/ ZTB04**	**8**	**8**	**8**	**30**	**65**	**20.5**	**0.60**	**7**	**9**	**8.0**
11	**PR22/ ZTB05**	**7**	**7**	**8**	**27**	**70**	**19.0**	**0.65**	**8**	**7**	**7.4**
12	**PR27/ ZTB06**	**9**	**8**	**8**	**25**	**65**	**19.0**	**0.55**	**8**	**7**	**8.0**
13	PR28	6	6	7	26	70	18.5	0.50	7	7	6.6
14	**PR34/ ZTB07**	**9**	**9**	**9**	**28**	**70**	**20.5**	**0.65**	**9**	**9**	**9.0**
15	PR35	6	6	7	26	65	16.5	0.55	7	7	6.6
16	PR36	6	8	7	25	70	16.5	0.50	7	7	7.0
17	PR42	7	7	6	28	65	17.0	0.45	6	6	6.4

Assessed traits: overwintering (PZ), the energy of spring regrowth (ENOD), regrowth after mowing (ODR), beginning of inflorescence (PKŁ), length of longest stem (WYS), length of flag leaf (DLF), width of flag leaf (SLF), the condition before winter (SPZ), overall aspect (OA).

Table 9. Characteristics of the chosen traits of tested perennial ryegrass genotypes inhabited by endophytic fungus *Epichloë festucae* var. *loli* (season 2025).

No.	Gen. code	PZ	ENOD	ODR	PKŁ (May)	WYS [cm]	DLF [cm]	SLF [cm]	OA	Mean (3,4,5,10,11)
1	2	3	4	5	6	7	8	9	11	12
1	PR1	6	7	7	26	65	15.0	0.60	8	7.00
2	**PR5/ ZTB01**	**8**	**7**	**8**	**27**	**70**	**18.5**	**0.65**	**8**	**7.75**
3	PR8	6	7	6	30	70	17.0	0.65	7	6.50
4	PR11	7	6	7	31	65	18.0	0.60	7	6.75
5	PR12	6	6	6	31	65	15.0	0.60	8	6.50
6	PR13	7	7	7	30	75	19.5	0.65	8	7.25
7	**PR14/ ZTB02**	**8**	**7**	**8**	**28**	**75**	**20.0**	**0.75**	**9**	**8.00**
8	**PR15/ ZTB03**	**9**	**8**	**9**	**31**	**75**	**20.0**	**0.70**	**8**	**8.50**
9	PR16	7	6	5	02.06	65	17.0	0.65	8	6.50
10	**PR21/ ZTB04**	**8**	**8**	**8**	**31**	**70**	**20.0**	**0.65**	**8**	**8.00**
11	**PR22/ ZTB05**	**8**	**7**	**8**	**29**	**70**	**20.0**	**0.70**	**8**	**7.75**
12	**PR27/ ZTB06**	**8**	**7**	**8**	**30**	**75**	**20.5**	**0.65**	**7**	**7.50**
13	PR28	6	6	6	28	65	18.5	0.50	7	6.25
14	PR34/ ZTB07	9	8	8	31	75	20.5	0.60	9	8.50
15	PR35	6	6	6	27	65	18.0	0.50	6	6.00
16	PR36	7	6	7	30	70	17.0	0.50	7	6.75
17	PR42	7	6	6	30	60	18.0	0.55	6	6.25

Assessed traits: overwintering (PZ), the energy of spring regrowth (ENOD), regrowth after mowing (ODR), beginning of inflorescence (PKŁ), length of longest stem (WYS), length of flag leaf (DLF), width of flag leaf (SLF), overall aspect (OA).

5.4. Effect of Cold Atmospheric Plasma on endophytes

5.4.1. Eradication effectiveness of DBD plasma applied on seeds

In the study evaluating the eradication effectiveness of CAP against *E. festucae* var. *loli*, plants selected during earlier stages of the research were used: ZTB01, ZTB02, ZTB03, ZTB04, ZTB05, ZTB06, and ZTB07. These plants demonstrated optimal performance parameters and relatively high endophyte infestation in tillers. Initial experiments were conducted across the widest possible range of DBD generator power settings: 200W, 300W, 400W, 500W, and 600W. Lower and higher values were found to be ineffective during preliminary trials. Similarly, exposure times of 10 s, 20 s, and 30 s were selected. Higher generator power levels and exposure durations exceeding 30 s caused kernel damage and negatively affected germination. During the application process, occasional streamers were also observed, which slightly burned the seed husks. At lower power levels and shorter application times, this had no effect on seed germination. The effectiveness of DBD plasma was assessed by quantifying the number of endophyte hyphae in leaf sheaths after four weeks of growth following plasma treatment.

A three-way factorial ANOVA was conducted to determine the effects of exposure time, power, and grass genotype on hyphal count, including all interaction terms. The analysis revealed that all three factors had highly significant effects on number of hyphae. As shown in Table 10, exposure duration had a strong influence, F (2, 315) = 179.29, p < .001, with a large effect size (ω^2 = 0.168, 95% CI [0.097, 0.239]). Both power and grass genotype also produced significant effects: F (4, 315) = 100.17, p < .001, ω^2 = 0.186 [0.109, 0.256], and F(6, 315) = 71.26, p < .001, ω^2 = 0.198 [0.115, 0.264], respectively.

Several interaction effects were also significant. The interaction between exposure time and grass genotype was significant, F (12, 315) = 13.08, p < .001, with a small-to-moderate effect size (ω^2 = 0.068 [0.000, 0.095]). A similar significant interaction was observed between power and grass genotype, F (24, 315) = 12.09, p < .001, ω^2 = 0.125 [0.009, 0.137]. A smaller but still significant interaction between exposure time and power was also present, F (8, 315) = 3.35, p = .001, ω^2 = 0.009 [0.000, 0.007]. The three-way interaction among exposure time, power, and grass genotype reached significance, F (48, 315) = 3.16, p < .001, although the effect size was small (ω^2 = 0.049 [0.000, 0.000]). The residual variance was 2.99.

Table 10. ANOVA results on effect of Time of exposure, Power, and Grass genotype on the number of endophyte hyphae in plants, and their interactions under DBD plasma treatment of seed samples.

Cases	Sum of Squares	df	Mean Square	F	p	ω^2	95% CI for ω^2	
							Lower	Upper
Time of exposure	1,073.20	2	536.600	179.294	< .001	0.168	0.097	0.239
Power	1,199.20	4	299.800	100.172	< .001	0.186	0.109	0.256
Grass genotype	1,279.59	6	213.265	71.258	< .001	0.198	0.115	0.264
Time of exposure ✻ Power	80.23	8	10.029	3.351	.001	0.009	0.000	0.007
Time of exposure ✻ Grass genotype	469.87	12	39.156	13.083	< .001	0.068	0.000	0.095
Power ✻ Grass genotype	868.43	24	36.185	12.090	< .001	0.125	0.009	0.137
Time of exposure ✻ Power ✻ Grass genotype	453.54	48	9.449	3.157	< .001	0.049	0.000	0.000
Residuals	942.75	315	2.993					

Note. Type III Sum of Squares

Table 11 summarizes the mean number of fungal hyphae for each combination of grass genotype, cold plasma exposure time (10, 20, and 30 s), and plasma power (200–600 W).

Plasma power significantly influenced quantity of hyphae, in nearly all genotypes and exposure durations, the number of hyphae decreased as power increased. For example, in genotype ZTB01 at exposure duration of 10 s, the mean dropped from 15.0 ± 0.82 at 200 W to 8.5 ± 1.29 at 600 W, with the coefficient of variation (CV) rising from 0.05 to 0.15. Similar trends were observed in genotypes ZTB02, ZTB03, and ZTB05.

Exposure time had a modest effect, with longer durations generally reducing number of hyphae, though the impact varied by genotype. For instance, in genotype ZTB02 at 200 W, the mean declined from 17.5 at 10 s to 14.3 at 30 s. In contrast, genotypes like ZTB04 showed less consistent responses at intermediate power levels. The variation in genotypes influenced the number of hyphae. The genotype ZTB07 consistently recorded the highest number of hyphae at 200W across all exposure times (e.g., 20.8 ± 0.50 at 10 s), while genotypes ZTB03 and ZTB02 showed the lowest counts at higher powers and longer exposures (e.g., 5.3 ± 0.96 at 30 s, 400W for ZTB02). CVs were generally low (0.02–0.20) at lower powers and increased slightly with higher power or longer exposure, indicating greater variability in hyphal suppression under more intense plasma treatment.

Table 11. The mean number of endophyte hyphae for each combination of grass genotype, time of exposure (10, 20, and 30 s), and power levels (200–600 W) under DBD plasma treatment of seed samples.

Grass genotype	Time of exposure	Power	N	Mean	SD	SE	Coefficient of variation
ZTB01	10s	200W	4	15.000	0.816	0.408	0.054
		300W	4	12.500	1.291	0.645	0.103
		400W	4	11.000	2.160	1.080	0.196
		500W	4	10.000	1.414	0.707	0.141
		600W	4	8.500	1.291	0.645	0.152
	20s	200W	4	14.000	0.816	0.408	0.058
		300W	4	11.750	1.708	0.854	0.145
		400W	4	10.250	2.630	1.315	0.257
		500W	4	8.750	0.957	0.479	0.109

Grass genotype	Time of exposure	Power	N	Mean	SD	SE	Coefficient of variation
		600W	4	8.500	2.380	1.190	0.280
	30s	200W	4	13.000	1.826	0.913	0.140
		300W	4	11.000	2.160	1.080	0.196
		400W	4	8.750	1.258	0.629	0.144
		500W	4	11.250	0.957	0.479	0.085
		600W	4	9.750	2.630	1.315	0.270
ZTB02	10s	200W	4	17.500	0.577	0.289	0.033
		300W	4	12.000	0.816	0.408	0.068
		400W	4	10.750	0.957	0.479	0.089
		500W	4	8.750	1.258	0.629	0.144
		600W	4	9.750	0.957	0.479	0.098
	20s	200W	4	15.500	1.291	0.645	0.083
		300W	4	11.500	1.915	0.957	0.167
		400W	4	8.250	2.217	1.109	0.269
		500W	4	9.750	1.708	0.854	0.175
		600W	4	8.750	2.217	1.109	0.253
	30s	200W	4	14.250	2.500	1.250	0.175
		300W	4	8.750	0.500	0.250	0.057
		400W	4	5.250	0.957	0.479	0.182
		500W	4	5.500	0.577	0.289	0.105
		600W	4	5.500	0.577	0.289	0.105
ZTB03	10s	200W	4	13.750	0.957	0.479	0.070
		300W	4	8.500	0.577	0.289	0.068
		400W	4	5.750	1.258	0.629	0.219
		500W	4	9.000	0.816	0.408	0.091
		600W	4	10.750	1.500	0.750	0.140
	20s	200W	4	9.750	0.957	0.479	0.098

Grass genotype	Time of exposure	Power	N	Mean	SD	SE	Coefficient of variation
		300W	4	7.500	2.380	1.190	0.317
		400W	4	5.250	1.708	0.854	0.325
		500W	4	6.750	2.062	1.031	0.305
		600W	4	11.000	1.633	0.816	0.148
	30s	200W	4	10.250	1.258	0.629	0.123
		300W	4	6.250	0.957	0.479	0.153
		400W	4	5.500	1.291	0.645	0.235
		500W	4	5.750	0.957	0.479	0.167
		600W	4	8.000	1.826	0.913	0.228
ZTB04	10s	200W	4	17.000	0.816	0.408	0.048
		300W	4	10.500	1.915	0.957	0.182
		400W	4	14.750	1.893	0.946	0.128
		500W	4	14.250	1.708	0.854	0.120
		600W	4	16.500	1.291	0.645	0.078
	20s	200W	4	14.250	2.630	1.315	0.185
		300W	4	8.750	0.957	0.479	0.109
		400W	4	10.500	1.291	0.645	0.123
		500W	4	13.500	2.380	1.190	0.176
		600W	4	13.500	2.380	1.190	0.176
	30s	200W	4	11.250	1.500	0.750	0.133
		300W	4	14.000	2.160	1.080	0.154
		400W	4	10.250	0.957	0.479	0.093
		500W	4	11.500	1.915	0.957	0.167
		600W	4	9.000	2.582	1.291	0.287
ZTB05	10s	200W	4	16.000	0.816	0.408	0.051
		300W	4	10.000	1.414	0.707	0.141
		400W	4	12.000	1.826	0.913	0.152

Grass genotype	Time of exposure	Power	N	Mean	SD	SE	Coefficient of variation
		500W	4	13.500	1.732	0.866	0.128
		600W	4	10.500	1.291	0.645	0.123
	20s	200W	4	14.000	1.414	0.707	0.101
		300W	4	14.750	1.893	0.946	0.128
		400W	4	9.250	0.957	0.479	0.104
		500W	4	15.500	2.517	1.258	0.162
		600W	4	11.750	2.062	1.031	0.175
	30s	200W	4	11.250	3.403	1.702	0.303
		300W	4	10.750	2.217	1.109	0.206
		400W	4	7.750	1.708	0.854	0.220
		500W	4	9.750	0.957	0.479	0.098
		600W	4	7.750	2.500	1.250	0.323
ZTB06	10s	200W	4	19.250	0.500	0.250	0.026
		300W	4	14.000	3.367	1.683	0.240
		400W	4	18.000	0.816	0.408	0.045
		500W	4	20.000	0.816	0.408	0.041
		600W	4	13.500	2.380	1.190	0.176
	20s	200W	4	17.250	1.708	0.854	0.099
		300W	4	9.000	0.816	0.408	0.091
		400W	4	14.000	1.414	0.707	0.101
		500W	4	13.000	0.816	0.408	0.063
		600W	4	11.750	2.062	1.031	0.175
	30s	200W	4	13.250	2.217	1.109	0.167
		300W	4	5.250	0.957	0.479	0.182
		400W	4	11.750	2.062	1.031	0.175
		500W	4	10.000	1.414	0.707	0.141
		600W	4	8.000	1.414	0.707	0.177

Grass genotype	Time of exposure	Power	N	Mean	SD	SE	Coefficient of variation
ZTB07	10s	200W	4	20.750	0.500	0.250	0.024
		300W	4	20.000	4.082	2.041	0.204
		400W	4	18.250	1.258	0.629	0.069
		500W	4	15.500	1.291	0.645	0.083
		600W	4	13.500	2.380	1.190	0.176
	20s	200W	4	19.750	1.708	0.854	0.086
		300W	4	11.500	2.517	1.258	0.219
		400W	4	10.750	1.500	0.750	0.140
		500W	4	8.000	0.816	0.408	0.102
		600W	4	11.750	2.062	1.031	0.175
	30s	200W	4	14.000	2.160	1.080	0.154
		300W	4	13.500	1.291	0.645	0.096
		400W	4	10.000	1.633	0.816	0.163
		500W	4	8.500	2.380	1.190	0.280
		600W	4	8.000	1.414	0.707	0.177

- Effect of DBD plasma on the number of endophyte hyphae under 200W power

The results showed a general decline in the number of hyphae with increasing exposure time (Figure 16). All genotypes exhibited a gradual reduction from 10 to 30 seconds, indicating that longer exposure enhanced endophyte suppression, even at a relatively low plasma power (200 W). The effect was genotype dependent. For example, genotype ZTB07 consistently showed the highest number of hyphae (≈21 at 10 s, ≈14 at 30 s), while genotype ZTB06 also maintained relatively high levels (≈19 to ≈14). In contrast, genotype ZTB03 had the lowest counts, dropping from ≈14 to ≈10.

The rate of decline varied among genotypes. Genotype ZTB03 showed sharper reduction between 10 and 20 seconds, whereas genotypes ZTB07 and ZTB06 declined more gradually. These results demonstrated that both exposure time and genotype significantly influenced endophyte suppression at a constant plasma power of 200 W. Some genotypes, like ZTB03,

were more sensitive to treatment, while others, such as ZTB07 and ZTB06, were more resistant.

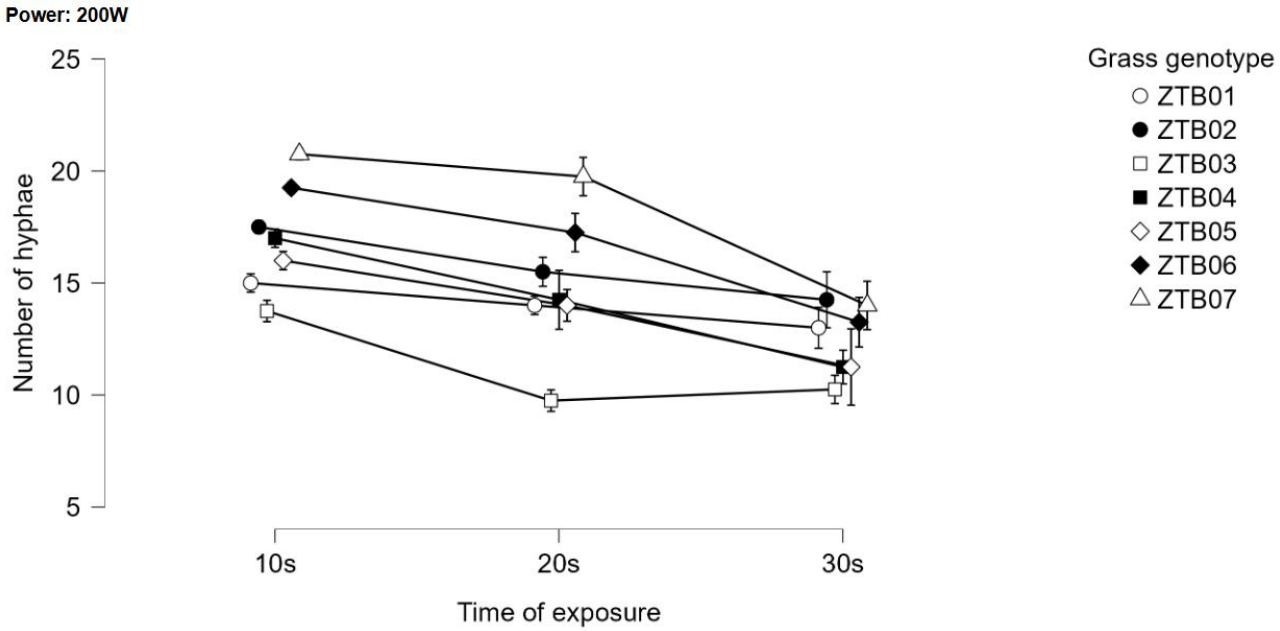

Figure 16. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under DBD plasma (200W) treatment of seed samples.

- Effect of DBD plasma on the number of endophyte hyphae under 300W power

Figure 17 illustrates a consistent decline in number of hyphae with increasing plasma exposure time across most genotypes. The magnitude of reduction varied by genotype. ZTB07 exhibited the highest initial count (~20 at 10 s), decreasing to ~12 at 30 s. Genotype ZTB06 showed a pronounced drop from ~13 to ~5, indicating high sensitivity to treatment. ZTB03 maintained low counts throughout, with a slight decline from ~9 to ~7. Genotypes such as ZTB01 and ZTB04 demonstrated greater tolerance, showing minimal changes. Crossing trends among genotypes (e.g., ZTB01, ZTB04, ZTB05) suggest variable suppression rates and occasional increases between 10 and 20 seconds.

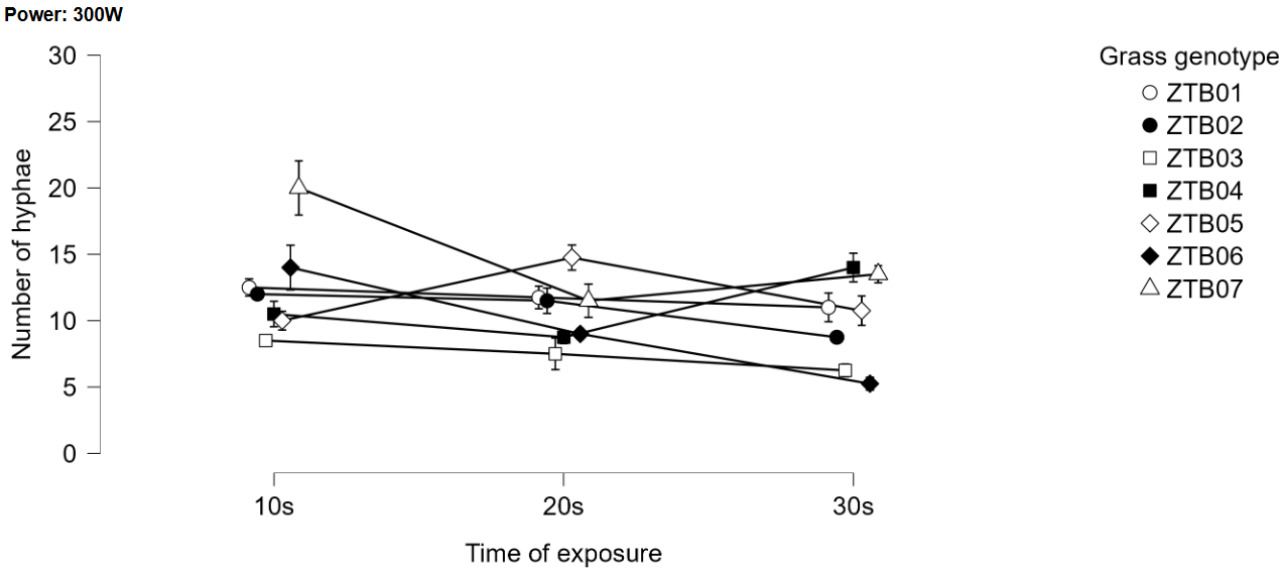

Figure 17. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under DBD plasma (300W) treatment of seed samples.

- Effect of DBD plasma on the number of endophyte hyphae under 400W power

At a plasma power of 400 W, all perennial ryegrass genotypes showed a clear reduction in the number of fungal hyphae as exposure time increased (Figure 18). Initial hyphal densities varied at 10 s, with ZTB06 showing the highest mean (~18 hyphae) and ZTB03 the lowest (~6 hyphae). By 30 s, hyphal counts converged to a narrower range of approximately 5–12 across genotypes, indicating a strong overall suppressive effect of the treatment. The decline was most pronounced in ZTB06, which declined by roughly 50%, while genotypes such as ZTB01, ZTB02, ZTB04, and ZTB05 showed moderate but steady reductions. Genotype ZTB03 maintained consistently low counts, suggesting either inherent sensitivity to plasma exposure or a lower baseline endophyte infection.

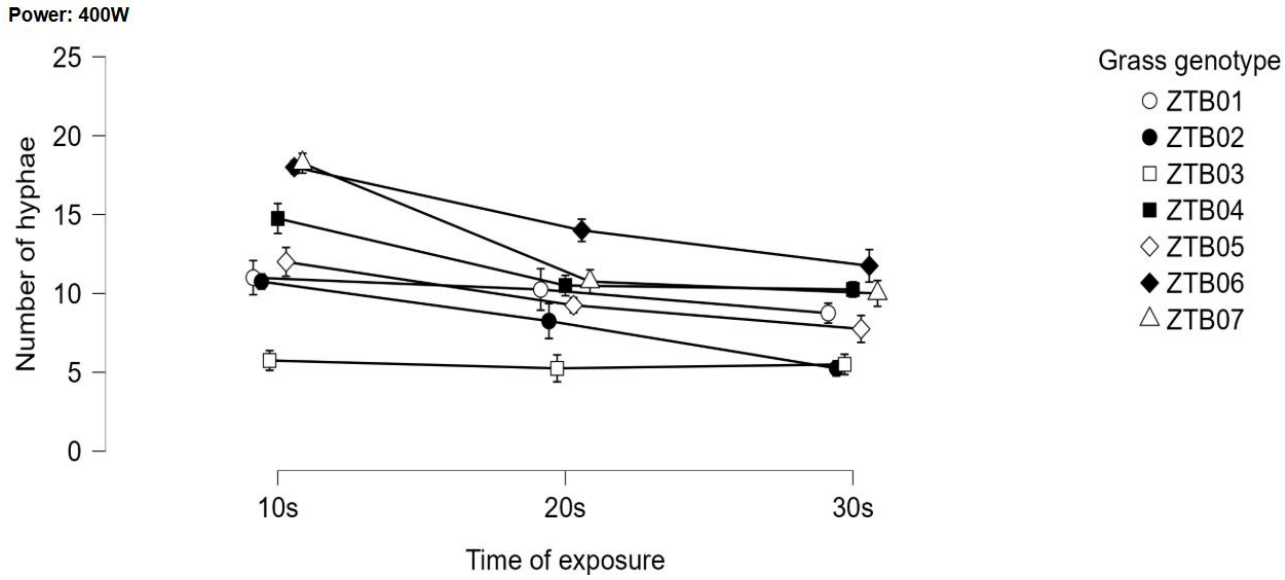

Figure 18. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under DBD plasma (400W) treatment of seed samples.

- Effect of DBD plasma on the number of endophyte hyphae under 500W power

Cold atmospheric plasma with power of 500W tends to reduce the number of fungal hyphae detected in several grass genotypes, indicating this power level enhanced endophytes suppression (Figure 19). The number of fungal hyphae generally started higher at 10 seconds exposure and tended to decrease or remain steady toward 30 seconds across most genotypes. The genotype ZTB06 started with the highest number of fungal hyphae around 20 at 10 seconds and showed a decrease to around 10 at 30 seconds, indicating strong sensitivity to the plasma treatment. Genotype ZTB03 showed a decrease in fungal hyphae from about 10 at 10 seconds to even fewer, around 5 number of hyphae at 30 seconds, indicating high sensitivity to the treatment or low initial infestation. Some genotypes like ZTB01, ZTB02, and ZTB07 fluctuated but stayed roughly in the range of ~7 and ~12 fungal hyphae at 30s. Genotypes ZTB04 and ZTB05 showed less variation and stayed closer to around 10 to 15 fungal hyphae throughout the time points. Genotypes like ZTB06 and ZTB07 exhibit steeper declines, while others like ZTB01 and ZTB04 show more gradual reductions, highlighting variation in genotype sensitivity.

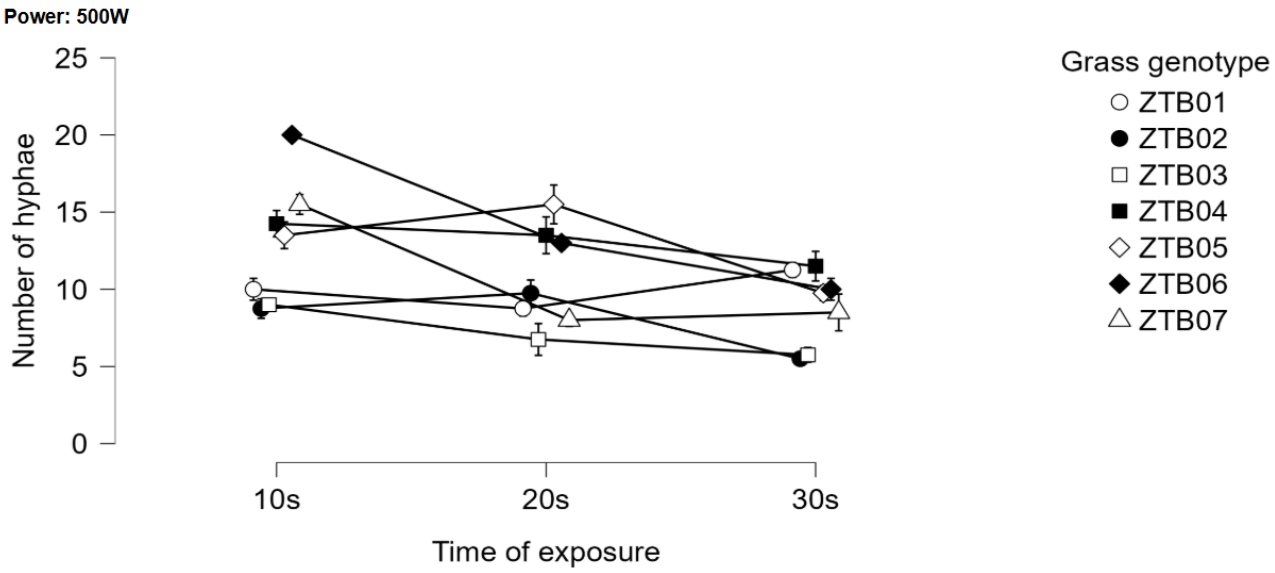

Figure 19. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under DBD plasma (500W) treatment of seed samples.

- Effect of DBD plasma on the number of endophyte hyphae under 600W power

Cold atmospheric plasma at 600W caused a decline in the number of hyphae over time in most grass genotypes. Most genotypes exhibited a downward trend in number of hyphae with increased exposure time (Figure 20). At 10 seconds of exposure, genotype ZTB04 showed the highest hyphal count (~17), which steadily decreased to ~8 by 30 seconds. Genotypes ZTB07 and ZTB05 start with approximately 13 and 11 number of hyphae, respectively, and showed slight decline by 30 seconds. Genotype ZTB02 began with ~10 hyphae and decreased more sharply to ~5 number of hyphae. Standard error bars indicated variability around the mean, suggesting consistency across replicates.

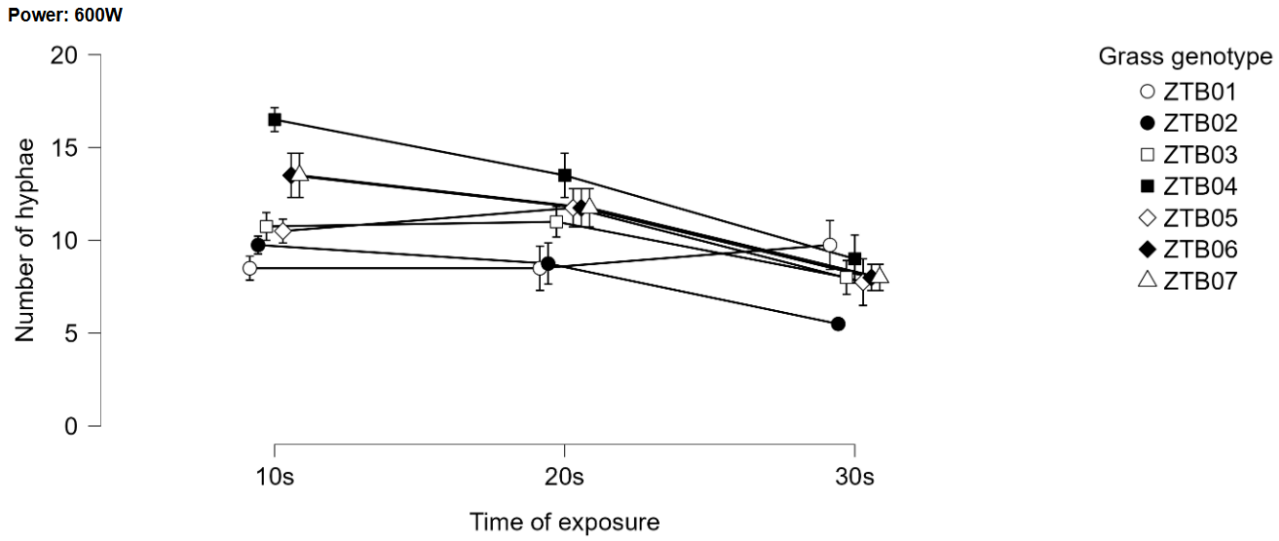

Figure 20. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under DBD plasma (600W) treatment of seed samples.

- The analysis of HSD in the number of endophyte hyphae among grass genotypes

The comparisons among grass genotypes based on mean differences in the number of hyphae revealed highly significant differences ($p < .001$) (Table 12). Several genotype pairs showed statistically significant differences, notably genotype ZTB03, which consistently exhibited lower hyphal counts compared to others, with large effect sizes (e.g., Cohen's $d >$ 2.5 vs. ZTB04, ZTB06, and ZTB07). Genotype ZTB06 vs. ZTB07 also differed significantly from most other genotypes, indicating strong plasma sensitivity or a lower baseline level of endophyte infection. Moderate differences were observed in comparisons such as ZTB01 vs ZTB03 (mean difference = 2.683, $d = 1.551$) and ZTB02 vs ZTB03 (mean difference = 1.867, $d =$ 1.079). Other comparisons showed no significant differences, suggesting similar hyphal levels among those genotype pairs.

Table 12. Pairwise comparison of mean differences on endophyte hyphae produced among grass genotypes (DBD plasma treatment of seed samples)

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
ZTB01	ZTB02	0.817	-0.121	1.754	0.316	315	2.586	0.472	-0.090	1.034	.134	.214
	ZTB03	2.683	1.746	3.621	0.316	315	8.496	1.551	0.961	2.141	< .001 *	< .001 *
	ZTB04	-1.700	-2.637	-0.763	0.316	315	-5.382	-0.983	-1.555	-0.411	< .001 *	< .001 *
	ZTB05	-0.700	-1.637	0.237	0.316	315	-2.216	-0.405	-0.966	0.157	.290	.575
	ZTB06	-2.267	-3.204	-1.329	0.316	315	-7.176	-1.310	-1.892	-0.729	< .001 *	< .001 *
	ZTB07	-2.650	-3.587	-1.713	0.316	315	-8.390	-1.532	-2.121	-0.942	< .001 *	< .001 *
ZTB02	ZTB03	1.867	0.929	2.804	0.316	315	5.910	1.079	0.505	1.654	< .001 *	< .001 *
	ZTB04	-2.517	-3.454	-1.579	0.316	315	-7.968	-1.455	-2.041	-0.868	< .001 *	< .001 *
	ZTB05	-1.517	-2.454	-0.579	0.316	315	-4.802	-0.877	-1.446	-0.307	< .001 *	< .001 *
	ZTB06	-3.083	-4.021	-2.146	0.316	315	-9.762	-1.782	-2.382	-1.182	< .001 *	< .001 *

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
	ZTB07	-3.467	-4.404	-2.529	0.316	315	-10.976	-2.004	-2.614	-1.394	<.001 ***	<.001 ***
ZTB03	ZTB04	-4.383	-5.321	-3.446	0.316	315	-13.878	-2.534	-3.173	-1.895	<.001 ***	<.001 ***
	ZTB05	-3.383	-4.321	-2.446	0.316	315	-10.712	-1.956	-2.564	-1.348	<.001 ***	<.001 ***
	ZTB06	-4.950	-5.887	-4.013	0.316	315	-15.672	-2.861	-3.521	-2.202	<.001 ***	<.001 ***
	ZTB07	-5.333	-6.271	-4.396	0.316	315	-16.886	-3.083	-3.757	-2.409	<.001 ***	<.001 ***
ZTB04	ZTB05	1.000	0.063	1.937	0.316	315	3.166	0.578	0.014	1.142	.028 *	.036 *
	ZTB06	-0.567	-1.504	0.371	0.316	315	-1.794	-0.328	-0.888	0.233	.553	1.000
	ZTB07	-0.950	-1.887	-0.013	0.316	315	-3.008	-0.549	-1.112	0.014	.045 *	.060
ZTB05	ZTB06	-1.567	-2.504	-0.629	0.316	315	-4.960	-0.906	-1.476	-0.336	<.001 ***	<.001 ***

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
	ZTB07	-1.950	-2.887	-1.013	0.316	315	-6.174	-1.127	-1.703	-0.551	<.001 ***	<.001 ***
ZTB06	ZTB07	-0.383	-1.321	0.554	0.316	315	-1.214	-0.222	-0.781	0.338	.889	1.000

* p < .05, *** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 7 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

Note. Results are averaged over the levels of: Time of exposure, Power

- The analysis of HSD in the number of endophyte hyphae among exposure durations

Post hoc comparisons of exposure time (10 s, 20 s, and 30 s), averaged across plasma power and grass genotypes, revealed a significant effect on fungal hyphal counts (Table 13). All pairwise comparisons were highly significant ($p < .001$), with number of hyphae increasing progressively from 10s to 20s, 20s to 30s, and most notably from 10s to 30s. The largest mean difference (3.914) and effect size (Cohen's d = 2.263) occurred between the 10s and 30s, indicating a strong time-dependent effect. Mean differences were 2.043 (10s vs 20s), 1.871 (20s vs 30s), and 3.914 (10s vs 30s), with all Cohen's d values exceeding 1, reflecting large effect sizes. Confidence intervals for both mean differences and effect sizes did not overlap zero, confirming the consistent and significant increase in hyphal counts with longer exposure durations.

Table 13. Pairwise comparison of mean differences on endophyte hyphae produced among different exposure durations (DBD plasma treatment of seed samples).

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
10s	20s	2.043	1.556	2.530	0.207	315	9.880	1.181	0.872	1.490	<.001 *	<.001 *
	30s	3.914	3.427	4.401	0.207	315	18.930	2.263	1.902	2.623	<.001 *	<.001 *
20s	30s	1.871	1.385	2.358	0.207	315	9.051	1.082	0.776	1.388	<.001 *	<.001 *

*** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 3 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

Note. Results are averaged over the levels of: Power, Grass genotype

- The analysis of HSD in the number of endophyte hyphae among power levels

Pairwise comparisons of plasma power levels (200W to 600W), averaged over exposure time and grass genotype, revealed significant differences in number of hyphae (Table 14). All comparisons between 200W and higher power levels (300W, 400W, 500W, and 600W) were highly significant ($p < .001$), with large mean differences and effect sizes. For instance, the comparison between 200W and 600W displayed the largest mean difference (4.524) and effect size (Cohen's $d = 2.615$), indicating a strong suppressive effect of higher plasma power

on fungal hyphae. Similarly, comparisons of 200W with 400W (*d* = 2.560), 500W (*d* = 2.271), and 300W (*d* = 2.181) also showed substantial reductions.

In contrast, comparisons among higher power levels (300W to 600W) showed smaller or non-significant differences. For example, 300W vs 600W was marginally significant (*p* = .042), with a small effect size (*d* = 0.434), while other contrasts, such as 300W vs 400W and 500W, and 400W vs 500W and 600W, were not statistically significant. These results suggest that while increasing plasma power beyond 200 W significantly enhances fungal suppression, further increases beyond 300 W offer a limited benefit.

Confidence intervals for mean differences and effect sizes that do not overlap zero reinforce the robustness of these findings.

Table 14. Pairwise comparison of mean differences on endophyte hyphae produced among different power levels (DBD plasma treatment of seed samples).

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
200 W	300 W	3.774	3.041	4.506	0.267	315	14.137	2.181	1.681	2.682	<.001 *	<.001 *
	400 W	4.429	3.696	5.161	0.267	315	16.590	2.560	2.037	3.083	<.001 *	<.001 *
	500 W	3.929	3.196	4.661	0.267	315	14.717	2.271	1.765	2.777	<.001 *	<.001 *

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
	600 W	4.524	3.791	5.256	0.267	315	16.947	2.615	2.089	3.141	<.001 ***	<.001 ***
300 W	400 W	0.655	-0.078	1.387	0.267	315	2.453	0.378	-0.060	0.817	.104	.147
	500 W	0.155	-0.578	0.887	0.267	315	0.580	0.089	-0.347	0.526	.978	1.000
	600 W	0.750	0.018	1.482	0.267	315	2.810	0.434	-0.005	0.872	.042 *	.053
400 W	500 W	-0.500	-1.232	0.232	0.267	315	-1.873	-0.289	-0.726	0.148	.334	.620
	600 W	0.095	-0.637	0.828	0.267	315	0.357	0.055	-0.381	0.491	.997	1.000
500 W	600 W	0.595	-0.137	1.328	0.267	315	2.230	0.344	-0.094	0.782	.171	.265

* p < .05, *** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 5 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

Note. Results are averaged over the levels of: Time of exposure, Grass genotype

5.4.2. Eradication effectiveness of DBD plasma applied on seedlings

The study evaluated the effectiveness of dielectric barrier discharge (DBD) plasma in eradicating *E. festucae* var. *loli* in perennial ryegrass seedlings. Effectiveness was determined by counting endophytic hyphae in leaf sheaths after four weeks of growth following plasma treatment. A three-way ANOVA revealed that grass genotype had a significant and substantial effect on hyphal counts ($F(6, 189) = 15.82$, $p < .001$, $\omega^2 = 0.164$, 95% CI [0.061, 0.243]), indicating notable differences among genotypes (Table 15). Exposure time also had a small but significant effect ($F(2, 189) = 3.19$, $p = .043$, $\omega^2 = 0.008$, 95% CI [0, 0.044]), as did plasma power ($F(2, 189) = 6.81$, $p = .001$, $\omega^2 = 0.022$, 95% CI [0, 0.072]).

Several two-way interactions were statistically significant, including genotype vs. time ($F(12, 189) = 3.23$, $p < .001$, $\omega^2 = 0.050$), genotype vs. power ($F(12, 189) = 6.17$, $p < .001$, $\omega^2 = 0.115$), and time vs. power ($F(4, 189) = 7.51$, $p < .001$, $\omega^2 = 0.048$), suggesting that the effect of one factor depends on the level of another. Moreover, the three-way interaction among genotype, exposure time, and power was also significant and substantial ($F(24, 189) = 3.87$, $p < .001$, $\omega^2 = 0.127$), indicating a complex combined influence on hyphal suppression. The residual variance was 4.335 ($df = 189$). While genotype emerged as the strongest individual predictor, the significant interaction effects, particularly the three-way interaction, demonstrated that hyphal reduction was influenced by the interplay of genotype, exposure duration, and plasma power rather than by any single factor alone.

Table 15. ANOVA results on effect of Time of exposure, Power, and Grass genotype on the number of endophyte hyphae in plants, and their interactions under DBD plasma treatment of seedlings.

							95% CI for ω²	
Cases	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**	**ω²**	**Lower**	**Upper**
Grass genotype	411.43	6	68.571	15.819	< .001	0.164	0.061	0.243

Cases	Sum of Squares	df	Mean Square	F	p	ω²	95% CI for ω²	
							Lower	Upper
Time of exposure	27.65	2	13.825	3.190	.043	0.008	0.000	0.044
Power	59.06	2	29.528	6.812	.001	0.022	0.000	0.072
Grass genotype ✻ Time of exposure	168.07	12	14.006	3.231	< .001	0.050	0.000	0.059
Grass genotype ✻ Power	321.17	12	26.764	6.174	< .001	0.115	9.399×10^{-4}	0.158
Time of exposure ✻ Power	130.16	4	32.540	7.507	< .001	0.048	0.000	0.104
Grass genotype ✻ Time of exposure ✻ Power	402.12	24	16.755	3.865	< .001	0.127	0.000	0.120
Residuals	819.25	189	4.335					

Note. Type III Sum of Squares

The results demonstrated that hyphae number was strongly genotype-dependent and modulated by both exposure time and power (Table 16). The descriptive statistics for the number of hyphae showed clear variation across grass genotypes (ZTB01–ZTB07), times of exposure (10, 20, and 30 seconds), and power levels (200W, 300W, 400W). Grass genotype strongly influenced hyphae number: for example, ZTB06 exhibited the highest means across several conditions such as 18.5 at 20s/200W and 18.25 at 30s/200W, whereas ZTB03 often showed the lowest counts (9.0 at 30s/300W). Time of exposure showed variable effects depending on genotype; ZTB01 peaked at 20s/300W (15.0) but declined at 30s/200W (10.0),

while ZTB07 increased at longer exposures under certain powers. Power level also interacted with genotype and time, as seen in ZTB02 where number of hyphae decreased from 14.75 at 200W to 9.75 at 400W for the 20s exposure, highlighting that higher power decreased fungal hyphae number. Variability within conditions was notable, with standard deviations ranging from 0.5 to 3.786 and coefficients of variation from 0.033 to 0.261, indicating that some genotype, time of exposure and power combinations elicit more heterogeneous responses.

Table 16. The mean number of endophyte hyphae for each combination of grass genotype, time of exposure (10, 20, and 30 s), and power levels (200–600 W) under DBD plasma treatment of seedlings.

Grass genotype	Time of exposure	Power	N	Mean	SD	SE	Coefficient of variation
ZTB01	10s	200W	4	12.750	0.957	0.479	0.075
		300W	4	12.750	1.893	0.946	0.148
		400W	4	11.000	0.816	0.408	0.074
	20s	200W	4	14.500	2.646	1.323	0.182
		300W	4	15.000	2.160	1.080	0.144
		400W	4	14.500	1.291	0.645	0.089
	30s	200W	4	10.000	0.816	0.408	0.082
		300W	4	15.000	2.944	1.472	0.196
		400W	4	14.750	2.630	1.315	0.178
ZTB02	10s	200W	4	17.750	1.708	0.854	0.096
		300W	4	12.750	0.957	0.479	0.075
		400W	4	14.750	0.957	0.479	0.065
	20s	200W	4	14.750	2.217	1.109	0.150
		300W	4	13.500	1.915	0.957	0.142
		400W	4	9.750	0.500	0.250	0.051
	30s	200W	4	9.750	2.363	1.181	0.242

Grass genotype	Time of exposure	Power	N	Mean	SD	SE	Coefficient of variation
		300W	4	15.500	2.887	1.443	0.186
		400W	4	12.250	2.754	1.377	0.225
ZTB03	10s	200W	4	11.250	2.217	1.109	0.197
		300W	4	11.750	2.062	1.031	0.175
		400W	4	11.250	1.708	0.854	0.152
	20s	200W	4	13.250	2.217	1.109	0.167
		300W	4	12.250	2.630	1.315	0.215
		400W	4	9.250	0.500	0.250	0.054
	30s	200W	4	10.500	1.732	0.866	0.165
		300W	4	9.000	0.816	0.408	0.091
		400W	4	10.500	2.517	1.258	0.240
ZTB04	10s	200W	4	15.250	1.708	0.854	0.112
		300W	4	16.000	2.944	1.472	0.184
		400W	4	15.250	0.500	0.250	0.033
	20s	200W	4	15.000	2.160	1.080	0.144
		300W	4	16.500	1.291	0.645	0.078
		400W	4	15.750	2.062	1.031	0.131
	30s	200W	4	17.500	2.380	1.190	0.136
		300W	4	10.750	0.957	0.479	0.089
		400W	4	17.000	0.816	0.408	0.048
ZTB05	10s	200W	4	12.500	1.915	0.957	0.153
		300W	4	14.250	2.630	1.315	0.185
		400W	4	14.000	2.944	1.472	0.210
	20s	200W	4	14.500	2.646	1.323	0.182
		300W	4	15.250	1.708	0.854	0.112
		400W	4	10.250	1.258	0.629	0.123
	30s	200W	4	11.000	2.708	1.354	0.246

Grass genotype	Time of exposure	Power	N	Mean	SD	SE	Coefficient of variation
		300W	4	11.250	0.957	0.479	0.085
		400W	4	10.500	2.380	1.190	0.227
ZTB06	10s	200W	4	16.250	1.500	0.750	0.092
		300W	4	13.000	2.160	1.080	0.166
		400W	4	13.000	0.816	0.408	0.063
	20s	200W	4	18.500	2.646	1.323	0.143
		300W	4	10.500	2.380	1.190	0.227
		400W	4	9.750	0.500	0.250	0.051
	30s	200W	4	18.250	2.217	1.109	0.121
		300W	4	10.250	2.062	1.031	0.201
		400W	4	14.500	3.786	1.893	0.261
ZTB07	10s	200W	4	17.250	2.630	1.315	0.152
		300W	4	13.750	2.062	1.031	0.150
		400W	4	13.250	2.754	1.377	0.208
	20s	200W	4	13.250	2.500	1.250	0.189
		300W	4	12.750	1.500	0.750	0.118
		400W	4	13.500	3.317	1.658	0.246
	30s	200W	4	11.750	1.708	0.854	0.145
		300W	4	16.000	2.160	1.080	0.135
		400W	4	16.750	2.217	1.109	0.132

- Effect of DBD plasma on the number of endophyte hyphae under 200W power

The graph represents an analysis of the number of hyphae produced by seven different grass genotypes (ZTB01 to ZTB07) plotted against time of exposure to a fixed power of 200W, with measurements taken at 10, 20, and 30 seconds. At 10 seconds, the initial fungal hyphae count was relatively high for all genotypes (Figure 21), with ZTB02 showing the highest beginning level near 18 hyphae and ZTB03 showing the lowest near 11 number of hyphae. Over time, most genotypes (ZTB01, ZTB02, ZTB03, ZTB05, ZTB07) exhibit a decline in hyphae

count, dropping to as low as around 10 hyphae after 30 seconds, indicating a probable inhibitory effect of prolonged exposure. In contrast, genotypes ZTB04 and ZTB06 reacted differently; ZTB04 began at about 15 hyphae and displayed an increasing trend reaching approximately 17 by 30 seconds, while ZTB06 started at around 16, peaked around 19 at 20 seconds, and maintains this elevated level at 30 seconds, suggesting a distinct response mechanism or higher tolerance to exposure in these genotypes. Error bars on each data point reflect the variability of measurements, which are relatively small and consistent across genotypes and times, supporting the reliability of observed trends.

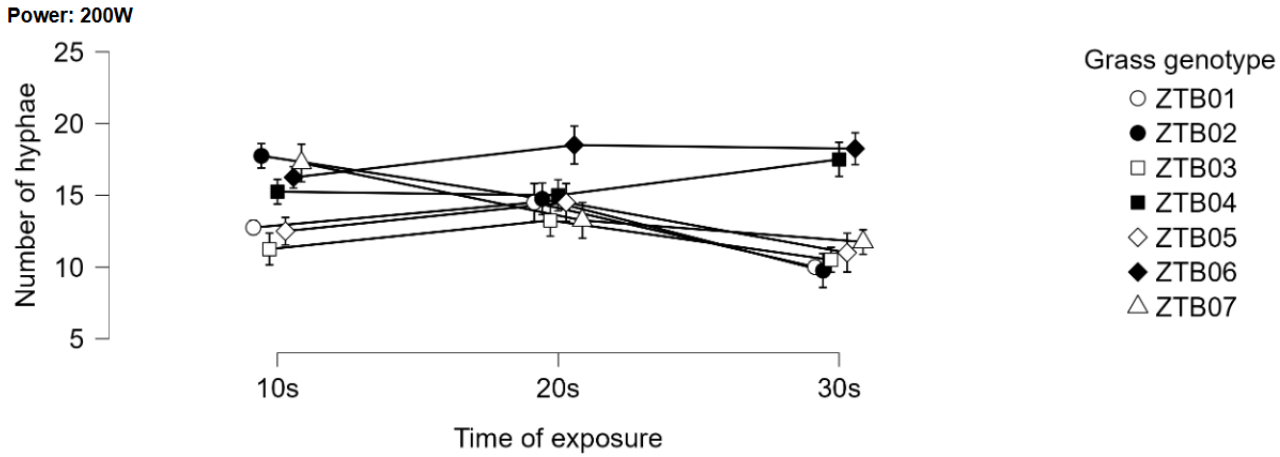

Figure 21. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under DBD plasma (200W) treatment of seedlings.

- Effect of DBD plasma on the number of endophyte hyphae under 300W power

Figure 22 presents a line graph illustrating the number of fungal hyphae produced by seven grass genotypes (ZTB01–ZTB07) across three exposure durations (10 s, 20 s, and 30 s) under a constant plasma power of 300 W. The results revealed genotype-specific responses to the treatment. At 10 s, genotype ZTB04 recorded the highest in number of hyphae (~16), followed by genotype ZTB05 (~14.5) and genotypes ZTB01/ZTB06 (~13), while genotypes ZTB02, ZTB03, and ZTB07 showed slightly lower numbers (~12.5, 11.5, and 12, respectively). By the 20s, genotype ZTB04 remained the most prolific (~16.5), with ZTB01 (~14) and ZTB05 (~15) also maintaining high levels. In contrast, genotype ZTB02 declined to ~10.5, indicating sensitivity to prolonged exposure. At 30 s, genotypes ZTB07 and ZTB02 showed increased hyphal production (~15.5), while ZTB01 remained stable (~15). Genotype ZTB04 dropped

to~9, implying that extended exposure significantly minimized its ability to grow in number of hyphae.

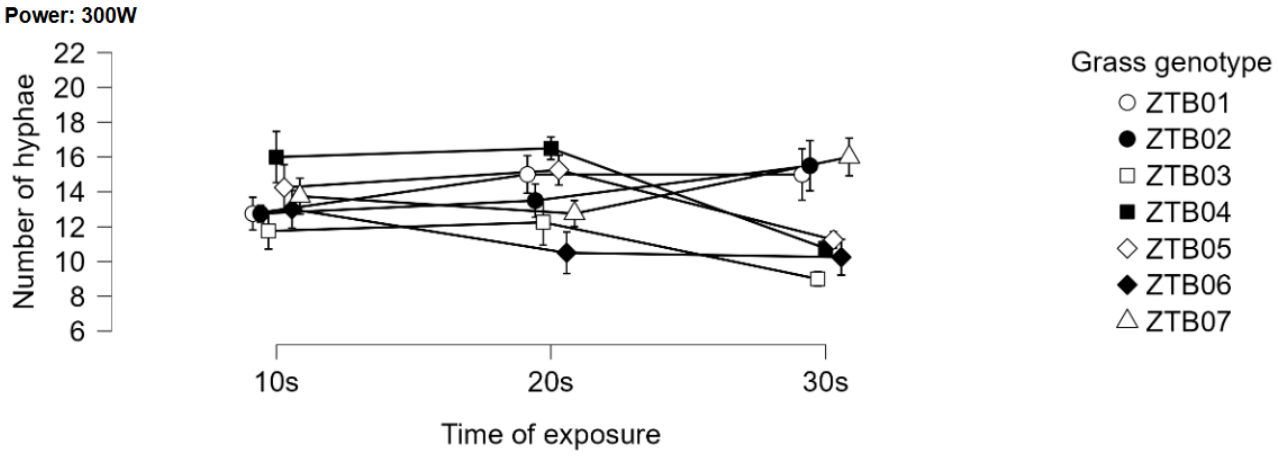

Figure 22. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under DBD plasma (300W) treatment of seedlings.

- Effect of DBD plasma on the number of endophyte hyphae under 400W power

The results demonstrated that hyphal proliferation is genotype-specific at a constant power plasma of 400 W, under different exposure duration (Figure 23). Across the three exposure intervals, the genotypes exhibited divergent hyphal responses: the genotype ZTB04 consistently maintained the highest number of hyphae, remaining near the upper end of the scale, whereas ZTB02 showed a progressive decline, dropping toward the lower range by 30 s; genotypes ZTB01 and ZTB07 displayed modest increases over time, while genotypes ZTB03, ZTB05, and ZTB06 fluctuated with overlapping error bars that indicate measurement variability. The genotypes ZTB04 and ZTB07 showed the most robust growth in fungal hyphae.

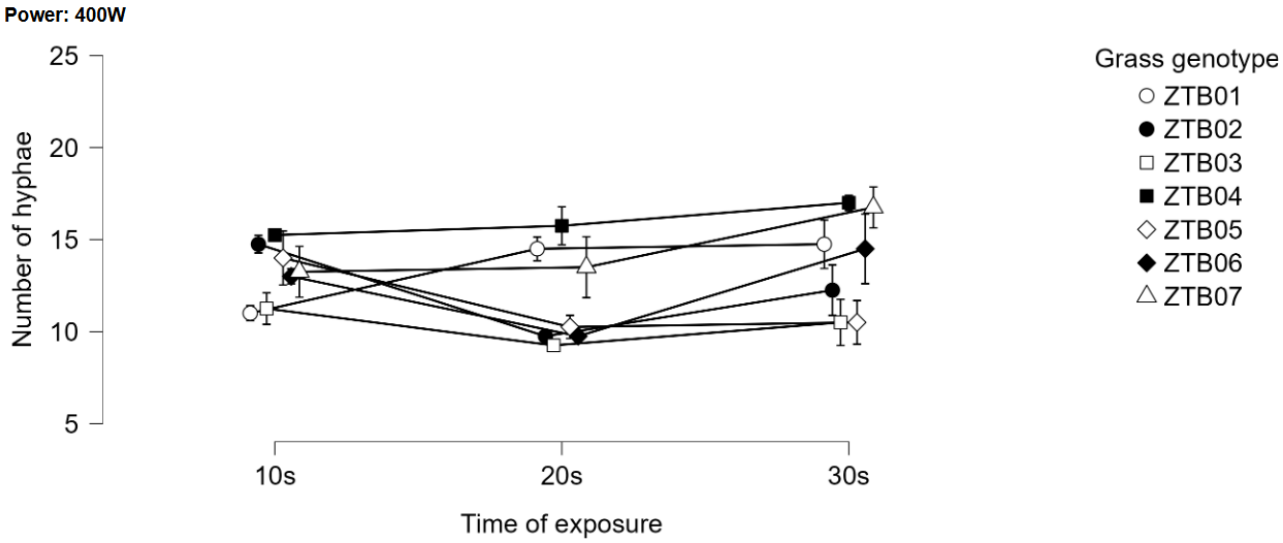

Figure 23. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under DBD plasma (400W) treatment of seedlings.

- The analysis of HSD in the number of endophyte hyphae among grass genotypes

The table 17 presents post hoc pairwise comparisons of fungal hyphal counts among seven grass genotypes (ZTB01–ZTB07), averaged across exposure time and plasma power. Several genotype pairs showed statistically significant differences, indicating genotype-specific responses to plasma treatment. Notably, ZTB03 consistently produced significantly higher number of hyphae compared to all other genotypes, with mean differences ranging from 2.361 (vs. ZTB01, $p < .001$) to 4.444 (vs. ZTB04, $p < .001$). Genotype ZTB04 also differed significantly from ZTB01 and ZTB02, showing lower number of hyphae in both cases ($p < .001$ and $p = .001$, respectively), but recorded significantly higher counts than genotypes ZTB05 and ZTB06 ($p < .001$ and $p = .014$, respectively). Genotype ZTB05 showed a significant difference only when compared to genotype ZTB07 (mean diff = -1.639, $p = .017$), while other comparisons involving genotypes ZTB01, ZTB02, ZTB05, ZTB06, and ZTB07 were not statistically significant ($p > .05$), indicating similar number of hyphae production among these genotypes. The most prolific genotype in terms of fungal hyphae was ZTBO3, while ZTB04 showed the greatest suppression.

Table 17. Pairwise comparison of mean differences on endophyte hyphae produced among grass genotypes (DBD plasma treatment of seedlings)

		Mean Difference	SE	df	t	p_{tukey}
ZTB01	ZTB02	-0.056	0.491	189	-0.113	.000
	ZTB03	2.361	0.491	189	4.811	< .001
	ZTB04	-2.083	0.491	189	-4.245	< .001
	ZTB05	0.750	0.491	189	1.528	.727
	ZTB06	-0.417	0.491	189	-0.849	.979
	ZTB07	-0.889	0.491	189	-1.811	.542
ZTB02	ZTB03	2.417	0.491	189	4.925	< .001
	ZTB04	-2.028	0.491	189	-4.132	.001
	ZTB05	0.806	0.491	189	1.642	.656
	ZTB06	-0.361	0.491	189	-0.736	.990
	ZTB07	-0.833	0.491	189	-1.698	.618
ZTB03	ZTB04	-4.444	0.491	189	-9.057	< .001
	ZTB05	-1.611	0.491	189	-3.283	.021
	ZTB06	-2.778	0.491	189	-5.661	< .001
	ZTB07	-3.250	0.491	189	-6.623	< .001
ZTB04	ZTB05	2.833	0.491	189	5.774	< .001
	ZTB06	1.667	0.491	189	3.396	.014
	ZTB07	1.194	0.491	189	2.434	.190
ZTB05	ZTB06	-1.167	0.491	189	-2.377	.214
	ZTB07	-1.639	0.491	189	-3.340	.017
ZTB06	ZTB07	-0.472	0.491	189	-0.962	.961

Note. P-value adjusted for comparing a family of 7 estimates.

Note. Results are averaged over the levels of: Time of exposure, Power

- The analysis of HSD in the number of endophyte hyphae among exposure durations

The Table 18 presents post hoc pairwise comparisons of fungal hyphal counts across three exposure durations 10 seconds, 20 seconds, and 30 seconds averaged over grass genotypes and plasma power. The results indicate that the difference between 10s and 30s was statistically significant (mean difference = 0.810, p = .033), indicating that longer exposure time led to a considerable increase in number of hyphae. In contrast, the comparisons between 10s and 20s (mean difference = 0.357, p = .508) and between 20s and 30s (mean difference = 0.452, p = .339) were not statistically significant, suggesting that the changes in hyphal numbers between these intermediate durations were not substantial. These findings imply a time-dependent effect, where the most pronounced increase in fungal hyphae occurred between the shortest and longest exposure durations, while shorter intervals may not produce significant changes. The intermediate exposure durations did not differ significantly from each other.

Table 18. Pairwise comparison of mean differences on endophyte hyphae produced among different exposure durations (DBD plasma treatment of seedlings).

		Mean Difference	SE	df	t	p_{tukey}
10s	20s	0.357	0.321	189	1.112	.508
	30s	0.810	0.321	189	2.520	.033
20s	30s	0.452	0.321	189	1.408	.339

Note. P-value adjusted for comparing a family of 3 estimates.

Note. Results are averaged over the levels of: Grass genotype, Power

- The analysis of HSD in the number of endophyte hyphae among power levels

The post hoc Tukey comparisons for power levels, averaged across all grass genotypes and exposure times, revealed that increasing power from 200W to higher levels significantly affected the number of fungal hyphae (Table 19). Specifically, 200W vs. 300W showed a mean difference of 0.845 hyphae (SE = 0.321), which was statistically significant (t (189) = 2.631, p =

0.025), indicating a modest increase in hyphae number at 300W compared with 200W. Similarly, 200W vs. 400W exhibited a larger and highly significant mean difference of 1.143 hyphae (SE = 0.321, t (189) = 3.557, p = 0.001), demonstrating that hyphae counts were significantly higher at 400W than at 200W. In contrast, 300W vs. 400W showed a non-significant difference of 0.298 hyphae (SE = 0.321, t (189) = 0.926, p = 0.624), suggesting that hyphae numbers at these two higher power levels did not differ appreciably. The results indicated that increasing power from 200W to 300W or 400W led to a significant increase in fungal hyphae number but further increased beyond 300W did not produce additional significant benefit.

Table 19. Pairwise comparison of mean differences on endophyte hyphae produced among different power levels (DBD plasma treatment of seedlings).

		Mean Difference	**SE**	**df**	**t**	**p_{tukey}**
200W	300W	0.845	0.321	189	2.631	.025
	400W	1.143	0.321	189	3.557	.001
300W	400W	0.298	0.321	189	0.926	.624

Note. P-value adjusted for comparing a family of 3 estimates.

Note. Results are averaged over the levels of: Grass genotype, Time of exposure

5.4.3. Eradication effectiveness of GA plasma applied on seeds

Experiments on the eradication effectiveness of GA plasma against *E. festucae* var. *loli* were conducted using a generator power of 200 W. Longer exposure times caused damage to the kernels and had a negative impact on their germination. The same exposure times were used as in the DBD plasma experiments: 10 s, 20 s, and 30 s. The effectiveness of GA plasma was measured by the number of endophyte mycelium hyphae in the leaf sheaths after 4 weeks of growth following plasma treatment.

The two-way ANOVA evaluated the effects of grass genotype, time of exposure, and their interaction on the number of hyphae. This indicated that the pattern of hyphal production across exposure times was consistent across all genotypes; no genotype showed

a unique response trend to longer or shorter exposure (Table 20). The genotype factor showed a highly significant effect: F (6, 63) = 7.28, p < .001. The effect size (ω^2) is 0.33 (95 % CI [0.10, 0.46]), indicating that roughly one-third of the variance in hyphal counts was attributable to differences among genotypes which was a large practical effect. Time did not significantly influence the number of hyphae: F (2, 63) = 1.20, p = .307. The ω^2 estimate was only 0.004 (95 % CI [0.000, 0.046]), effectively negligible, showing that exposure duration (10 s, 20 s, 30 s) contributed very little to the variability. The genotype and time interaction effect was also non-significant: F (12, 63) = 0.45, p = .937, with ω^2 = 0.000. The residual mean square was 7.04, representing within-group variability not explained by the model.

Table 20. ANOVA results on effect of Time of exposure and Grass genotype on the number of endophyte hyphae in plants, and their interactions under GA plasma treatment of seed samples.

							95% CI for ω^2	
Cases	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**	**ω^2**	**Lower**	**Upper**
Grass genotype	307.40	6	51.234	7.278	< .001	0.326	0.100	0.459
Time of exposure	16.93	2	8.464	1.202	.307	0.004	0.000	0.046
Grass genotype ✻ Time of exposure	37.74	12	3.145	0.447	.937	0.000	0.000	0.000
Residuals	443.50	63	7.040					

Note. Type III Sum of Squares

The results in table 21 are generated across all genotypes, mean hyphal counts range from about 12 to 21, with standard deviations (SD) between ~0.6 and ~4.5. Standard errors (SE) were proportionally small because of the fixed sample size. Coefficients of variation (CV), a measure of relative variability, spanned from very low (0.028) to moderate (~0.25), indicating that some genotype, time combinations were highly consistent while others showed more dispersion. Genotype ZTB07 consistently showed the largest number of hyphae, peaking at 20.5 ± 0.58 (SE 0.29) at 10 s and remaining comparatively high at 20 s (18.5 ± 3.51) and 30 s (16.5 ± 1.29). Genotype ZTB06 followed closely with means of 18.75 ± 1.26 at 10 s and a stable 18.0 at 20 and 30 s. we have noticed intermediate means in genotypes such as ZTB02 and ZTB04 had moderately high values, generally between 15 and 17.5. Genotype ZTB05 is similar but slightly lower, ranging from 14.75 to 15.5 across times. On the other hand, lowest means were observed in genotypes ZTB01 and ZTB03 produced the fewest hyphae, with means mostly between 12 and 14.5. Within most genotypes, changes over the three exposure durations were small relative to their standard deviations, suggesting no strong time trend. A slight decline from 10 s to 30 s was visible in ZTB07 (20.5 to 16.5) and ZTB02 (17 to 16), while ZTB04 showed a small increase (16.5 to 17.5). Variability often peaked at 20 s, as seen in ZTB06 (SD 4.55, CV 0.25) and ZTB04 (SD 3.70, CV 0.24). Genotype ZTB07 at 10 s was the most uniform condition (CV 0.028), whereas ZTB06 at 20 s showed the greatest relative spread (CV 0.253). In general, higher mean counts were not always associated with higher variability. Time of exposure introduced minor fluctuations but did not dramatically alter the ranking of genotypes.

Table 21. The mean number of endophyte hyphae for each combination of grass genotype and time of exposure (10, 20, and 30 s) under GA plasma treatment of seed samples.

Grass genotype	Time of exposure	N	Mean	SD	SE	Coefficient of variation
ZTB01	10s	4	14.50	1.732	0.866	0.119
	20s	4	13.75	3.304	1.652	0.240
	30s	4	14.25	2.630	1.315	0.185

Grass genotype	Time of exposure	N	Mean	SD	SE	Coefficient of variation
ZTB02	10s	4	17.00	1.414	0.707	0.083
	20s	4	15.25	3.500	1.750	0.230
	30s	4	16.00	2.160	1.080	0.135
ZTB03	10s	4	13.00	2.160	1.080	0.166
	20s	4	12.00	1.826	0.913	0.152
	30s	4	13.50	2.646	1.323	0.196
ZTB04	10s	4	16.50	1.732	0.866	0.105
	20s	4	15.50	3.697	1.848	0.239
	30s	4	17.50	2.517	1.258	0.144
ZTB05	10s	4	15.50	1.732	0.866	0.112
	20s	4	15.25	3.500	1.750	0.230
	30s	4	14.75	3.686	1.843	0.250
ZTB06	10s	4	18.75	1.258	0.629	0.067
	20s	4	18.00	4.546	2.273	0.253
	30s	4	18.00	2.160	1.080	0.120
ZTB07	10s	4	20.50	0.577	0.289	0.028
	20s	4	18.50	3.512	1.756	0.190
	30s	4	16.50	1.291	0.645	0.078

- Effect of exposure time on the number of endophyte hyphae under treatment by GA plasma under 200W

Across the experiment, grass genotype, is the primary driver of fungal-hyphae abundance. The results indicate that exposure duration up to 30 seconds does not meaningfully alter fungal hyphal numbers, nor does it change how different genotypes behave relative to one another (Figure 24). Genotypes ZTB07 and ZTB06 consistently exhibit the greatest number of hyphae. Genotype ZTB07 begins around 20–21 number of hyphae and ends near 17, while genotype ZTB06 stays near 18–19 throughout. Other genotypes considered as intermediate produces of number of hyphae. For instance, genotype ZTB02, ZTB04, and ZTB05 form a middle group, generally between 15 and 17 hyphae across times. On the other hand, another group of genotypes such as ZTB01 and ZTB03 remain the lowest, typically 12–15. Variation across exposure time is inconsistent and means within each genotype shift only slightly between 10, 20, and 30 seconds, and the directions of change are inconsistent. Some genotypes such as ZTB07 and ZTB02 showed small decreases over time. Others such as ZTB04 and ZTB06 showed a mild increase or remain stable. Statistical testing shows no significant main effect of time (F = 1.20, p = .307, $\omega^2 \approx 0.004$) and no genotype × time interaction (F = 0.45, p = .937).

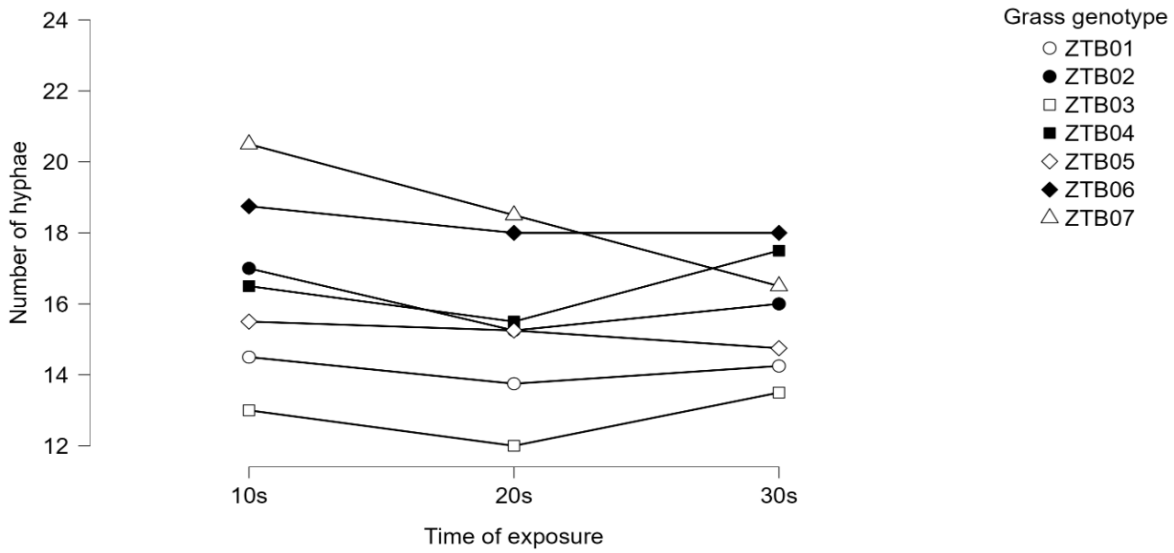

Figure 24. Effect of exposure time (10, 20, and 30 s) on the number of endophyte hyphae in plants across grass genotypes (ZTB01–ZTB07) under GA plasma (200W) treatment of seed samples.

- The analysis of HSD in the number of endophyte hyphae among grass genotypes

The post hoc Tukey comparisons reveal several noteworthy differences among the seven grass genotypes (ZTB01–ZTB07), after averaging across exposure times and adjusting confidence intervals and p-values for multiple testing (Table 22). Compared with ZTB01, both ZTB06 and ZTB07 showed the strongest and most consistent decreases in the measured response. ZTB06 differed from ZTB01 by a mean of –4.08 units (95 % CI [–7.38, –0.78]) with a moderate to large negative effect size (Cohen's d = –1.54, 95 % CI [–2.90, –0.18]), significant at p = .006 (Tukey) and p = .008 (Bonferroni). ZTB07 shows a very similar pattern (mean difference –4.33, 95 % CI [–7.63, –1.03]; d = –1.63, 95 % CI [–3.01, –0.26]), significant at p = .003 and p = .004. Other contrasts with ZTB01 (vs. ZTB02, ZTB03, ZTB04, ZTB05) are not significant, indicating that only the genotypes ZTB06 and ZTB07 are clearly lower than ZTB01. No pairwise comparison involving ZTB02 reaches the adjusted significance thresholds. The largest positive difference is against ZTB03 (mean = 3.25, 95 % CI [–0.05, 6.55], p = .056), which approaches but does not reach significance after correction. Genotype ZTB03 stands out for several significant negative differences when compared with later genotypes. Relative to ZTB04 the mean difference is –3.67 (95 % CI [–6.97, –0.37]; d = –1.38), significant at p = .020 (Tukey) and p = .026 (Bonferroni). The contrasts with ZTB06 and ZTB07 are even stronger: ZTB06 is lower by –5.42 (95 % CI [–8.72, –2.12]; d = –2.04) and ZTB07 by –5.67 (95 % CI [–8.97, –2.37]; d = –2.14), both highly significant (p < .001 for both correction methods). These results indicate that ZTB03 exhibited substantially higher response values than ZTB06 and ZTB07. Most pairwise comparisons among ZTB04, ZTB05, and ZTB06 did not reach significance, except for a marginal effect between ZTB05 and ZTB07 (mean difference –3.33, 95 % CI [–6.63, –0.03], p = .046 Tukey, p = .065 Bonferroni), which is only significant under the Tukey adjustment. The final contrast, ZTB06 vs. ZTB07, was negligible (mean –0.25, 95 % CI [–3.55, 3.05]), confirming that these two genotypes perform similarly.

Table 22. Pairwise comparison of mean differences on endophyte hyphae produced among grass genotypes (GA plasma treatment of seed samples).

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
ZTB01	ZTB02	-1.917	-5.216	1.382	1.083	63	-1.769	-0.722	-2.031	0.586	.573	1.000
	ZTB03	1.333	-1.966	4.632	1.083	63	1.231	0.503	-0.798	1.803	.879	1.000
	ZTB04	-2.333	-5.632	0.966	1.083	63	-2.154	-0.879	-2.196	0.437	.335	.736
	ZTB05	-1.000	-4.299	2.299	1.083	63	-0.923	-0.377	-1.674	0.920	.967	1.000
	ZTB06	-4.083	-7.382	-0.784	1.083	63	-3.770	-1.539	-2.903	-0.175	.006 **	.008 **
	ZTB07	-4.333	-7.632	-1.034	1.083	63	-4.001	-1.633	-3.005	-0.261	.003 **	.004 **
ZTB02	ZTB03	3.250	-0.049	6.549	1.083	63	3.000	1.225	-0.113	2.563	.056	.081
	ZTB04	-0.417	-3.716	2.882	1.083	63	-0.385	-0.157	-1.450	1.136	.000	1.000

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
	ZTB05	0.917	-2.382	4.216	1.083	63	0.846	0.345	-0.951	1.642	.979	1.000
	ZTB06	-2.167	-5.466	1.132	1.083	63	-2.000	-0.817	-2.130	0.496	.425	1.000
	ZTB07	-2.417	-5.716	0.882	1.083	63	-2.231	-0.911	-2.229	0.407	.294	.614
ZTB03	ZTB04	-3.667	-6.966	-0.368	1.083	63	-3.385	-1.382	-2.732	-0.032	.020 *	.026 *
	ZTB05	-2.333	-5.632	0.966	1.083	63	-2.154	-0.879	-2.196	0.437	.335	.736
	ZTB06	-5.417	-8.716	-2.118	1.083	63	-5.001	-2.042	-3.457	-0.626	< .001 *	< .001 *
	ZTB07	-5.667	-8.966	-2.368	1.083	63	-5.232	-2.136	-3.562	-0.710	< .001 *	< .001 *
ZTB04	ZTB05	1.333	-1.966	4.632	1.083	63	1.231	0.503	-0.798	1.803	.879	1.000
	ZTB06	-1.750	-5.049	1.549	1.083	63	-1.616	-0.660	-1.965	0.646	.673	1.000

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
	ZTB07	-2.000	-5.299	1.299	1.083	63	-1.846	-0.754	-2.064	0.556	.523	1.000
ZTB05	ZTB06	-3.083	-6.382	0.216	1.083	63	-2.847	-1.162	-2.496	0.171	.082	.125
	ZTB07	-3.333	-6.632	-0.034	1.083	63	-3.077	-1.256	-2.597	0.084	.046 *	.065
ZTB06	ZTB07	-0.250	-3.549	3.049	1.083	63	-0.231	-0.094	-1.387	1.199	.000	1.000

* p < .05, ** p < .01, *** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 7 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

Note. Results are averaged over the levels of: Time of exposure

- The analysis of HSD in the number of endophyte hyphae among exposure durations

The Tukey post hoc comparison in Table 23 examines differences in the number of fungal hyphae among the three exposure times (10 s, 20 s, and 30 s), averaging across all grass genotypes. The results exhibited that no pair of exposure times differed significantly in the number of hyphae, because all confidence intervals for the mean differences included zero and all adjusted *p*-values were well above 0.05. 10 s vs. 20 s: the mean difference was 1.07 hyphae (95 % CI [–0.63, 2.77]), $t(63) = 1.51$. The effect size was small (Cohen's $d = 0.40$, 95 % CI [–0.26, 1.07]). Both the Tukey-adjusted $p = 0.293$ and Bonferroni-adjusted $p = 0.407$

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indicated no significant difference. 10 s vs. 30 s: the mean difference was 0.75 (95 % CI [–0.95, 2.45]), $t(63) = 1.06$, $d = 0.28$ (95 % CI [–0.38, 0.94]), again non-significant (Tukey $p = 0.544$; Bonferroni $p = 0.883$). 20 s vs. 30 s: the mean difference was –0.32 (95 % CI [–2.02, 1.38]), $t(63) = –0.45$, $d = –0.12$ (95 % CI [–0.78, 0.54]); Tukey $p = 0.893$ and Bonferroni $p = 1.000$.

Table 23. Pairwise comparison of mean differences on endophyte hyphae produced among time of exposure (GA plasma treatment of seed samples).

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}
10 s	20 s	1.071	-0.631	2.774	0.709	63	1.511	0.404	-0.259	1.067	.293	.407
	30 s	0.750	-0.952	2.452	0.709	63	1.058	0.283	-0.378	0.943	.544	.883
20 s	30 s	-0.321	-2.024	1.381	0.709	63	-0.453	-0.121	-0.779	0.537	.893	1.000

Note. P-value and confidence intervals adjusted for comparing a family of 3 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

Note. Results are averaged over the levels of: Grass genotype

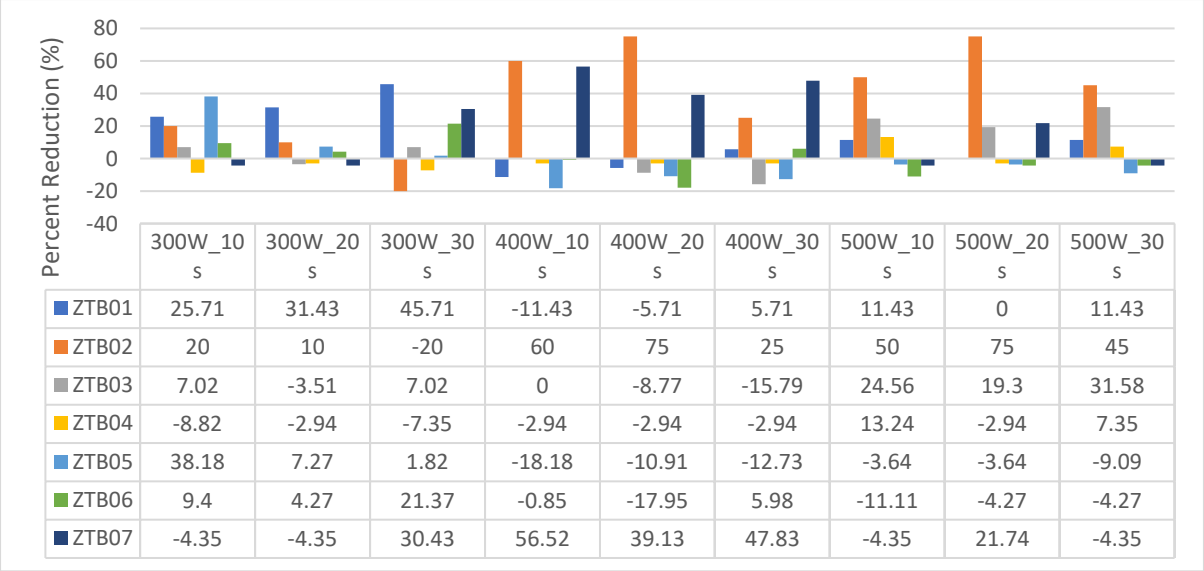
5.5. Effect of cold plasma on seeds infestation by fungi

This study explored the influence of DBD plasma treatment on the total number of microorganisms isolated from seeds of chosen genotypes of perennial ryegrass. The test was conducted under varying power levels (300W, 400W, and 500W) and exposure durations (10s, 20s, and 30s), including a non-plasma-treated seeds as a control combination for comparison.

In general, the number of fungal colonies considerably declined after plasma exposure. The untreated seeds had the highest number of colonies (Figure 25). To figure out the power and the time of exposure that stimulated the higher reduction in microorganisms, the percentage reduction was estimated:

Reduction (%) = (Control Value−Treated Value) / Control Value) ×100

Bars above zero represent positive values, which indicate a percentage reduction in microbial organisms. Contrarily, the bars below zero represent negative values that indicate an increase in microbial organisms instead of a reduction. In most cases, the microbial percentage was decreased compared to the control; for example, ZTB02 under 500 W at 20 s reached 75% of reduction, while ZTB07 under 400 W at 10 s reached 56% of reduction. This proved that DBD plasma was influencing the reduction of microbial load. However, some genotypes were demonstrated to consistently retain higher microbial counts even if higher power and exposure time were applied; the results revealed that these genotypes were less affected by DBD plasma. Power and exposure time did not always lead to significant microbial reduction. For instance, genotype ZTB06 under 400W for 20 seconds increased microorganisms by up to 17.95%, and genotype ZTB05 under 400W for 10 seconds increased them by 18.18%. In contrast, genotype ZTB02 consistently showed significant reductions across various power levels and exposure durations, with an increase of up to 20% of microorganisms at 30 seconds under 300W. The percentage of the microbial counts sometimes boosted compared to the control in such genotypes probably required an alternative treatment. Figure 26 is an example of high inhibiting effect of DBD plasma treatment on the development of fungal colonies in some cases. There was observed higher number of colonies growing on the seeds from control combination than on seeds treated with DBD plasma (ZTB02, 500 W, 20 s).



	300W_10 s	300W_20 s	300W_30 s	400W_10 s	400W_20 s	400W_30 s	500W_10 s	500W_20 s	500W_30 s
ZTB01	25.71	31.43	45.71	-11.43	-5.71	5.71	11.43	0	11.43
ZTB02	20	10	-20	60	75	25	50	75	45
ZTB03	7.02	-3.51	7.02	0	-8.77	-15.79	24.56	19.3	31.58
ZTB04	-8.82	-2.94	-7.35	-2.94	-2.94	-2.94	13.24	-2.94	7.35
ZTB05	38.18	7.27	1.82	-18.18	-10.91	-12.73	-3.64	-3.64	-9.09
ZTB06	9.4	4.27	21.37	-0.85	-17.95	5.98	-11.11	-4.27	-4.27
ZTB07	-4.35	-4.35	30.43	56.52	39.13	47.83	-4.35	21.74	-4.35

Figure 25. Percentage reduction of microorganisms' colonies on PDA medium isolated from seeds of ZTB01-ZTB07 genotypes under DBD plasma (300-500W, 10-30s) treatment compared to the control.

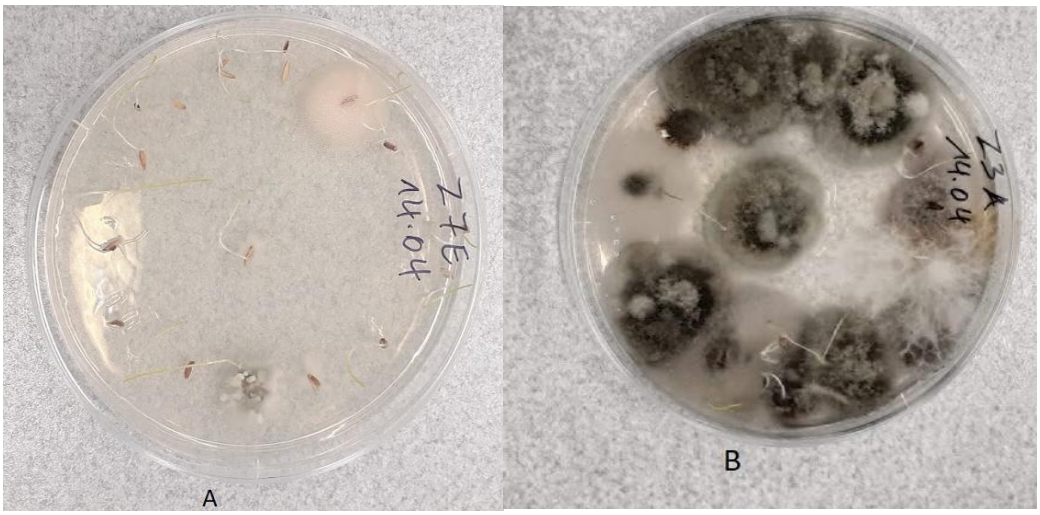


Figure 26. Colonies of microorganisms on PDA medium isolated from seeds of ZTB02 genotype under DBD plasma (500W, 20s) treatment (A) and from untreated seeds (B).

5.6. Effect of CAP treatment on the activity of chosen PR enzymes

5.6.1. Effect of DBD plasma treatment on glucanases activity

The variance analysis of grass genotype demonstrated high significance. The ω^2 (size effect) value of 0.051 suggests that about 5.1% (Table 24) of the total variance in GLU content was explained by differences among grass genotypes, a small to moderate effect size. The time

of exposure also significantly affected the GLU activity level but explains a smaller portion of variance (~0.9%). The lower bound of the confidence interval touching zero suggests this effect was weaker but still statistically significant. Power had the largest effect on GLU activity, explaining approximately 23.7% of the variance, indicating it was the most influential factor among the three. The two-way interaction of grass genotype and time of exposure was significant and explains about 10.7% of the variance, indicating that the effect of grass genotype on GLU activity depended on the time of exposure. While grass genotype and power interaction explains about 4.7% of variance, it indicates that the genotype effect varied with power levels. On the other hand, the time of exposure and power interaction was not statistically significant, indicating no strong combined effect of time of exposure and power that influenced the amount of GLU activity. The three-way interaction of grass genotype, time of exposure, and power explains about 10.8% of the variance, showing that the influence of the combined effect of genotype and time of exposure on GLU depended on the power level. All main effects except for the interaction between time of exposure and power were statistically significant ($p < 0.001$), indicating robust influences on the GLU activity. In this study, power was the most significant factor influencing GLU activity, accounting for nearly a quarter of the variance; therefore, to effectively influence the GLU outcome, we need to adjust the power level. Significant interactions, in which the effect of one factor depends on the levels of other factors, particularly the three-way interaction, signify complex relationships. For instance, the influence of grass genotype on the amount of GLU activity changed based on both duration of exposure and power. The effect sizes (ω^2) gave perspective on practical significance: while some effects were statistically significant, their contribution to total variance varied from small (~1%) to moderate (~24%). Since there was a large residual variance, the other factors not included in the model or inherent variability contributed substantially to GLU activity variation.

Table 24. ANOVA results on effect of Time of exposure, Power, and Grass genotype on glucanases activity in plants, and their interactions under DBD plasma treatment.

							95% CI for ω^2	
Variables	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**	**ω^2**	**Lower**	**Upper**
Grass genotype	403.75	6	67.29	15.76	< .001	0.051	0.018	0.080

Time of exposure	78.50	3	26.17	6.13	< .001	0.009	0.000	0.024
Power	1759.15	2	879.58	205.96	< .001	0.237	0.184	0.289
Grass genotype x Time of exposure	868.11	18	48.23	11.29	< .001	0.107	0.047	0.132
Grass genotype x Power	400.12	12	33.34	7.81	< .001	0.047	0.008	0.066
Time of exposure x Power	46.96	6	7.83	1.83	0.090	0.003	0.000	0.004
Grass genotype x Time of exposure x Power	948.75	36	26.35	6.17	< .001	0.108	0.026	0.110
Residuals	2869.82	672	4.27					

- The effect of DBD plasma treatment on GLU activity under 300W power

The study revealed that across all genotypes, GLU activity remained within a relatively narrow range of approximately 10–18 units throughout the exposure period, implying small time-based variation (Figure 27). Most genotypes displayed small, non-directional fluctuations rather than a clear increasing or decreasing trend. For example, genotype ZTB02 began at the upper end of the range near 18 units, declined slightly at 10–20 seconds, and then recovered to a similar level by time of exposure 30 s. In contrast, genotype ZTB04 showed a gradual decrease from about 13 units at the start to around 10 units at 30 s, while genotype ZTB06 dipped to its lowest point at 20 seconds before a modest rebound. Other genotypes (ZTB01, ZTB03, ZTB05, ZTB07) hover consistently between 12 and 15 units with only slight, irregular changes over time. Importantly, the error bars representing standard error overlap extensively among all genotypes and time points, suggesting that differences observed were not statistically robust. The results displayed that 300 W did not uniformly affect GLU activity. Due to more fluctuations in GLU activity, genotype did not prove to be a crucial factor.

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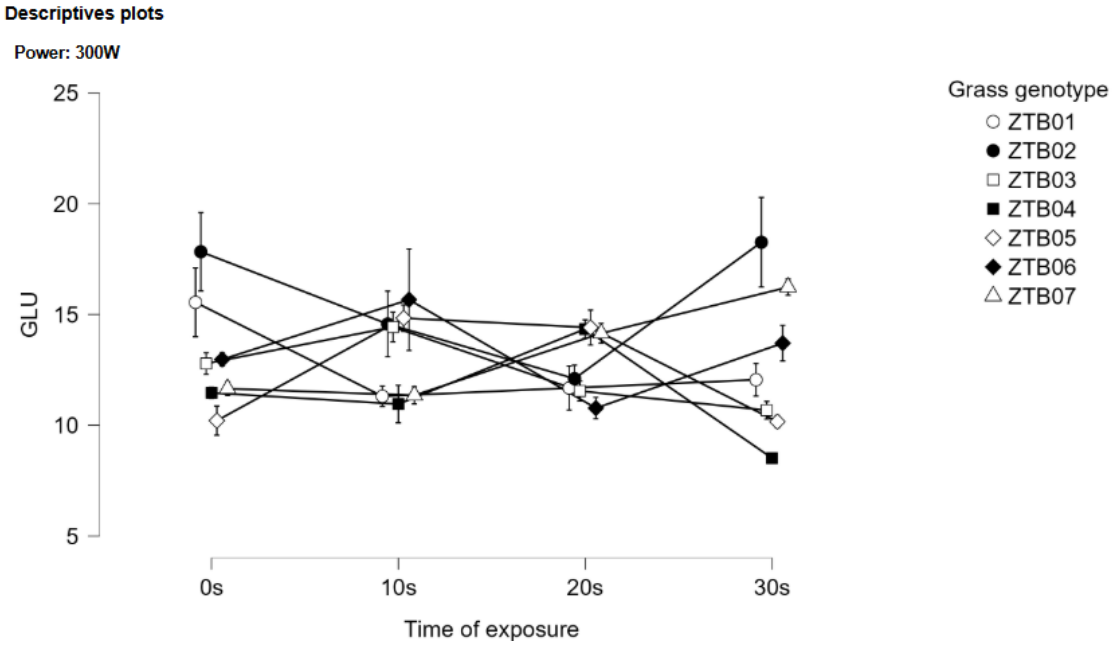

Figure 27. Variation in glucanase activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (300W) treatment.

- The effect of DBD plasma treatment on GLU activity under 400W power

The Figure 28 represents the variation of glucanase activity across seven genotypes (ZTB01-ZTB07) of perennial ryegrass treated by CAP at a constant power of 400W under different times of exposure (untreated 0s, 10s, 20s, and 30s). Most genotypes showed relatively stable glucanase activity across time points, clustering between 8 and 12 units. The genotype ZTB07 stands out, exhibiting a sharp peak at 20s, getting the highest glucanase activity before it returned to the baseline at 30s. The genotype ZTB01 showed a slight dip at 10s and 20s, then a small increase at 30s. On the other hand, the genotypes ZTB03, ZTB04, ZTB06, and ZTB05 displayed minor fluctuations but no dramatic changes. Genotype ZTB02 remained relatively flat throughout. Except for genotype ZTB07, which might be particularly sensitive or responsive to this treatment time, this figure proves that plasma treatment at 400W had a largely stable effect on GLU activity for most perennial ryegrass genotypes.

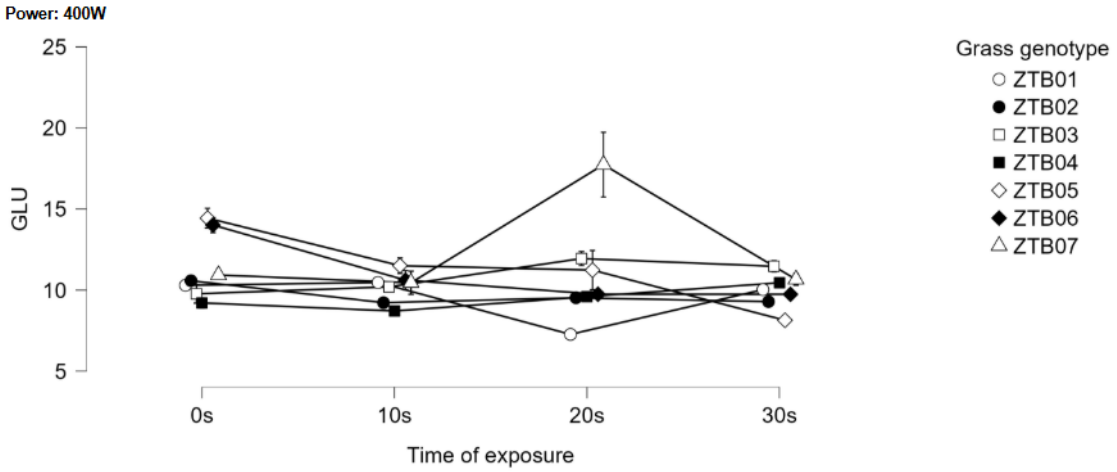

Figure 28. Variation in glucanase activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (400W) treatment.

- The effect of DBD plasma treatment on GLU activity under 500W power

Seven perennial ryegrasses were treated by CAP with a power of 500 W under 4 different durations (0 s, 10 s, 20 s, 30 s) (Figure 29). The genotype ZTB06 started with the highest GLU activity at 0s (approximately 12.5), dipped at 10s, then rose again at 20s before returning to baseline at 30s. This pattern can suggest that CAP treatment was negatively affecting the glucanase activity in genotype ZTB06, whereas genotype ZTB07 showed a peak at 20s (~12) but otherwise fluctuated around the mean, and ZTB03 rose to a maximum at 20s and then dropped at 30s. On the other hand, ZTB04 remained relatively stable across all time points, with a slight increase, while ZTB01 declined in the 20s and returned to the base in the 30s, and ZTB05 exhibited small changes but no dramatic peaks or troughs. Across all genotypes, the figure shows that there was no consistent variation in the GLU activioty level with increased exposure time. Peaks at 20s for most genotypes (ZTB03, ZTB05, and ZTB07) showed their highest GLU values at 20s, suggesting a possible optimal exposure duration for these genotypes under 500W plasma treatment. These peaks suggest that optimizing plasma exposure time could enhance glucanase activity, but this effect was not universal across all tested genotypes. Most GLU activities fall between 8 and 12 across all genotypes and time points. GLU activity was comparatively constant across all genotypes, suggesting that CAP treatment at 500 W was not harmful within the tested time frame. The reaction of genotype

ZTB02 with a flat line indicates that this genotype was less responsive to CAP treatment at 500 W.

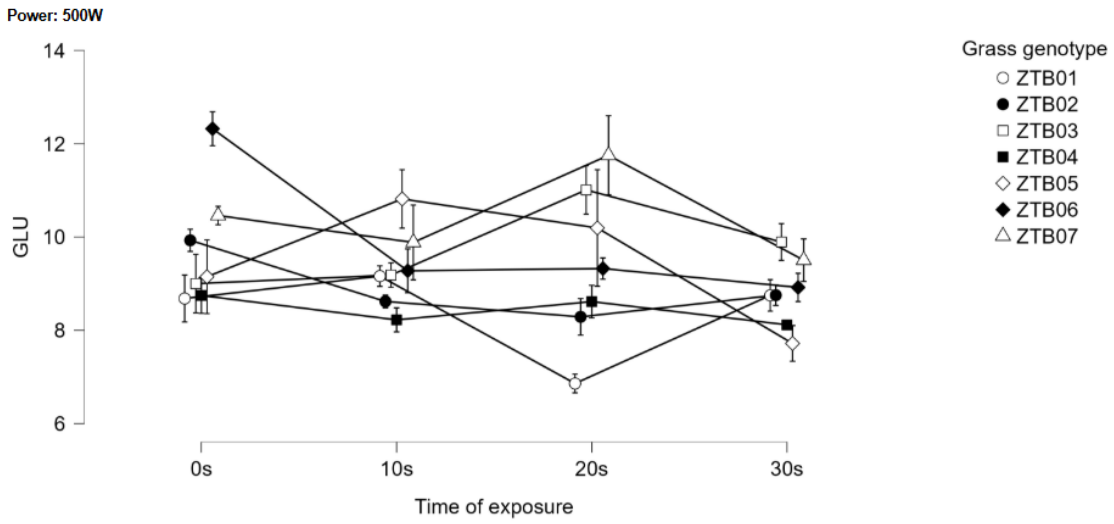

Figure 29. Variation in glucanase activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (500W) treatment.

- The analysis of HSD in the GLU activity among grass genotypes

The results of post hoc are presented in pairwise comparisons across seven perennial ryegrass genotypes (ZTB01 to ZTB07) following an ANOVA, using Tukey's Honestly Significant Difference (HSD) test (Table 25). The comparisons assess differences in mean GLU activity averaged over time based on exposure and power levels. Significant differences among genotypes are proved. Several genotype pairs, such as ZTB01 vs ZTB02, ZTB01 vs ZTB05, ZTB01 vs ZTB06, and ZT01 vs ZTB07, showed statistically significant differences in mean GLU activities, indicated by p-values adjusted for multiple comparisons (ptukey and pbonf) and marked with asterisks (* $p < .05$, ** $p < .01$, and *** $p < .001$) (table 19). The largest and most consistent differences involve genotype ZTB07, which differed significantly from most other genotypes (e.g., ZTB01, ZTB02, ZTB03, ZTB04, and ZTB05) with mean differences ranging from approximately -0.65 to -1.89 and very strong significance ($p < .001$).

Other significant differences included: ZTB01 differed significantly from ZTB02, ZTB05, ZTB06, and ZTB07, respectively. The genotype ZTB04 differed significantly from ZTB02, ZTB05,

ZTB06, and ZTB07. ZTB03 differed significantly from both ZTB04 and ZTB07. Another significant difference available to ZTB05 differs from ZTB07. Some comparisons, such as ZTB02 vs. ZTB03, ZTB02 vs. ZTB05, and ZTB05 vs. ZTB06, were not significant, indicating similar GLU activity means. Cohen's effect sizes quantified the magnitude of differences between genotypes. In the comparisons of genotype ZTB07 versus others, the large effect size was observed, where the effect size d > 0.8, such as d = -1.336 (ZTB01 vs. ZTB07). Moderate effect sizes (d ~ 0.4 to 0.8) appeared in comparisons like ZTB02 vs. ZTB04 (d = 0.808) and ZTB01 vs. ZTB05 (d = -0.432). Low or negligible effect sizes corresponded to non-significant comparisons. The range of reasonable values provided by 95% CIs and Cohen's d (effect size) supported the interpretation of statistical significance. For instance, there was a significant difference confirmed by the mean difference between ZTB01 and ZTB07, which ranged from -2.72 to -1.06. The mean GLU activities of ZTB07 were consistently lower than those of most other genotypes. The genotypes ZTB04 and ZTB01 showed clear differences from other tested genotypes, indicating unique physiological or biochemical profiles. Some genotypes showed GLU activities in a group without a noticeable variation. These results demonstrate that the tested perennial ryegrass genotypes exhibited distinct genetic variation in GLU.

Table 25. Pairwise comparison of mean differences in GLU activity among grass genotypes under DBD plasma treatment.

			95% CI for Mean Difference						95% CI for Cohen's d					
		Mean Difference	**Lower**	**Upper**	**SE**	**df**	**t**	**Cohen's d**	**Lower**	**Upper**	p_{tukey}		p_{bonf}	
ZTB01	ZTB02	-1.239	-2.071	-0.408	0.281	672	-4.407	-0.600	-1.018	-0.182	< .001	***	< .001	***
	ZTB03	-0.818	-1.649	0.014	0.281	672	-2.907	-0.396	-0.812	0.021	0.058		0.079	
	ZTB04	0.430	-0.402	1.261	0.281	672	1.528	0.208	-0.207	0.623	0.728		1.000	
	ZTB05	-0.892	-1.724	-0.060	0.281	672	-3.172	-0.432	-0.848	-0.015	0.026	*	0.033	*
	ZTB06	-1.247	-2.079	-0.415	0.281	672	-4.435	-0.603	-1.022	-0.185	< .001	***	< .001	***
	ZTB07	-1.889	-2.720	-1.057	0.281	672	-6.715	-0.914	-1.336	-0.492	< .001	***	< .001	***

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ZTB02	ZTB03	0.422	-0.410	1.253	0.281	672	1.500	0.204	-0.211	0.619	0.745		1.000	
	ZTB04	1.669	0.837	2.501	0.281	672	5.935	0.808	0.387	1.228	< .001	***	< .001	***
	ZTB05	0.347	-0.484	1.179	0.281	672	1.235	0.168	-0.247	0.583	0.880		1.000	
	ZTB06	-0.008	-0.839	0.824	0.281	672	-0.028	-0.004	-0.419	0.411	1.000		1.000	
	ZTB07	-0.649	-1.481	0.182	0.281	672	-2.308	-0.314	-0.730	0.102	0.241		0.447	
ZTB03	ZTB04	1.247	0.416	2.079	0.281	672	4.435	0.604	0.186	1.022	< .001	***	< .001	***
	ZTB05	-0.074	-0.906	0.757	0.281	672	-0.264	-0.036	-0.451	0.379	1.000		1.000	
	ZTB06	-0.430	-1.261	0.402	0.281	672	-1.527	-0.208	-0.623	0.208	0.728		1.000	
	ZTB07	-1.071	-1.903	-0.239	0.281	672	-3.808	-0.518	-0.935	-0.101	0.003	**	0.003	**
ZTB04	ZTB05	-1.322	-2.153	-0.490	0.281	672	-4.700	-0.640	-1.058	-0.221	< .001	***	< .001	***
	ZTB06	-1.677	-2.509	-0.845	0.281	672	-5.963	-0.811	-1.232	-0.391	< .001	***	< .001	***
	ZTB07	-2.318	-3.150	-1.487	0.281	672	-8.244	-1.122	-1.547	-0.696	< .001	***	< .001	***
ZTB05	ZTB06	-0.355	-1.187	0.476	0.281	672	-1.263	-0.172	-0.587	0.243	0.869		1.000	
	ZTB07	-0.997	-1.828	-0.165	0.281	672	-3.544	-0.482	-0.899	-0.065	0.008	**	0.009	**
ZTB06	ZTB07	-0.641	-1.473	0.190	0.281	672	-2.281	-0.310	-0.726	0.105	0.255		0.480	

* p < .05, ** p < .01, *** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 7 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

- The analysis of HSD in GLU activity among exposure durations

The mean GLU actvities for each exposure group at different times of exposure (0s, 10s, 20s, and 30s) is shown pairwise in Table 26. All grass genotypes and power levels are averaged in the results. Multiple comparisons were conducted by adjusting p-values and confidence intervals for both mean differences and the effect size, using Tukey's HSD test.

Significant increase in the comparison of pairs of 30s vs 0s was observed with a moderate effect size (Cohen's d = 0.437); the mean GLU at 30 seconds of exposure was roughly 0.9 units higher than at the untreated control (0 seconds). The adjusted p-value was highly significant (<.001), and neither the mean difference nor the effect size had confidence intervals that include zero. This implies that, in comparison to untreated control, CAP treatment for 30 seconds considerably rose GLU activities. On the contrary, the comparisons of 30s vs 10s and 30s vs 20s showed positive mean differences (0.4 and 0.5 units, respectively) with moderate effect sizes (~0.2–0.25), but these differences were not statistically significant after adjustment (p-values 0.234 and 0.064). In addition, the differences among 0s, 10s, and 20s exposure times were small and not statistically significant, indicating that shorter CAP exposures did not significantly alter GLU compared to no exposure. The data demonstrated that 30 seconds was the minimum time for exposure to CAP treatment to have a significant increase in GLU activity; on the other hand, 10 and 20 seconds were short exposure times and did not produce statistically significant changes in GLU activity.

Table 26. Pairwise comparison of mean differences in GLU activity among time of exposure under DBD plasma treatment.

			95% CI for Mean Difference						95% CI for Cohen's d					
		Mean Difference	**Lower**	**Upper**	**SE**	**df**	**t**	**Cohen's d**	**Lower**	**Upper**	**p_{tukey}**		**p_{bonf}**	
0s	10s	0.501	-0.046	1.049	0.213	672	2.358	0.243	-0.030	0.515	0.086		0.112	
	20s	0.376	-0.172	0.923	0.213	672	1.767	0.182	-0.091	0.454	0.290		0.466	
	30s	0.903	0.355	1.450	0.213	672	4.246	0.437	0.163	0.711	< .001	***	< .001	***
10s	20s	-0.126	-0.673	0.422	0.213	672	-0.591	-0.061	-0.333	0.211	0.935		1.000	
	30s	0.401	-0.146	0.949	0.213	672	1.888	0.194	-0.078	0.467	0.234		0.357	
20s	30s	0.527	-0.021	1.074	0.213	672	2.478	0.255	-0.018	0.528	0.064		0.081	

*** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 4 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

- The analysis of HSD in GLU activity among power levels

Table 27 shows pairwise comparisons of mean GLU across three CAP power levels: 300W, 400W, and 500W. The results are averaged over all grass genotypes and time of exposure levels. Tukey's HSD test was used to adjust for multiple comparisons, with adjusted p-values and confidence intervals reported for both mean differences and effect sizes (Cohen's d). Each pairwise comparison showed a statistically significant difference in mean GLU between power levels with p-values < .001 after adjustment for multiple testing. According to the data presented in table 27, when the power of plasma shifted from 300W to 400W, the GLU activities increased by approximately 2.39 units, a substantial effect size (Cohen's d = 1.16), implying a strong and meaningful increase in GLU activity. Increasing power further from 400W to 500W resulted in an additional increase of about 1.29 units (moderate effect size, d = 0.62). Changing power from 400W to 500W added an extra 1.29 units (moderate effect size, d = 0.62). There was a significant variation observed between 300W and 500W, where the mean increased by 3.68 units, resulting in a substantial effect size (d = 1.78). The highest significant effect was observed between 300W and 500W based on Cohen's d effect size values. This post hoc analysis demonstrates that increasing CAP power from 300W to 400W and then to 500W significantly elevated GLU activity, with large and meaningful effect sizes. The results revealed that the power level was a key driver since CAP power has a strong dose-dependent effect on GLU activity.

Table 27. Pairwise comparison of mean differences in GLU activity among power levels under DBD plasma treatment.

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}	p_{bonf}

300 W	400 W	2.39	1.96	2.83	0.18	672	12.99	1.16	0.93	1.38	< .001	***	< .001	***
	500 W	3.68	3.25	4.11	0.18	672	20.00	1.78	1.54	2.03	< .001	***	< .001	***
400 W	500 W	1.29	0.86	1.72	0.18	672	7.00	0.62	0.41	0.84	< .001	***	< .001	***

*** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 3 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

5.6.2. Effect of DBD plasma treatment on chitinases activity

The results of the study indicated that different grass genotypes differ significantly in CHI activity (Table 28). Grass genotype had a significant effect on CHI ($F = 14.125$, $p < .001$)**.** Time of exposure had a significant influence ($F = 5.265$, $p = 0.001$). Small effect size ($\omega^2 = 0.007$), explaining less than 1% of variance. Suggests that exposure time influenced CHI but to a lower extent. Power had a highly significant effect ($F = 229.319$, $p < .001$). The largest effect size ($\omega^2 = 0.259$), explaining about 26% of the variance, made power the dominant factor influencing CHI activity.

Two-way interactions of grass genotype and time of exposure had a significant interaction ($F = 11.273$, $p < .001$). Moderate effect size ($\omega^2 = 0.105$). Indicated that the effect of exposure time on CHI activity varied by genotype. While grass genotype and power had a significant interaction ($F = 7.846$, $p < .001$). A small to moderate effect size ($\omega^2 = 0.047$) suggests that the effect of power on CHI depended on genotype**.** Contrary to the time of exposure and power, it had a smaller significant effect ($F = 3.131$, $p = 0.005$). A minimal effect size ($\omega^2 = 0.007$) indicated some interactions between exposure time and power on CHI activity.

The three-way interaction of grass genotype, time of exposure, and power was significant ($F = 6.063$, $p < .001$). Moderate effect size ($\omega^2 = 0.103$) showed that the combined effect of genotype and exposure time on CHI activity depended on the power level. With power accounting for roughly 25% of the variance, it was the most significant factor influencing CHI activity. Although they had less of an impact separately, genotype and time of

exposure also had an impact on CHI activity. The interactions highlighted that the impact of one component was dependent on the levels of other factors. The moderate effect sizes for interactions imply that CHI's reaction to CAP treatment was genotype-specific and changes with power and exposure duration. The high residual variance indicated that CHI activity might be significantly influenced by other variables.

Table 28. ANOVA results on effect of Time of exposure, Power, and Grass genotype on chitinases activity in plants, and their interactions under DBD plasma treatment.

							95% CI for ω^2	
Variables	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**	**ω^2**	**Lower**	**Upper**
Grass genotype	296.648	6	49.441	14.125	< .001	0.045	0.013	0.071
Time of exposure	55.287	3	18.429	5.265	0.001	0.007	0.000	0.021
Power	1605.337	2	802.668	229.319	< .001	0.259	0.204	0.310
Grass genotype x Time of exposure	710.270	18	39.459	11.273	< .001	0.105	0.045	0.129
Grass genotype x Power	329.557	12	27.463	7.846	< .001	0.047	0.008	0.065
Time of exposure x Power	65.765	6	10.961	3.131	0.005	0.007	0.000	0.015
Grass genotype x Time of exposure x Power	763.993	36	21.222	6.063	< .001	0.103	0.022	0.104
Residuals	2352.147	672	3.500					

- The effect of DBD plasma treatment on CHI activity under 300W power

Figure 30 displays the variation CHI activity in seven perennial ryegrass genotypes (ZTB01–ZTB07) at four different plasma exposure times (0s, 10s, 20s, 30s), all of them treated under a constant power of 300W. Each line represents a genotype, and error bars indicate the standard error of the mean. The highest CHI activities among the tested genotypes were recorded at the genotype ZTB02 under an exposure time of 30 s. However, the genotype ZTB07 also increases at 30s, though less dramatically than ZTB02. In contrast,

ZTB04 demonstrated a sharp fall in CHI activities at 30s, ending with the lowest value among all the tested genotypes. On the other hand, ZTB06 and ZTB01 displayed relatively stable CHI activity across all exposure times, with minor fluctuations, while ZTB03 and ZTB05 exhibited moderate variability; however, there was no clear upward or downward trend. Across all the different times of exposure, under plasma treatment at 300w, genotypes ZTB01 and ZTB06 were constantly stable. Time of exposure did not show the uniform trend across genotypes; some increased, some decreased, and some remained stable as exposure time increased. Longer plasma exposure at 300 W amplified genotypic differences in CHI activity, as evidenced by the greatest genotypic divergence at the 30-second exposure mark. The CHI activity of some genotypes, such as ZTB02 and ZTB07, might be positively affected by plasma, while others, like ZTB04, might be negatively affected by longer plasma exposure under 300W.

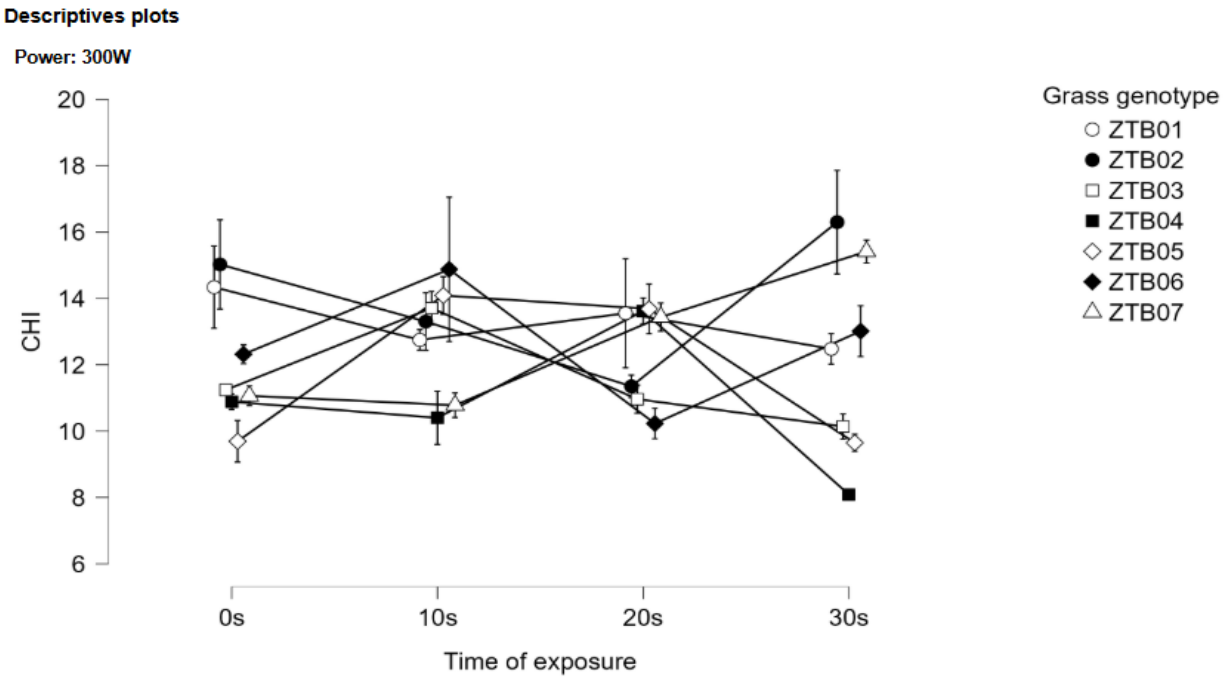

Figure 30. Variation in chitinases activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (300W) treatment.

- The effect of DBD plasma treatment on CHI activity under 400W power

The variation in CHI activity among seven grass genotypes (ZTB01–ZTB07), treated by constant power plasma at 400 W with different exposure durations (0 s, 10 s, 20 s, 30 s), is displayed in Figure 31. The pronounced peak of CHI activity among all the tested genotypes is observed at the genotype ZTB07 with an exposure time of 20s, followed by a sharp fall at 30s. Most other tested genotypes (ZTB01, ZTB02, ZTB03, ZTB04, ZTB05, ZTB06) exhibited a

relatively stable or minor fluctuation in CHI activity across all different exposure durations. The genotype ZTB05 started relatively high at the untreated control (0s) but declined and then stabilized. The genotype ZTB01 displayed a remarkable decrease in CHI activity at the exposure time of 20s and recovered at 30s of exposure; on the other hand, the CHI activity in genotypes ZTB02 and ZTB06 remained consistent under all times of exposure to plasma treatment. The spike in CHI activity displayed at the genotype ZTB07 under an exposure time of 20s suggests a unique, genotype-specific response to plasma exposure. Considering other tested genotypes, the data suggested a limited sensitivity to exposure time at 400 W of plasma treatment, since the data from other genotypes were clustered between 8 and 13 units. Based on the interaction of genotypes and time of exposure, most genotypes exhibited little variation in CHI activity, except for the genotype ZTB07 with high responsiveness at 20s, suggesting that the influence of plasma on CHI activity was not uniform; rather, it was genotype-time-dependent.

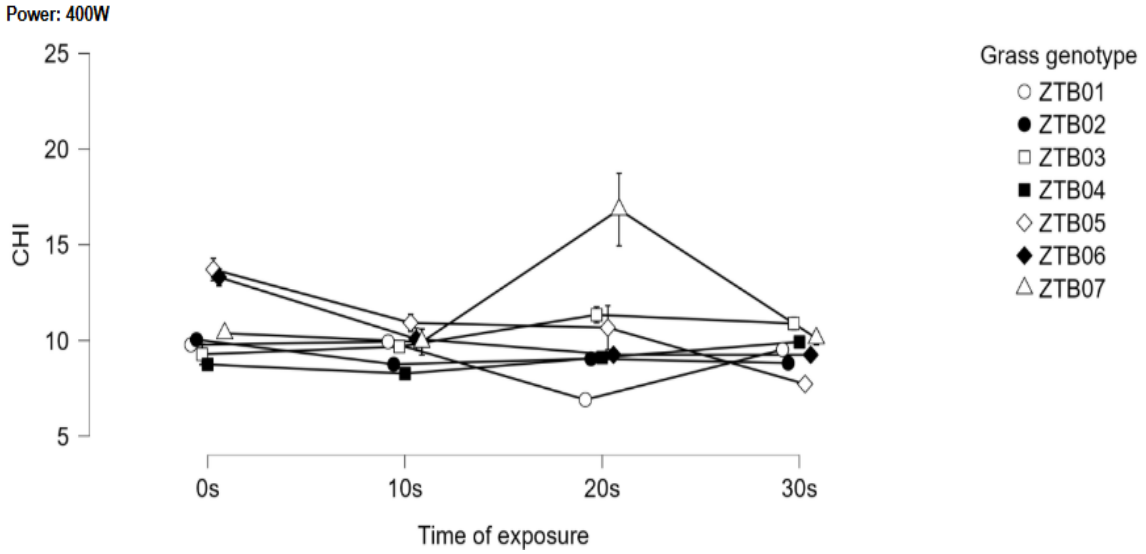

Figure 31. Variation in chitinases activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (400W) treatment.

- The effect of DBD plasma treatment on CHI activity under 500W power

Figure 32 demonstrates the genotype ZTB06 started with the highest CHI activity in the untreated sample at 0s, drops sharply when samples were exposed for 10s to plasma treatment under 500W, recovered at 20s, and fallen when exposed for 30s under the same power of plasma treatment. This showed different stages of responding to plasma treatment by a strong initial sensitivity, the transient recovery, and the final decrease as exposure time

lasted longer. Both genotypes, ZTB03 and ZTB07, showed a rising trend at the exposure time of 20s, and both fell as the exposure time was increased to 30s. Contrary to the genotypes, ZTB01 and ZTB02 experienced a minor fluctuation across all times of exposure with the exception of CHI activities at ZTB01 when it was exposed for 20s under plasma treatment, which led to higher decrease in CHI activity. However, at the exposure time of 30 s, both ZTB01 and ZTB02 returned at the same CHI activity level, whileZTB04 displayed modest, relatively stable CHI activity, with minor rises or falls but no considerable shift.

The time of exposure did not show a uniform trend across all genotypes. A pronounced peak at the 20s was observed in some genotypes, such as ZTB07, ZTB05, and ZTB03. On the other hand, ZTB01 exhibited a strong fall at the same time of exposure. The clustering of most genotypes between CHI activity of 8 and 10 units suggests that 500 W plasma treatment tended to keep CHI activities within a moderate range, with only a few genotypes showing strong responses. The sharp initial drop and partial recovery in ZTB06 may suggest a stress response to high plasma power, with possible adaptation or damage over time. This figure demonstrates that the response of CHI activity to high-power plasma treatment (500 W) in perennial ryegrass was highly dependent on genotype and exposure time.

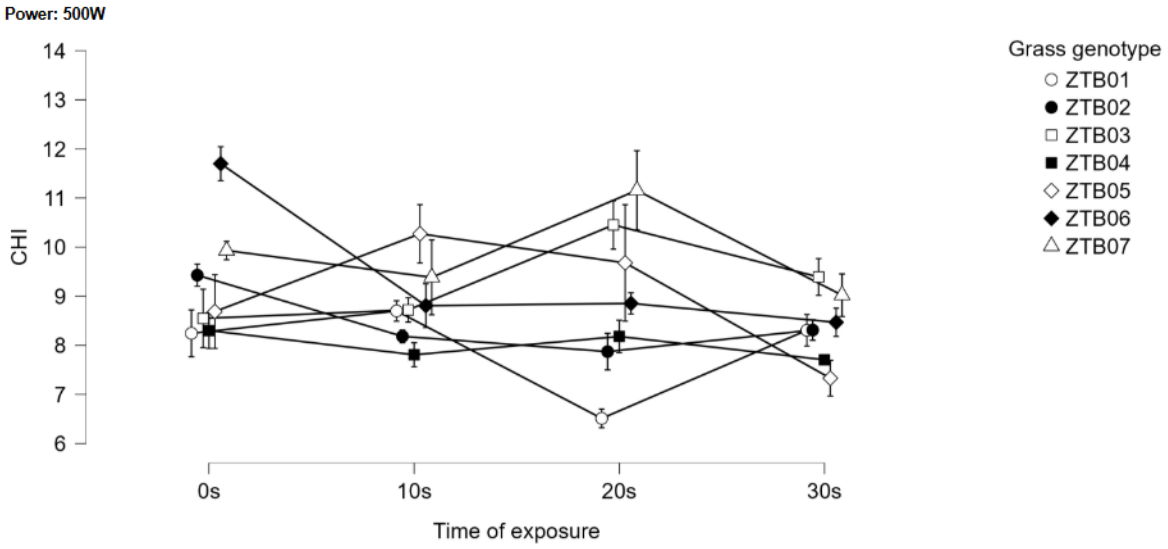

Figure 32. Variation in chitinases activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (500W) treatment.

- The analysis of HSD in CHI activity among grass genotypes

The results proved significant differences among genotypes (Table 29). The comparison between the genotype ZTB07 and other tested genotypes of perennial ryegrass

showed significantly lower CHI activity, while ZTB04 and ZTB06 showed intermediate levels. The genotype ZTB07, compared to ZTB01: mean difference = -1.369, p < .001, large effect size (d = -0.732). Compared to ZTB02: Mean difference = -0.917, p = 0.006, moderate effect size (d = -0.490). Compared to ZTB03: Mean difference = -1.091, p < .001, large effect size (d = -0.583). Compared to ZTB04: Mean difference = -2.20, p < .001, huge effect size (d = -1.18). Compared to ZTB05: Mean difference = -0.95, p = 0.004, moderate effect size (d = -0.51).

ZTB04 also differed significantly from several genotypes, generally showing lower CHI activity than ZTB01, ZTB02, and ZTB03: The mean difference was found to be 0.83, with a p-value of 0.02. Mean difference = 1.283, p < .001. Mean difference = 1.109, p < .001, respectively. While ZTB04 vs. ZTB05: Mean difference = -1.26, p < .001 (ZTB05 higher) and ZTB04 vs. ZTB06: Mean difference = -1.59, p < .001 (ZTB06 higher). The genotype ZTB06 showed significantly lower CHI activity than genotype ZTB01 (p = 0.05) and significantly higher CHI activity than genotypes ZTB04 and ZTB07. On the other hand, some genotype comparisons demonstrated non-significant differences, such as ZTB01 vs. ZTB02, ZTB03, and ZTB05, which showed no significant differences. The genotypes ZTB02 vs. ZTB03 and ZTB05 were similar. At the same time, the genotype ZTB05 vs. ZTB06 was not significant, and ZTB06 vs. ZTB07 showed a trend but was not statistically significant after correction. Effect sizes (Cohen's d) demonstrated large effect sizes (|d| > 0.8), which were observed in comparisons involving ZTB07 vs. ZTB04 (-1.18), ZTB07 vs. ZTB01 (-0.73), and ZTB04 vs. ZTB06 (-0.851), indicating substantial differences in CHI activity. Contrary moderate effect sizes (0.4 < |d| < 0.8) appeared in several other significant comparisons, could be reflecting meaningful biological differences. Small effect sizes correspond to non-significant differences. The 95% CIs for mean differences and Cohen's d that did not cross zero correspond to significant comparisons. For example, the mean difference between genotypes ZTB07 and ZTB04 ranged from -2.95 to -1.45, confirming a robust difference.

Table 29. Pairwise comparison of mean differences in CHI activity among grass genotypes under DBD plasma treatment.

			95% CI for Mean Difference						95% CI for Cohen's d			
		Mean Difference	**Lower**	**Upper**	**SE**	**df**	**t**	**Cohen's d**	**Lower**	**Upper**	**p_{tukey}**	**p_{bonf}**

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ZTB 01	ZTB 02	-0.45	-1.20	0.30	0.255	672	-1.773	-0.24	-0.66	0.17	0.57		1.00	
	ZTB 03	-0.28	-1.03	0.48	0.255	672	-1.090	-0.14	-0.56	0.27	0.93		1.00	
	ZTB 04	0.83	0.08	1.59	0.255	672	3.267	0.45	0.03	0.86	0.02	*	0.02	*
	ZTB 05	-0.42	-1.18	0.33	0.255	672	-1.665	-0.23	-0.64	0.19	0.64		1.00	
	ZTB 06	-0.76	-1.51	-0.01	0.255	672	-2.988	-0.41	-0.82	0.01	0.05	*	0.06	
	ZTB 07	-1.37	-2.12	-0.62	0.255	672	-5.376	-0.73	-1.15	-0.31	< .001	***	< .001	***
ZTB 02	ZTB 03	0.17	-0.58	0.93	0.255	672	0.683	0.09	-0.32	0.51	0.994		1.000	
	ZTB 04	1.28	0.53	2.04	0.255	672	5.040	0.69	0.27	1.11	< .001	***	< .001	***
	ZTB 05	0.03	-0.73	0.78	0.255	672	0.107	0.02	-0.40	0.43	1.000		1.000	
	ZTB 06	-0.30	-1.06	0.44	0.255	672	-1.215	-0.17	-0.58	0.25	0.888		1.000	
	ZTB 07	-0.92	-1.67	-0.16	0.255	672	-3.603	-0.49	-0.91	-0.07	0.006	**	0.007	**
ZTB 03	ZTB 04	1.11	0.36	1.86	0.255	672	4.357	0.59	0.18	1.01	< .001	***	< .001	***
	ZTB 05	-0.15	-0.90	0.61	0.255	672	-0.576	-0.08	-0.49	0.34	0.997		1.000	
	ZTB 06	-0.48	-1.24	0.27	0.255	672	-1.898	-0.26	-0.67	0.16	0.482		1.000	
	ZTB 07	-1.09	-1.84	-0.38	0.255	672	-4.286	-0.58	-1.00	-0.17	< .001	***	< .001	***
ZTB 04	ZTB 05	-1.26	-2.01	-0.50	0.255	672	-4.933	-0.67	-1.09	-0.25	< .001	***	< .001	***
	ZTB 06	-1.59	-2.35	-0.84	0.255	672	-6.255	-0.85	-1.27	-0.43	< .001	***	< .001	***
	ZTB 07	-2.20	-2.95	-1.45	0.255	672	-8.643	-1.18	-1.60	-0.75	< .001	***	< .001	***

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ZTB 05	ZTB 06	-0.34	-1.09	0.42	0.255	672	-1.323	-0.18	-0.60	0.24	0.841		1.000	
	ZTB 07	-0.95	-1.70	-0.19	0.255	672	-3.711	-0.50	-0.92	-0.09	0.004	**	0.005	**
ZTB 06	ZTB 07	-0.61	-1.36	0.15	0.255	672	-2.388	-0.33	-0.74	0.09	0.205		0.362	

* p < .05, ** p < .01, *** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 7 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

- The analysis of HSD in CHI activity among exposure durations

The results of pairwise comparisons of CHI activity extracted from seven genotypes of perennial ryegrass after being treated by plasma at different times of exposure (0s, 10s, 20s, 30s) are presented in Table 30. The analysis used Tukey's HSD test for multiple comparisons, with adjusted p-values, confidence intervals, mean difference, and effect size. Results exhibited the significant rise of CHI activity at the exposure time of 30s compared to the untreated control (0s) by approximately 0.7 units, with a small to moderate effect size (Cohen's d = 0.38). The CI for both the mean difference and effect size excluded zero, and the adjusted p-value was highly significant (p = 0.002). The results implied that plasma treatment with an exposure time of 30s significantly increased CHI activity compared to no exposure. A similar significant rise was observed at the comparison of 30s and 20s, where we had about 0.61 units (d = 0.33), with a significant adjusted p-value (p = 0.01). The results demonstrated that increasing exposure duration from 20s to 30s positively influenced CHI activity. On the other hand, the differences among 0s, 10s, and 20s exposure times were small and statistically non-significant, indicating that shorter plasma exposures did not significantly alter CHI activity compared to no exposure. The positive mean value of 0.45 and moderate effect size of 0.24 were observed at the comparison of 10s and 30s; however, the correction p=0.09 confirmed that it was not statistically significant. The data from the tested time of exposure suggested that 30 seconds of plasma exposure was the minimum effective duration to significantly increase CHI activity, while shorter exposure durations (10 s, 20 s) did not produce statistically significant variations.

Table 30. Pairwise comparison of mean differences in CHI activity among time of exposure under DBD plasma treatment.

			95% CI for Mean Difference						95% CI for Cohen's d					
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}		p_{bonf}	
0s	10s	0.25	-0.24	0.747	0.19	672	1.31	0.14	-0.14	0.41	0.56		1.00	
	20s	0.09	-0.403	0.59	0.19	672	0.48	0.05	-0.22	0.32	0.96		1.00	
	30s	0.70	0.208	1.20	0.19	672	3.66	0.38	0.10	0.65	0.002	**	0.002	**
10s	20s	-0.16	-0.655	0.34	0.19	672	-0.83	-0.08	-0.36	0.19	0.84		1.00	
	30s	0.45	-0.044	0.95	0.19	672	2.35	0.24	-0.03	0.51	0.09		0.11	
20s	30s	0.61	0.115	1.11	0.19	672	3.18	0.33	0.05	0.60	0.01	**	0.01	**

** p < .01

Note. P-value and confidence intervals adjusted for comparing a family of 4 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

- The analysis of HSD in CHI activity among power levels

The Table 31 demonstrates the pairwise comparisons of CHI activity extracted from seven genotypes of perennial ryegrass after being treated by plasma with different power levels (300 W, 400 W, 500 W). The analysis used Tukey's HSD test for multiple comparisons, with adjusted p-values, confidence intervals, mean difference, and effect size. The comparison of each pairwise demonstrated a statistically significant difference in mean

between different levels of power, with p-values < .001. When the power was increased from 300W to 400W, the CHI activity rose approximately 2.29 units with a large effect size of d=1.23, which indicates a considerable increase. At the same time, when the power increased from 400W to 500W, it resulted in an additional increase of about 1.22 units with a moderate effect size of d=0.65. However, the strong difference was between 300W and 500W, which increased the mean up to 3.52 units and had a substantial effect size of d=1.88. This higher effect size suggests that power level was a major element influencing CHI activity. The data revealed that the CAP power has a strong dose-dependent effect on CHI activity, with higher power levels producing significantly greater CHI activity levels. Rising power from 300W to 500W might enhance the outcome of the application, aiming to maximize the CHI activity level.

Table 31. Pairwise comparison of mean differences in CHI activity among power levels under DBD plasma treatment.

			95% CI for Mean Difference						95% CI for Cohen's d					
		Mean Difference	Lower	Upper	SE	df	t	Cohen's d	Lower	Upper	p_{tukey}		p_{bonf}	
300 W	400 W	2.29	1.90	2.68	0.167	672	13.749	1.23	0.996	1.453	< .001	***	< .001	***
	500 W	3.52	3.12	3.91	0.167	672	21.094	1.88	1.633	2.126	< .001	***	< .001	***
400 W	500 W	1.22	0.83	1.66	0.167	672	7.345	0.65	0.436	0.872	< .001	***	< .001	***

*** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 3 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

5.6.3. Effect of DBD plasma treatment on guaiacol peroxidase activity

The results of ANOVA demonstrated that grass genotypes affect GPOX activity significantly, where F = 19.257 and p < .001 and effect size ($\omega^2 = 0.053$) explains that 5.3% of

the variance suggests a moderate effect (Table 32). All tested genotypes of perennial ryegrass displayed significantly different GPOX activity. The time of exposure had a significant effect ($F = 9.311$, $p < .001$). Smaller effect size ($\omega^2 = 0.012$), explaining about 1.2% of variance. Exposure time influenced GPOX content but less strongly than genotype or power. At the same time, the power showed a highly significant effect ($F = 240.748$, $p < .001$). Largest effect size ($\omega^2 = 0.232$), describing roughly 23% of variance. Power was the dominant factor influencing GPOX content. The two-way interactions between grass genotype and time of exposure demonstrated a significant interaction ($F = 19.152$, $p < .001$). The study found a large effect size of 0.158, indicating a significant change in the effect of exposure duration on GPOX activity based on genotypes. The Grass genotype and Power showed a significant interaction ($F = 4.404$, $p < .001$). The effect size was found to be smaller, with a value of 0.020. This suggests that there were genotype-specific responses to different power levels. Contrary to the time of exposure and power, it was not statistically significant at $p < .05$ ($p = 0.073$). A minimal effect size ($\omega^2 = 0.003$) indicates minimal interaction between exposure time and power on GPOX. The three-way interaction of grass genotype, time of exposure, and power demonstrated a significance ($F = 9.952$, $p < .001$). A large effect size ($\omega^2 = 0.156$) exhibited an intricate merged effect of genotype, exposure duration, and power on GPOX activity. Genotype and time of exposure also significantly influenced GPOX activity, but to a lower extent individually. Significant interactions, especially involving genotype, suggest that the response of GPOX activity to plasma treatment depended heavily on genetic background and treatment conditions. The non-significant interaction between time and power alone suggests these factors weakly interacted without considering genotype. The large three-way interaction indicated that optimal plasma treatment for enhancing GPOX activity must consider at the same time the genotype, particularly the exposure duration, and the level of power used for treatment.

Table 32. ANOVA results on effect of Time of exposure, Power, and Grass genotype on guaiacol peroxidase activity in plants, and their interactions under DBD plasma treatment.

Variables	**Sum of Squares**	**df**	**Mean Square**	**F**	**p**	**ω^2**
Grass genotype	5.83	6	0.97	19.26	< .001	0.053

Time of exposure	1.41	3	0.47	9.31	< .001	0.012
Power	24.28	2	12.14	240.75	< .001	0.232
Grass genotype * Time of exposure	17.38	18	0.97	19.15	< .001	0.158
Grass genotype * Power	2.67	12	0.22	4.40	< .001	0.020
Time of exposure* Power	0.59	6	0.09	1.93	0.073	0.003
Grass genotype * Time of exposure * Power	18.07	36	0.50	9.95	< .001	0.156
Residuals	33.89	672	0.050			

- The effect of DBD plasma treatment on GPOX activity under 300W power

The genotypes demonstrated different specific responses across different times of exposure under 300 W (Figure 33). The genotype ZTB01 started with the highest GPOX activity at the untreated control 0s, dropped sharply at 10s and 20s, then rose again at 30s to the highest value among all genotypes. The genotype ZTB02 remained relatively stable, with a slight dip at 10s and 30s and a peak at 20s. Other genotypes, such as ZTB03, were stable, with a slight increase at 10s, then a gradual decrease, whereas ZTB04 showed a modest decline from 0s to 30s, with the lowest value at 30s. On the other hand, ZTB05 demonstrated a minor fluctuation, with a slight increase at 10s and 20s, then a decrease at 30s. The genotype ZTB06 rose from 0s to 10s, dipped at 20s, then rose again at 30s. Contrary to ZTB07, it gradually increased, peaking at 30s. There was no consistent trend across all genotypes based on the time of exposure. Some increased, some decreased, and some fluctuated with exposure time. Peaks at 30s on ZTB01 and ZTB07 showed their highest GPOX activity at the longest exposure, whereas ZTB04 and ZTB05 reached their lowest values at 30s. The figure highlights that the effect of CAP exposure on GPOX activity was highly genotype dependent. Longer exposures stimulated some genotypes (ZTB01, ZTB07), while they inhibited others (ZTB04, ZTB05). The CAP power of 300 W was sufficient to raise GPOX activity in genotypes such as ZTB01 and ZTB02. Conversely, genotypes like ZTB04 may require shorter exposures to avoid enzyme

suppression. The complex interaction between genotype and exposure time was a crucial element to influence the GPOX activity in tested perennial ryegrass at 300 W of plasma treatment.

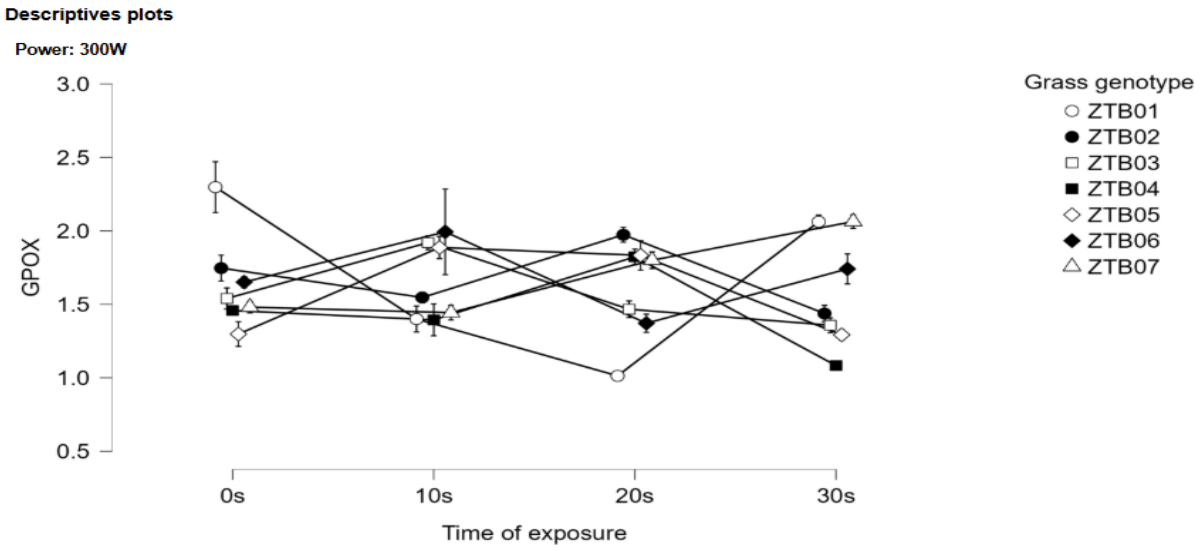

Figure 33. Variation in GPOX activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (300W) treatment.

- The effect of DBD plasma treatment on GPOX activity under 400W power

Figure 34 demonstrates the response of GPOX activity in different tested genotypes of perennial ryegrass (ZTB01-ZTB07) at a constant plasma power of 400 W under different times of exposure (10 s, 20 s, 30 s) compared to the untreated control (0 s). The plot demonstrated that, for the tested genotypes, GPOX activity was highly genotype dependent. The genotype ZTB07 displayed a higher response across all tested genotypes in GPOX activity at the exposure duration of 20 s. This pattern is accompanied by a larger error bar, indicating more variability or possible outliers. The genotype ZTB05 had many changes in GPOX activity from the untreated control up to 30s with lower GPOX activity, while ZTB01 remained at the same axis except for the slight dip at 20s of exposure time. Genotypes such as ZTB02, ZTB03, ZTB04, and ZTB06 were clustered with medium changes, displaying relatively stable GPOX activity levels in all tested exposure times. Therefore, most genotypes did not show a clear increase or decrease in GPOX activity with longer exposure. Instead, the lines were mostly flat or slightly undulating. This plot demonstrated that under 400W plasma power, GPOX activity in perennial ryegrass was generally stable across genotypes and exposure times, with the

notable exception of ZTB07, which exhibited a dramatic, transient increase at 20s. This points to the importance of considering both genotype and exposure duration when applying plasma treatments to modulate GPOX activity.

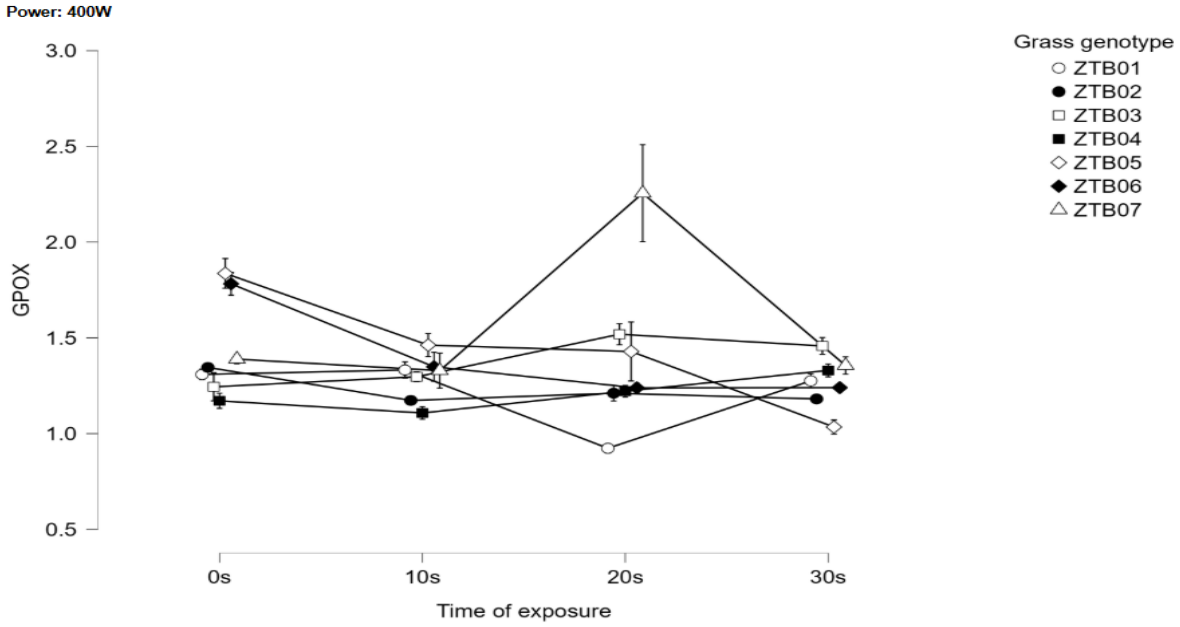

Figure 34. Variation in GPOX activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (400W) treatment.

- The effect of DBD plasma treatment on GPOX activity under 500W power

Figure 35 displayed that the plasma treatment under the power of 500w and the GPOX activity of the tested samples (ZTB01-ZTB07) were highly genotype and exposure duration dependent. Some genotypes exhibited a transient change of GPOX activity at specific exposure durations, while others remained largely unaffected. These results highlight that there should be CAP treatment protocols for specific genotypes and optimization of the time of exposure to achieve a desired GPOX activity outcome, since different genotypes respond differently to the CAP treatment. The genotype ZTB06 displayed different responses to plasma treatment, depending on the exposure time, where it showed the highest GPOX activity at no treatment (0s), followed by a sharp peak at 10s, and then stayed almost stable in GPOX activity at 20s and at 30s. These findings may suggest that, in this genotype, the plasma treatment negatively affected the GPOX activity.

The genotype ZTB07 peaked in the 20s, fluctuated slightly from the 0s to the 10s, and then declined in the 30s. This indicates that moderate plasma exposure may stimulate GPOX activity in this genotype. ZTB05 showed a mild increase from 0s to 10s, then a slight decline

up to 30s; the results may suggest the moderate time of exposure was beneficial to its physiological activities. However, the longer they were exposed to CAP treatment, it started to affect their physiological activities, such as maintaining the level of GPOX activity. At the same time, the genotype ZTB03 rose from 0s to 20s, peaking at 20s, then dropping at 30s. Conversely, ZTB01, ZTB02, and ZTB04 showed lines that were mostly flat or small changes, indicating that GPOX activity was less sensitive to exposure time at this high power. If the goal is to enhance GPOX activity (for improved oxidative stress defence), genotypes like ZTB07 and ZTB03 may benefit from 20s exposure, while ZTB06 responds best when it is not treated (0s). For other genotypes, plasma at this power level did not provide a clear benefit. Most enzyme activity values clustered between 1.0 and 1.4, with only a few peaks above 1.5. Regardless, genotypes ZTB07 and ZTB01 at 20s showed slightly higher variability; error bars are generally small, suggesting reliable measurements.

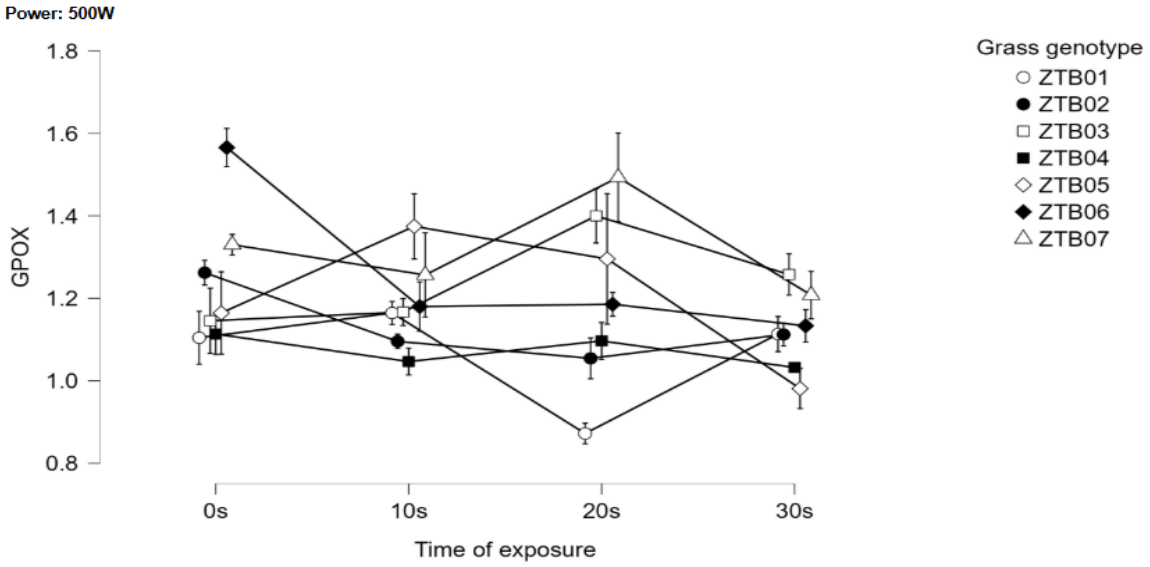

Figure 35. Variation in GPOX activity for perennial ryegrass genotypes (ZTB01–ZTB07) across time of exposure (0s, 10s, 20s, 30s) under DBD plasma (500W) treatment.

- The analysis of HSD in GPOX activity among grass genotypes

Results exhibited significant differences in GPOX activity among the various grass genotypes under the study (Table 33). The grass genotype ZTB07 had less of the enzyme, while the grass genotypes ZTB01, ZTB02, and ZTB03 had more GPOX activity. These findings indicated that the genotype was a crucial factor in influencing the enzyme activities. There was no notable difference when genotype ZTB01 was compared to genotype ZTB02 (p=0.990), genotype ZTB03 (p=0.173), and genotype ZTB04 (p=0.100). Conversely, genotype ZTB01

exhibited substantial disparities with genotype ZTB06 ($p < .001$, $d = -0.580$) and genotype ZTB07 ($p < .001$, $d = -0.941$), indicating that genotype ZTB01 possessed greater GPOX activity compared to ZTB06 and ZTB07. When compared to the other examined genotypes, genotype ZTB02 had more GPOX activity than genotypes ZTB04 ($p=0.011$, $d=0.468$), ZTB06 ($p=0.008$, $d=-0.479$), and ZTB07 ($p < .001$, $d=-0.840$). The GPOX content in genotype ZTB02 exhibited no significant variations when compared to ZTB03 or ZTB05. The GPOX activity for genotype ZTB03 was substantially greater than that of ZTB04 ($p < .001$, $d=0.703$) and ZTB07 ($p < .001$, $d=-0.606$). But ZTB05 and ZTB06 were not too different. In contrast, ZTB04 was much lower than ZTB05, ZTB06, and ZTB07 (all $p < .001$), with high effect sizes (d from -0.746 to -1.309). In the case of the genotype ZTB05, the GPOX activity was substantially higher than that of ZTB07 ($p < .001$, $d = -0.563$), whereas no significant difference was observed with ZTB06. Simultaneously, no significant difference was seen between genotypes ZTB06 and ZTB07 after correction ($p = 0.112$). Genotype ZTB07 had greater effect sizes ($|d| > 0.8$) in comparison to genotypes ZTB01, ZTB02, ZTB04, and ZTB05, indicating significant differences. Most comparisons showed moderate effect sizes ($0.4 < |d| < 0.8$), which means that the biological differences were not massive. The comparisons showed minor or no effect sizes, meaning the differences were insignificant. The genotype ZTB07 may possess diminished antioxidant capacity, as it had the lowest GPOX activity level. The genotypes ZTB01, ZTB02, and ZTB03 have more GPOX activity than the genotypes ZTB04, ZTB06, and ZTB07. These findings imply that genetic variation influenced GPOX activity expression. Genotypes containing more enzymes, such as ZTB01, ZTB02, and ZTB03, may be better at dealing with oxidative stress.

Table 33. Pairwise comparison of mean differences in GPOX activity among grass genotypes under DBD plasma treatment.

			95% CI for Mean Difference						**95% CI for Cohen's d**			
		Mean Difference	**Lower**	**Upper**	**SE**	**df**	**t**	**Cohen's d**	**Lower**	**Upper**	**p_{tukey}**	**p_{bonf}**

ZTB 01	ZTB 02	-0.02	-0.113	0.068	0.031	672	-0.742	-0.101	-0.516	0.314	0.990		1.000	
	ZTB 03	-0.08	-0.166	0.015	0.031	672	-2.466	-0.336	-0.752	0.080	0.173		0.292	
	ZTB 04	0.08	-0.008	0.173	0.031	672	2.700	0.367	-0.049	0.784	0.100		0.149	
	ZTB 05	-0.09	-0.175	0.005	0.031	672	-2.782	-0.379	-0.795	0.038	0.081		0.117	
	ZTB 06	-0.13	-0.221	-0.040	0.031	672	-4.263	-0.580	-0.998	-0.162	< .001	***	< .001	***
	ZTB 07	-0.21	-0.302	-0.121	0.031	672	-6.918	-0.941	-1.364	-0.519	< .001	***	< .001	***
ZTB 02	ZTB 03	-0.05	-0.143	0.038	0.031	672	-1.724	-0.235	-0.650	0.181	0.600		1.000	
	ZTB 04	0.11	0.015	0.196	0.031	672	3.442	0.468	0.052	0.885	0.011	*	0.013	*
	ZTB 05	-0.06	-0.153	0.028	0.031	672	-2.039	-0.278	-0.693	0.138	0.391		0.878	
	ZTB 06	-0.11	-0.198	-0.017	0.031	672	-3.521	-0.479	-0.896	-0.062	0.008	**	0.010	**
	ZTB 07	-0.19	-0.279	-0.098	0.031	672	-6.175	-0.840	-1.261	-0.419	< .001	***	< .001	***
ZTB 03	ZTB 04	0.16	0.068	0.248	0.031	672	5.166	0.703	0.284	1.122	< .001	***	< .001	***

	ZTB 05	-0.01	-0.100	0.081	0.031	672	-0.315	-0.043	-0.458	0.372	1.000		1.000	
	ZTB 06	-0.06	-0.145	0.035	0.031	672	-1.797	-0.245	-0.660	0.171	0.551		1.000	
	ZTB 07	-0.14	-0.226	-0.046	0.031	672	-4.451	-0.606	-1.024	-0.188	< .001	***	< .001	***
ZTB 04	ZTB 05	-0.17	-0.258	-0.077	0.031	672	-5.481	-0.746	-1.166	-0.326	< .001	***	< .001	***
	ZTB 06	-0.21	-0.303	-0.122	0.031	672	-6.963	-0.948	-1.370	-0.525	< .001	***	< .001	***
	ZTB 07	-0.29	-0.384	-0.204	0.031	672	-9.617	-1.309	-1.738	-0.880	< .001	***	< .001	***
ZTB 05	ZTB 06	-0.05	-0.136	0.045	0.031	672	-1.482	-0.202	-0.617	0.214	0.756		1.000	
	ZTB 07	-0.13	-0.217	-0.036	0.031	672	-4.136	-0.563	-0.980	-0.145	< .001	***	< .001	***
ZTB 06	ZTB 07	-0.08	-0.171	0.009	0.031	672	-2.654	-0.361	-0.777	0.055	0.112		0.171	

* p < .05, ** p < .01, *** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 7 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

- The analysis of HSD in GPOX activity among exposure durations

The results of post hoc analysis showed significant differences in some situations and no significant differences in others, depending on whether the exposure time during CAP

treatment influenced the enzyme content (Table 34). The exposure times are clustered to make comparisons. The results displayed that plasma exposure durations significantly influenced the GPOX activity, with a notable rise at exposure durations of 10s and 30s when compared to the outcome of GPOX activity with no exposure duration (0s) (table 34). The GPOX activity reached the peak at 30s after plateauing at 10s and 20s, indicating a time-dependent stimulatory effect of plasma treatment. The small statistically significant variation in GPOX activity was observed between no exposure 0 s and 10 s (mean difference = 0.063, Cohen's d = 0.279); this suggests early plasma exposure started to raise GPOX activity level. There was a stabilization in GPOX activity, and there were no significant differences during the interval between 0s and 20s or between 10s and 20s.

The GPOX activity at 30 seconds was significantly higher than both the levels at 0 seconds and 20 seconds (mean difference = 0.118, d = 0.527). Additionally, enzyme activity at 30 seconds was significantly greater than that at 0 seconds (mean difference = 0.118, d = 0.527) and also higher than at 20 seconds (mean difference = 0.082, d = 0.366), indicating a pronounced stimulatory effect of longer plasma exposure. The increase from 10s to 30s showed a positive trend (p = 0.076), suggesting enzyme activity level may continue to rise with longer exposure but requires further research for confirmation. Among the tested range of exposure durations, 30s of plasma exposure appeared to be optimum for maximizing GPOX activity.

Table 34. Pairwise comparison of mean differences in GPOX activity among time of exposure under DBD plasma treatment.

			95% CI for Mean Difference						95% CI for Cohen's d					
		Mean Difference	**Lower**	**Upper**	**SE**	**df**	**t**	**Cohen's d**	**Lower**	**Upper**	p_{tukey}		p_{bonf}	
0s	10s	0.063	0.003	0.122	0.023	672	2.714	0.279	0.006	0.552	0.034	*	0.041	*
	20s	0.036	-0.023	0.096	0.023	672	1.564	0.161	-0.112	0.433	0.400		0.709	

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	30 s	0.118	0.059	0.178	0.023	672	5.124	0.527	0.252	0.802	< .001	***	< .001	***
10 s	20 s	-0.027	-0.086	0.033	0.023	672	-1.150	-0.118	-0.391	0.154	0.659		1.000	
	30 s	0.056	-0.004	0.115	0.023	672	2.410	0.248	-0.025	0.521	0.076		0.097	
20 s	30 s	0.082	0.023	0.142	0.023	672	3.559	0.366	0.093	0.640	0.002	**	0.002	**

* p < .05, ** p < .01, *** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 4 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

- The analysis of HSD in GPOX activity among power levels

Table 35 demonstrates the pairwise comparisons of GPOX activity extracted from seven grass genotypes after being treated by plasma with different power levels (300W, 400W, 500W). The analysis used Tukey's HSD test for multiple comparisons, with adjusted p-values, confidence intervals, mean difference, and effect size. The results exhibited that the rising of power plasma from 300W to 400W, then up to 500W, significantly increased GPOX activity, with large and meaningful effect sizes. The power level was a crucial element influencing GPOX activity responses to plasma treatment. After adjusting for multiple tests, the data revealed that all comparisons were statistically significantly different with p-values < .001. When the power was increased from 300W to 400W, the GPOX activity rose approximately 0.271 units with a large effect size of d=1.205, which indicates a considerable increase. At the same time, when the power increased from 400W to 500W, it resulted in an additional increase of about 0.164 units with a moderate effect size of d=0.731. However, the most significant difference was observed between 300W and 500W, resulting in an increase of the mean by 0.435 units and a substantial effect size of d=1.936. This higher effect size suggests that power level was a major element influencing GPOX activity. The data revealed that plasma power had a strong dose-dependent effect on GPOX activity, with higher power

levels producing significantly greater activity. Rising power from 300W to 500W might enhance the outcome of the application, which aims to maximize GPOX activity.

Table 35. Pairwise comparison of mean differences in GPOX activity among power levels under DBD plasma treatment.

			95% CI for Mean Difference						95% CI for Cohen's d					
		Mean Difference	**Lower**	**Upper**	**SE**	**df**	**t**	**Cohen's d**	**Lower**	**Upper**	p_{tukey}		p_{bonf}	
300 W	400 W	0.271	0.224	0.318	0.020	672	13.524	1.205	0.977	1.433	< .001	***	< .001	***
	500 W	0.435	0.388	0.482	0.020	672	21.727	1.936	1.687	2.184	< .001	***	< .001	***
400 W	500 W	0.164	0.117	0.211	0.020	672	8.202	0.731	0.512	0.950	< .001	***	< .001	***

*** p < .001

Note. P-value and confidence intervals adjusted for comparing a family of 3 estimates (CI for mean difference corrected using the Tukey method; CI for effect size corrected using the Bonferroni method).

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5.7. Assumptions of the technology of using CAP plasma for the eradication of endophytes from perennial ryegrass

The results obtained in the studies of the influence of cold plasma on the development of the endophyte mycelium in the tillers of perennial ryegrass confirmed the working hypothesis that CAP significantly inhibits the development of the symbiont in the plant. However, the type of plasma used is important. The use of GA Plasma turned out to be inefficient. With this type of low-temperature plasma, there was no significant impact on inhibition of the endophyte development in plants with the generator work parameters used. The effect of a strong inhibition of the endophyte's mycelium growth was achieved when treating seeds with DBD Plasma at 300, 400 and 500W power levels and an exposure time of 20 and 30 s. However, in any of the studied combinations, no complete inhibition of the symbiont's development was obtained. Therefore, to obtain the effect of total eradication of the endophyte from the plant, it is necessary to treat seeds one of the traditional methods. Preliminary tests and literature data indicate that use of a chemical method (tebuconazole and thiram), treatment with higher temperature or storage in adjusted conditions of the temperature, air humidity and length of the period can be chosen. The main criteria for choosing the exact method should be safety for the environment and period of available time for processing.

Therefore, based on the obtained results, guidelines for the prototype design of the endophytes eradication station using low -temperature plasma with an algorithm of conduct were developed. The project of prototype technology for the eradication of an endophyte from grass seeds is presented on Figure 36.

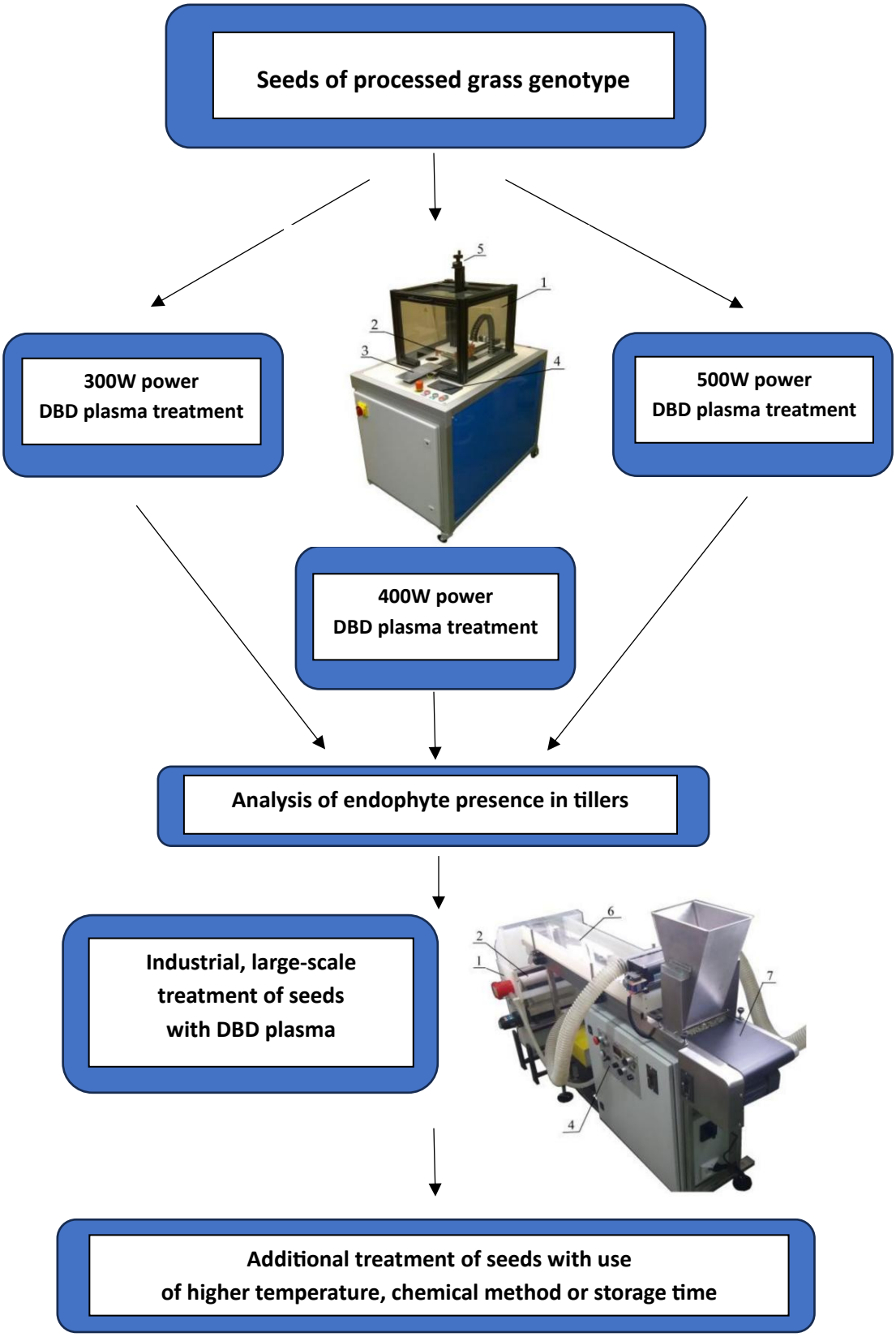

Figure 36. The guidelines for the project of prototype technology for the eradication of an endophyte from grass seeds.

Based on obtained results the following algorithm of conduct the process of eradication of the endophyte from grass seeds to be implemented into technology of production of symbiotically modified cultivars is proposed:

1. Seeds of the processed grass genotype should be treated with DBD plasma under laboratory conditions at generator power levels of 300 W, 400 W, and 500 W, and exposure times of 10 s, 20 s, and 30 s.
2. The treated seeds should then be sown in pots for a period of 4 weeks.
3. Developed plants should be examined under microscope for the presence of endophyte mycelium by staining the leaf sheaths with Rose Bengal.
4. Combinations of generator power and exposure time that minimize the most the number of endophyte hyphae should be selected for large-scale industrial seed treatment.
5. For mass treatment of large quantities of seeds, a prototype with a conveyor belt and multiple sets of electrodes must be designed to ensure high process efficiency. An example station for this type of DBD plasma treatment is shown in Figure 36 (Mućko et al. 2022).
6. Plasma-treated seeds should then be further treated with one of the following methods: chemical treatment (e.g., tebuconazole and thiram), treatment at a higher temperature, or storage under adjusted conditions of temperature, air humidity, and storage period.

When designing DBD plasma seed treatment stations, consideration should be given to using technical solutions that eliminate streamers that negatively impact seed quality after plasma treatment. This could include, for example, the use of reduced atmospheric pressure, which would allow for lower power and longer exposure time. This would enable the delivery of a more uniform plasma characterized by a higher energy density.

6. Discussion

The level of infection in perennial ryegrass is widely changing. In our study, the infection level in perennial ryegrass was 36.2%; this is considered as a medium infection. Panka (2008) reported less than 50% moderate infection of perennial ryegrass in Poland. According to Lewis (2001), the moderate infection was observed throughout Europe. The study conducted in 22 countries around Europe revealed that the average infection of plants and seeds was 49% in natural grassland, and fewer grasses and cultivars were used in agricultural systems (Faeth et al. 2001, Wäli et al. 2001). Pańka and Łukanowski (2001) and Pańka and Sadowski (2002) both revealed similar information about infection on natural and cultivated grasses in Poland. The surveys conducted in areas of New South Wales, Victoria, and Tasmania in Australia showed that the level of endophytes infection is higher in the older pastures than new pasture. The old pasture has higher infection level due to the build-up and persistent of infected seeds and infected plants over time (Kemp et al., 2007).

In European countries, meadow fescue is a grass species of high economic importance. In our collection of genotypes, the percentage of infected genotypes was 29.6%, which considered to be medium infection. According to Leuchtmann (1992) 290 grass species being infected by *Neotyphodium*/*Epichloë* endophytes and about 20–30% of all grasses were infected by these fungi. The study conducted in France on 237 species of European grasses, showed that only 22 species were infected (Leyronas and Raynal 2001). Zabalgogeazcoa et al. (2003) also found that 22% of grass species in semiarid grassland habitats in western Spain were infected with endophytes. The revel of infection in meadow fescue grasses can be higher, the study of Panka et al (2013) found that, the percentage of infection in meadow fescue was 74.5%. The similar results in meadow fescue were reported by Wiewióra (2011). Lewis 1994 revealed that fescues had very high infection rate, for red fescue the percentage was 77% of sampled sites, whereas for meadow and tall fescue was 100%. The range of infection varied from 0 to 100% for red fescue and 96-100% for meadow and tall fescue. The higher infection percentage is, the more grasses may become dangerous to the livestock. Leuchtmann (1992) revealed that, majority of fungal endophytes release poisonous alkaloids in the infected grasses. A significant proportion of endophytes associated with meadow fescue and red fescue in Poland exhibited the capacity to synthesize ergovaline, occasionally to a significant magnitude (Pańka et al., 2013). Lastly, the mechanism to implant a non-toxic endophyte into

the plant must be invented and conditions which guarantee symbiont's continuous presence in plant tissue must be determined. Novel endophytes association do not last long, due to the usually lower degree of compatibility to their host (Easton et al. 2001, Bouton et al. 2002). In the study by Panka (2011) on grass seeds infection by endophytes, 242 samples of seeds of 50 different grass varieties collected in Poland, including 124 samples of 20 cultivars perennial ryegrass, 61 samples of 10 cultivars of meadow fescue and 57 samples of 20 cultivars of red fescue, were examined their level of infection. Fungal endophytes were exclusively confirmed in the seeds of perennial ryegrass and meadow fescue. No fungal infection was detected in samples of red fescue. The endophytic infection was confirmed in 33 samples of 5 cultivars of perennial ryegrass with different levels of infection ranging from 4% up to 8%. Fungal infection in meadow fescue was confirmed in 15 samples of 2 cultivars with higher percentage of infection 24 to 52% for the first cultivar and 46 to 96% for the second cultivar.

A key factor in determining the effectiveness of eradicating an undesirable endophyte from grass is the strength and compatibility of the association. This largely depends on both the host and endophyte genotypes. In our study, the strength of the association expressed as the number of endophyte hyphae per 1 mm of leaf sheath width, ranging from 4.8 (PR42) to 20.8 (PR34/ZTB07). The mean value for all genotypes was 11.8. In the study by Pańka et al. (2013), the density of endophyte hyphae in the leaf sheaths of tall fescue was much higher, ranging from 48.17 to 64.02, depending on the plant genotype. A higher number of hyphae in the tillers indicates a higher strength of the association. In the conducted study, perennial ryegrass genotypes characterized by the highest association strength and the highest endophyte mycelium density were selected for testing. The lack of complete effectiveness of DBD plasma treatment may have resulted from this fact. In the case of genotypes with high performance parameters and lower mycelium density in the tissue, the eradication effectiveness of DBD plasma could be significantly higher. This indicates the need for further research with a larger number of genotypes with varying degrees of endophyte mycelium infestation of the tillers.

Molecular analysis of perennial ryegrass genotypes using Real-Time PCR with use of specific primers allowed the identification of the endophyte *Epichloë festucae* var. *loli* in 10 tested samples. Due to their specificity, modern molecular methods enable precise identification of fungal biomarkers associated with endophytic colonization (Christensen et al. 1993; Rasmussen et al. 2007). However, low endophyte mycelium content in the plant can

result in false negative results. In such cases, traditional methods of endophyte detection, including hyphae staining or serological tests, can be used. These methods allow to detect the presence of a symbiont, but in many cases, they cannot be used to clearly identify the species (Hiatt III et al., 1999, Saha et al., 1988). Their advantage, however, is their relatively low cost, and although they require considerable experience, are relatively time-consuming, and sometimes produce false positive results, they are still widely used. The conducted studies utilized all mentioned methods, taking of their advantages depending on the expected results.

In the conducted study the molecular analysis by Real-Time PCR confirmed the presence of *Epichloë festucae* var. *lolii* endophyte in 10 tested perennial ryegrass genotypes. Based on molecular tests using ITS region sequence analysis, two isolated *Epichloë festucae* var. *lolii* endophyte isolates showed no variation in the ITS sequence. They were identical with some *E. festucae* sequences deposited in the NCBI Gen Bank and with other sequences they show similarity above 99%. With other *Epichloë* species similarity was lower i.e. 99.12% (*E. coenophiala*), 97.86% (*E. baconii*). Due to low percentage of success during isolation of endophytes from perennial ryegrass the analysis of genetic diversity among isolates was limited.

In our study, on the effect of CAP on the number of endophytes, revealed that cold plasma could not eradicate the total number of endophytes, however there was significant differences in number of fungal hyphae, that proved the reduction at some extent. For some perennial ryegrass genotypes such as ZTB01 treated by plasma with power of 200W and time of exposure 10s the mean difference was 15±0.82. In this case plasma treatment reduced almost 45% of endophytes when for the same grass genotype treated by plasma at 600W, the mean difference was 8±1.29. A similar response was observed in another grass genotype, ZTB02. When it was treated with plasma at 200 W, the growth of the endophyte number of hyphae was reduced by nearly 55% compared to treatment at 600 W. The reduction in fungal mycelium was caused by different factors depending how cold atmospheric plasma was inhibiting the ability of multiplication, changing cell wall morphology, or cell wall destruction, etc. This is parallel with the study conducted on the effect of cold atmospheric plasma on common buckwheat and Tartary buckwheat revealed that the extent of seed invasion was higher, as determined by the percentage of Petri dish plate that were overwhelmed by fungi after one week. The 120 second CAP exposures lowered the degree of fungal infection in both buckwheat species to the similar extent as the seeds that were treated with 30% H_2O_2. The

longest exposure duration of 120s led to a reduced average number of fungal morphotype for each plate in both species. In the control seeds samples of both species, there were about 1.5 fungal morphotypes on each Petri dish. After 120 s of CAP treatment, the number of fungal morphotypes was reduced up to 0.5. The absolute fungal distribution showed that there were fewer fungi in seeds of Common buckwheat than in seeds of Tartary buckwheat. In Common buckwheat, there was a clear change from filamentous fungi, which were more common in shorter CAP exposures, to yeasts, which were more common in extended CAP exposure durations. In TB seeds, there was also a reduction in filamentous fungi. It's interesting that seeds of Tartary buckwheat had a little yeast incidence (2.9% of all fungi) compared with the seeds of common buckwheat (29.3%) (Mravlje et al., 2021). In the study by Yanga et al. (2011) on oral bacterial deactivation using a low-temperature atmospheric argon plasma brush, examined the morphological and structural changes on *Streptococcus mutans* and *Lactobacillus acidophilus* revealed a significant alteration in size and morphology of *S. mutans* cells. The plasma exposure of 15 s resulted in large amount of debris formation. Considering *L. acidophilus* cells, longer plasma exposure of 60s led to cell wall damages. Changing and destroying cell wall structures is the part of plasma inactivation endophytes, that could lead to reduction in fungal hyphae in grass genotype of our study, since their multiplication in mycelium was humped by structural disturbance. The morphological changes were confirmed by Denga et al. (2010) where the leakage of substance from cells observed as a direct physical impact on *Bacillus subtilis* after DBD-APP jet treatment. Similar morphological variation was noted by Hong et al. (2009), when *Escherichia coli* was exposed at 75W under 2 minutes, there as a leakage of bacterial DNA caused by a serious bacterial cytoplasmic deformation. Authors revealed that oxidative stress further disrupts cellular metabolic processes and the cellular membrane, along with its critical DNA, protein, and lipid constituents, leading to diminished fungal growth and decreased toxin release (Zhao et al., 2024). The study on tomatoes revealed that plasma treatment inactivated *Fusarium oxysporum* spores after 10 minutes of exposure (Panngom et al. 2014).

In the study by Tamošiūnė et al. (2020) on how CAP treatment of sunflower seeds modulates plant-associated microbiome and stimulates root and lateral organ growth, metagenomic research indicated that treating sunflower seedlings with CAP lowered the diversity of microbes that live inside the plants. In another study by Tamošiūnė et al. (2020)

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on cold plasma treatment of *Arabidopsis thaliana*, the metagenomic study revealed that the growth-stimulating CAP treatment greatly reduced the number of actinobacteria in the *Mycobacteriaceae* family.

Several research highlighted that CAP could reach log reductions ranging from 1 to 5 in bacterial and fungal counts, depends on factors such as the power of plasma treatment, the exposure duration, the gas composition and the distance between the source of treatment and the target (Seget et al., 2014; Scholtz et al., 2015). Cold plasma demonstrated the ability to minimize surface and internal microbial load without compromising plant viability in seeds and plant tissue status. CAP can reduce fungal contamination such as *Fusarium* spp., on wheat without affecting the germination ability of the seeds (Nedyalkova et al. 2019). In the study by Fang et al. (2023) the CAP did not show pivotal impact on population of endophytes, however it exhibited reduction of fungi of the genera *Penicillium*, *Aspergillus*, and *Acremonium*.

Cold atmospheric plasma can affect endophytes, especially fungal endophytes in plants. It has also antimicrobial properties that can change the fungus population that lives inside seeds and plants. CAP generates reactive oxygen and nitrogen species that have been demonstrated to lower the number and variety of fungi, especially those that live on seeds and plants' surfaces. In the study, generally, the microbial population considerably declines after DBD plasma exposure. The untreated seeds had the highest number of colonies, suggesting that the seeds of untreated control samples naturally contained epiphytic and potentially endophytic microorganisms. For example, genotype ZTB02 under 500 W at 20 s reached 75% of reduction in microorganisms compared to the control, while genotype ZTB07 under 400 W at 10 s reached 56% of reduction in microorganisms, compared to the control combination. These results are in line with those of other researchers who showed that CAP is very effective at killing filamentous fungi. Świecimska et al. (2020) found that plasma activity against *F. oxysporum* was quite strong, and the results showed that a 3-second plasma treatment was the best way to stop the pathogen from growing. Avramidis et al. (2010) utilized DBD plasma (20 kV, air) to inactivate *F. culmorum*, demonstrating that total suppression of hyphal development could be achieved within 360 seconds of exposure. Panngom et al. (2014) utilized dielectric barrier discharge plasma (0.75 kV, air, or argon) to inactivate *F. oxysporum* f. sp*. lycopersici*, revealing that less than 10% of fungal spores were capable of germination following 10 minutes of plasma treatment. In the results of study by

Abarghuei et al. (2021), lowest microbial load, measuring 2.42 CFU based on logarithm 10, was observed after treating basil seeds with 10 kV for 30 minutes. In contrast, the highest microbial load, 3.86 CFU, was found in the untreated control group. This is lined with our research where more colonies were observed in control and after treatment by plasma reduced contamination by microorganisms up to 75% in genotype ZTBO2. However, power and exposure time did not always lead to significant microbial reduction in all studied genotypes. The influence of plasma on fungal contamination, was genotype dependent. For instance, genotype ZTB06 under 400W for 20 seconds increased microorganisms by up to 17.95%, and genotype ZTB05 under 400W for 10 seconds increased them by 18.18%. There is not much direct evidence, however there are reasonable ways that cold plasma could enhance fungal growth or endophyte activity in certain situations. Low or sub-lethal plasma doses might cause mild stress that triggers compensatory responses such as increased enzyme secretion, accelerated growth recovery after damage in fungi. In the study by Shapourzdeh et al. (2016) general CAP treatments humped the development of the dermatophyte, the authors observed a moderate increase in keratinase enzyme activity at sublethal exposure durations. In other words, some fungal metabolic activities were stimulated even while net growth was suppressed. Another way could plasma treatment stimulating fungal growth is antioxidant defences in fungi might buffer oxidative stress from plasma, permitting survival and possible enhanced growth once initial ROS damage is dealt with. In such genotypes revealed stimulating fungal activity, may require an alternative treatment.

The obtained results exhibited that DBD plasma has a remarkable effect on the activity of glucanase. The power of the plasma has a paramount influence than other elements considered in the study such as exposure duration and different genotypes. Generally, our results revealed that the GLU activity increased up to 3.68 units when power was changed from 300W up to 500W. Peťková et al. (2021) reported that after treating seedlings with cold plasma, the glucanase activity increased, and associated with enhanced store protein mobilization and activation of defensive mechanisms in germination period. This suggests that plasma can activate glucanase as part of larger biochemical changes that support seedling growth and deal with stress. CAP treatment enhanced glucanase activity by stimulating the secretion of enzyme and upregulating gene expression in fungi, as well as by initiating biochemical process in plants that elevate the glucanase activity in the initial stage of the plant

growth. In our study we noticed that the influence of CAP treatment on GLU activity depended on different factors such grass genotype, power level and exposure duration. For instance, some genotypes showed greater increases in enzyme activities, such as, genotype ZTB05 increased by 5 units of enzyme activities when grass seeds were exposed to 300W for 10 seconds. This is parallel with the research conducted by Burak et al. (2024). Their study revealed that the effect of CAP treatment on proteins, lipids, carbohydrates, and organoleptic parameters of plant's material is primarily influenced by the specific type of plant material treated by CAP and the parameters of treatment such as exposure duration, intensity of power, frequence and gas flow rate. In the study by Guo et al. (2024) CAP caused mutation of *Lysobacter enzymogenes* that may generate proteolytic enzymes which are a category of enzymes including β-1,3-Glucanases. The mutant strains had a significant rise in proteolytic activity that reaches 1.94 times higher than that of the wild type of strain. To optimize the enzyme synthesis without causing harm, the optimal exposure duration is required, and the response depends on power level (Yu et al., 2022).

The significant structural ingredient of cell walls known as chitin is melted by hydrolytic enzyme Chitinase which is classified as a pathogenesis-related (PR) protein and usually increase when the plant is responding to pathogens (Khan et al., 2024). Our results revealed that the CHI activity was affected by plasma, however the power dominated other influencing factors. For instance, the CHI activity was increased 2.29 units when power increased from 300W up to 400W. the CHI activity was increased 3.52 units when the power shifted from 300W up to 500W. Since CAP produces reactive oxygen and nitrogen species (RONS), UV photons, and charged particles, exposure to these reactive species can simulate biotic stress conditions, leading to the activation of signalling cascades involving salicylic acid (SA), jasmonic acid (JA), and ethylene hormones known to regulate PR protein expression, including chitinases. All of them can serve as abiotic elicitors of plant defence mechanisms (Guo et al., 2019; Thirumdas et al., 2018). For certain genotypes, such as ZTB06, CHI activity increased to 15 units when treated with plasma at 300 W for 10 seconds, compared to 12 units in the control. In the grass genotype ZTB07, CHI activity was 10 units when exposed to 400 W for 10 seconds but increased to 17 units when the exposure time was extended to 20 seconds. The specificity and timing of the plasma treatment are critical, as short exposure appears sufficient to trigger a protective response without inducing oxidative stress beyond

the plant's tolerance threshold (Adhikari et al., 2020). Furthermore, the noted rise in chitinase activity could be probably linked to decline in fungal endophyte populations or epiphytic microbes partially, because CAP may selectively neutralize specific microbes while allowing the plant to reallocate metabolic supplies to defence relevant gene expression as reviewed by Zhu et al (2025). This microbial management could affect how microbes and host collaborate, leading to intermittent state of microbial disorder which plant identifies as an indication of stress which results in creation of PR substances. CAP is not only effective as physical disinfectant but also as a method to improve plant immune system. These results reinforce the prospective incorporation of plasma technology in combined diseases control strategies and seed priming techniques for turf grass and animal feed.

Cold plasma has become a potential technique for influencing physiological and biochemical processes in plant biology. Cold plasma treatment may increase guaiacol peroxidase activity, which would improve the plant's ability to eliminate ROS and deal with abiotic challenges like lack of humidity or excessive salt. This is validated by comprehensive reviews demonstrating that cold plasma can stimulate systemic signalling pathways and enhance the expression of stress-responsive genes and enzymes across diverse plant species (Karimi et al.,2024). In our study, the GPOX activity increased by 0.44 units when the power increased from 300W to 500W. Considering some genotypes such as ZTB05 increased from 1.2 units at untreated samples up to 1.4 units when treated by plasma at 500W exposed 10s. These results are parallel with the findings of other researchers, for instance, the findings exhibited that the activity of GPOX enhanced in seedlings of maize after plasma treatment, indicating improved antioxidant feedback (Bokor, 2021). According to another study, treating seeds by CAP enhanced physiological and biochemical characteristics, such as GPOX and other enzymes involved in oxidative stress defence (Mildaziene & Sera, 2022). Plasma activated water (PAW), that generated by CAP exhibited to improve seed germination, plant vigour, and enzyme activities such as peroxidases in crops like black gram (Vigna mungo) (Billah, 2019). The plant defence mechanisms, gene expression and health, most likely by modifying enzymes such as GPOX can be enhanced by treatment with PAW. The CAP treatment in diabetic samples, markedly enhanced the activity of antioxidant enzymes such as guaiacol peroxidase (GPOX), superoxide dismutase (SOD), and catalase (CAT) (Martirosyan et al., 2021). Although this research examined human samples, the higher levels of antioxidant enzymes are consistent with research on plants, which shows that plasma causes oxidative

stress reactions, such as enhanced GPOX activity. To maintain cellular homeostasis under stress, peroxidases support cell wall fortification, lignin biosynthesis, and detoxification of reactive oxygen species (ROS), which are essential in this process (Shigeto et al., 2016). In this study, the increase peroxidases indicates that CAP produces reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are messenger molecules which initiate developing systematic resistance (Almagro et al., 2009; Thirumdas et al., 2018).

7. Conclusions

- Dielectric Barrier Discharge plasma significantly inhibit growth of *Epichloë festucae* var. *lolii* endophyte in tested perennial ryegrass genotypes.
- The colonization of the collection of perennial ryegrass genotypes by *Epichloë festucae* var. *lolii* endophyte is at an average level of 36.2%.
- The colonization of the collection of meadow fescue genotypes by *Epichloë* endophyte is at an average level of 29.6%.
- The strength of endophyte/perennial ryegrass associations in the genotypes collection expressed as mycelium density in sheaths is low to average and is at the level of 4.8 to 20.8 of hyphae per 1mm of width of leaf sheath.
- Molecular analysis by Real-Time PCR confirms the presence of *Epichloë festucae* var. *lolii* endophyte in 10 tested perennial ryegrass genotypes.
- Based on molecular tests using ITS region sequence analysis, two isolated *Epichloë festucae* var. *lolii* endophyte isolates show no variation in the ITS sequence. They are identical with some *E. festucae* sequences deposited in the NCBI Gen Bank and with other sequences they show similarity above 99%. With other *Epichloë* species similarity is lower i.e. 99.12% (*E. coenophiala*), 97.86% (*E. baconii*).
- In the collection of perennial ryegrass genotypes colonized by the endophyte, seven selected genotypes have a relatively high utility value determined on the basis of selected traits and constitute a potential starting material for further breeding.
- The obtained results indicate that the genotype of perennial ryegrass, the power level (200W, 300Ws, 400W, 500W, 600W) of the Dielectric Barrier Discharge plasma generator, and the time of seed exposure (10 s, 20 s, and 30 s) to the plasma have a significant effect on the density of the endophyte mycelium in plants. For most genotypes, increasing power significantly reduces the mycelium content in plants. Exposure time has a smaller and ambiguous effect, although the general trend is a decrease in the endophyte mycelium content in the plant with increasing exposure time to the plasma. DBD plasma has the greatest inhibitory effect on endophyte development in plants in the power range from 300W to 600W.

- The obtained results indicate that the genotype of perennial ryegrass, the power level (200W, 300Ws, 400W) of the Dielectric Barrier Discharge plasma generator, and the time of seedlings exposure (10 s, 20 s, and 30 s) to the plasma have a significant effect on the density of the endophyte mycelium in plants. Genotype has the greatest influence on endophyte development in the plant. Power level and exposure time have a much lower effect on mycelium density.
- Application of Gliding Arch plasma (200W, at 10 s, 20 s, and 30 s) to perennial ryegrass seeds does not have a significant inhibitory effect on endophyte development in the plant, within individual genotypes.
- Application of Dielectric Barrier Discharge plasma to perennial ryegrass seeds has a significantly greater inhibitory effect on the development of endophyte mycelia in the plant than application of DBD plasma to 3-day-old seedlings or application of Gliding Arch plasma to seeds.
- The percentage reduction in contamination of perennial ryegrass seeds by microscopic fungi after application of Dielectric Barrier Discharge plasma (300W, 400W, 500W, at 10s, 20s, 30s) to seeds is high for most tested perennial ryegrass genotypes, reaching nearly 80%. However, in some cases, plasma application may also stimulate fungal growth. Due to the large variation between combinations of different power levels and exposure times, it is impossible to determine a clear relationship and effect of each factor on seed contamination by microscopic fungi.
- The obtained results indicate that the genotype of perennial ryegrass and the power level (200W, 300Ws, 400W) of the Dielectric Barrier Discharge plasma generator have a significantly the highest effect on stimulating of glucanases, chitinases and guaiacol peroxidase activity in plants after seeds treatment. Time of seeds exposure (10 s, 20 s, and 30 s) to plasma has the lower influence on the activity of these enzymes.
- The obtained results indicate that Dielectric Barrier Discharge plasma used in the power ranges of 200W, 300Ws, 400W, 500W, 600W of the Dielectric Barrier Discharge plasma generator and the time of seed exposure (10 s, 20 s, and 30 s) significantly reduces the colonization of perennial ryegrass by the endophyte, however, it does not allow for complete eradication of the symbiont. Therefore, plasma-treated seeds should then be further treated with one of the following methods: chemical treatment (e.g., tebuconazole and thiram), treatment at a higher temperature, or storage under adjusted conditions of

temperature, air humidity, and storage period. In connection with the above, the guidelines for the prototype design of the endophytes eradication station using low-temperature plasma with an algorithm of conduction were developed.

8. Literature

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9. English abstract

Endophytes eradication technology in the production of symbiotically modified grasses

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Keywords: Cold plasma, endophyte, grasses, eradication

Due to the presence of the *Epichloë* endophytes, grasses are usually more resistant to numerous stress factors, both abiotic and biotic. Selected isolates of endophytic fungi of the genus *Epichloë*/*Neotyphodium*, the so-called "novel endophytes" can be used for symbiotic modification of grasses (SMG) to provide plants with greater resistance to stress factors. However, the introduction of these isolates into the plant requires the prior removal of wild endophytes from the variety to be symbiotically modified. The eradication process is very difficult and time-consuming. It is usually carried out using fungicides or high temperature and the efficiency of those methods is very low.

Therefore, the main purpose of the conducted research was to determine the suitability of low-temperature plasma for the eradication of wild endophytes from plants in order to develop a proposal for an innovative, effective, and environmentally friendly technology for removing undesirable endophytes from grasses and then using them in the process of symbiotic modification of new varieties. Based on literature data and previous research, a working hypothesis was formulated that due to antibacterial and antifungal activity of low-temperature plasma growth of *Epichloë festucae* endophyte can be significantly inhibited after treatment by Dielectric Barrier Discharge plasma and Gliding Arch plasma. Following specific objectives were formulated to achieve the main objective: (i) to collect grass genotypes and isolates of endophytic fungi inhabiting different genotypes available commercially as well as ecotypes and to characterize the endophyte infected genotypes based on the chosen parameters, (ii) to identify the collected isolates of endophytes and to determine their genetic diversity, (iii) to determine the boundary and optimal operating parameters of the DBD plasma and GA plasma generator ensuring the highest level of endophyte eradication, (iv) to check the overall performance between DBD plasma and GA plasma, (v) to determine the effect of Cold Atmospheric Plasma (DBD plasma and GA plasma) on seeds contamination by fungi, (vi) to determine the effect of Cold

Atmospheric Plasma (DBD plasma and GA plasma) on the activity of chosen enzymes (Pathogenesis Related Proteins (PRP), (vii) to propose the guidelines for the prototype design of the endophytes' eradication technology using Cold Atmospheric Plasma.

The obtained results indicated that Dielectric Barrier Discharge plasma significantly inhibited growth of *Epichloë festucae* var. *lolii* endophyte in tested perennial ryegrass genotypes. The colonization of the gathered collection of perennial ryegrass genotypes by *Epichloë festucae* var. *lolii* endophyte was at an average level of 36.2% and the colonization of the collection of meadow fescue genotypes by *Epichloë* endophyte was at an average level of 29.6%. The strength of endophyte/perennial ryegrass associations in the genotypes collection expressed as mycelium density in sheaths was low to average and ranged from 4.8 to 20.8 of hyphae per 1mm of width of leaf sheath. Molecular analysis by Real-Time PCR confirmed the presence of *E. festucae* var. *lolii* endophyte in 10 tested perennial ryegrass genotypes. Based on molecular tests using ITS region sequence analysis, two isolated endophyte isolates showed no variation in the ITS sequence. They were identical with some *E. festucae* sequences deposited in the NCBI Gen Bank and with other sequences they showed similarity above 99%. In the collection of perennial ryegrass genotypes colonized by the endophyte, seven selected genotypes showed a relatively high utility value determined on the basis of selected traits and constitute a potential starting material for further breeding. The obtained results indicate that the genotype of perennial ryegrass, the power level (200W, 300W, 400W, 500W, 600W) of the Dielectric Barrier Discharge plasma generator, and the time of seed exposure (10 s, 20 s, and 30 s) to the plasma had a significant effect on the density of the endophyte mycelium in plants. For most genotypes, increasing power significantly reduced the mycelium content in plants. Exposure time had a lower and ambiguous effect, although the general trend was a decrease in the endophyte mycelium content in the plant with increasing exposure time to the plasma. DBD plasma has the greatest inhibitory effect on endophyte development in plants in the power range from 300W to 600W. The genotype of perennial ryegrass, the power level (200W, 300W, 400W) of DBD plasma generator, and the time of seedlings exposure (10 s, 20 s, and 30 s) had also a significant effect on the density of the endophyte mycelium in plants. Genotype had the greatest influence on endophyte development in the plant. Power level and exposure time had a much lower effect on mycelium density. Application of Gliding Arch plasma (200W, at 10 s, 20 s, and 30 s) to perennial ryegrass seeds did not have a significant inhibitory effect on endophyte

development in the plant, within individual genotypes. In the case of DBD plasma its application to perennial ryegrass seeds had a significantly greater inhibitory effect on the development of endophyte mycelia in the plant than application of DBD plasma to 3-day-old seedlings or application of Gliding Arch plasma to seeds. The percentage reduction in contamination of perennial ryegrass seeds by microscopic fungi after application of DBD plasma (300W, 400W, 500W, at 10s, 20s, 30s) to seeds was high for most tested perennial ryegrass genotypes, reaching nearly 80%. However, in some cases, plasma application stimulated fungal growth. Due to the large variation between combinations of different power levels and exposure times, it is impossible to determine a clear relationship and effect of each factor on seed contamination by microscopic fungi. Moreover, the obtained results indicated that the genotype of perennial ryegrass and the power level (200W, 300W, 400W) of DBD plasma had a significantly the highest effect on stimulating of glucanases, chitinases and guaiacol peroxidase activity in plants after seeds treatment. Time of seeds exposure (10 s, 20 s, and 30 s) to plasma had the lower influence on the activity of these enzymes.

The obtained results indicate that DBD plasma used in the power level of 200W, 300W, 400W, 500W, 600W and the time of seeds exposure (10 s, 20 s, and 30 s) significantly reduced the colonization of perennial ryegrass by the endophyte, however, it did not allow for complete eradication of the symbiont. Therefore, plasma-treated seeds should then be further treated with one of the following methods: chemical treatment (e.g., tebuconazole and thiram), treatment at a higher temperature, or storage under adjusted conditions of temperature, air humidity, and storage period. In connection with the above, the guidelines for the prototype design of the endophyte eradication station using DBD plasma with an algorithm of conduction were developed.

10. Polish abstract

Technologia eradykacji endofitów w produkcji traw modyfikowanych symbiotycznie

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Słowa kluczowe: zimna plazma, endofit, trawy, eradykacja

Dzięki obecności endofitów *Epichloë*, trawy są zazwyczaj bardziej odporne na liczne czynniki stresowe, zarówno abiotyczne, jak i biotyczne. Wybrane izolaty grzybów endofitycznych z rodzaju *Epichloë*/*Neotyphodium*, tzw. „novel endophytes", mogą być wykorzystane do symbiotycznej modyfikacji traw (SMG), zapewniając roślinom większą odporność na czynniki stresowe. Wprowadzenie tych izolatów do rośliny wymaga jednak wcześniejszego usunięcia dzikich endofitów z odmiany, która ma być symbiotycznie modyfikowana. Proces eradykacji jest bardzo trudny i czasochłonny. Zazwyczaj przeprowadza się go za pomocą fungicydów lub wysokiej temperatury, a skuteczność tych metod jest bardzo niska. Dlatego głównym celem przeprowadzonych badań było określenie przydatności plazmy niskotemperaturowej do eradykacji dzikich endofitów z roślin w celu opracowania propozycji innowacyjnej, skutecznej i przyjaznej dla środowiska technologii usuwania niepożądanych endofitów z traw, a następnie wykorzystania ich w procesie symbiotycznej modyfikacji nowych odmian.

Na podstawie danych literaturowych i wcześniejszych badań sformułowano hipotezę roboczą, że dzięki działaniu przeciwbakteryjnemu i przeciwgrzybiczemu plazmy niskotemperaturowej wzrost endofitu *Epichloë festucae* może zostać znacząco zahamowany po zastosowaniu plazmy DBD (Dielectric Barrier Discharge) oraz GA (Gliding Arch). Następujące szczegółowe cele zostały sformułowane w celu osiągnięcia celu głównego: (i) zebranie genotypów traw i izolatów grzybów endofitycznych zasiedlających różne genotypy dostępne komercyjnie, jak również ekotypy i scharakteryzowanie genotypów zasiedlonych przez endofity na podstawie wybranych parametrów, (ii) identyfikacja zebranych izolatów endofitów i określenie ich zróżnicowania genetycznego, (iii) określenie granicznych i optymalnych parametrów pracy generatora plazmy DBD i plazmy GA zapewniających najwyższy poziom eradykacji endofitów, (iv) porównanie efektywności plazmy DBD i plazmy GA, (v) określenie wpływu zimnej plazmy atmosferycznej (plazmy DBD i GA) na kontaminację

nasion grzybami mikroskopowymi, (vi) określenie wpływu plazmy DBD i GA) na aktywność wybranych enzymów (białek związanych z patogenezą (PRP), (vii) zaproponowanie wytycznych dla prototypu projektu technologii eradykacji endofitów przy użyciu zimnej plazmy.

Uzyskane wyniki wykazały, że plazma DBD istotnie hamowała wzrost *Epichloë festucae* var. *lolii* w badanych genotypach życicy trwałej. Kolonizacja zebranej kolekcji genotypów życicy trwałej przez endofita wyniosła średnio 36,2%, a kolonizacja kolekcji genotypów kostrzewy łąkowej 29,6%. Siła asocjacji endofit/życica trwała w kolekcji genotypów, wyrażona jako gęstość grzybni w pochwach liściowych, była niska do średniej i wahała się od 4,8 do 20,8 strzępek na 1 mm szerokości pochew liściowych. Analiza molekularna metodą Real-Time PCR potwierdziła obecność E. *festucae* var. *lolii* w 10 genotypach życicy trwałej. Na podstawie testów molekularnych z wykorzystaniem analizy sekwencji regionu ITS, dwa wyizolowane izolaty endofita nie wykazały zmienności w sekwencji ITS. Były one identyczne z niektórymi sekwencjami *E. festucae* zdeponowanymi w Banku Genów NCBI, a z innymi sekwencjami wykazywały podobieństwo powyżej 99%. W kolekcji genotypów życicy trwałej zasiedlonych przez endofita, siedem wybranych genotypów wykazało stosunkowo wysoką wartość użytkową, określoną na podstawie wybranych cech i może stanowić materiał wyjściowy w hodowli nowych odmian. Uzyskane wyniki wskazują, że genotyp życicy trwałej, poziom mocy (200 W, 300 W, 400 W, 500 W, 600 W) generatora plazmy DBD oraz czas ekspozycji nasion (10 s, 20 s i 30 s) na działanie plazmy miały istotny wpływ na zagęszczenie grzybni endofita w roślinach. Dla większości genotypów, zwiększenie mocy znacząco zmniejszyło zawartość grzybni w roślinach. Czas ekspozycji miał mniejszy i niejednoznaczny wpływ, chociaż ogólną tendencją był spadek zawartości grzybni endofitów w roślinie wraz ze wzrostem czasu ekspozycji na plazmę. Plazma DBD ma największy efekt hamujący na rozwój endofitów w roślinach w zakresie mocy od 300 W do 600 W. Genotyp życicy trwałej, poziom mocy (200 W, 300 W, 400 W) generatora plazmy DBD oraz czas ekspozycji siewek (10 s, 20 s i 30 s) również miały istotny wpływ na zagęszczenie grzybni endofitów w roślinach. Genotyp miał największy wpływ na rozwój endofitów w roślinie. Poziom mocy i czas ekspozycji miały znacznie mniejszy wpływ na zagęszczenie grzybni. Zastosowanie plazmy GA (200 W, przez 10 s, 20 s i 30 s) na nasiona życicy trwałej nie miało istotnego efektu hamującego rozwój symbionta w roślinie, w obrębie poszczególnych genotypów. W przypadku plazmy DBD jej aplikacja na nasiona życicy trwałej miało istotnie większy efekt hamujący na rozwój grzybni

endofitów w roślinie niż zastosowanie plazmy DBD na 3-dniowe siewki lub zastosowanie plazmy GA na nasiona. Procentowa redukcja kontaminacji nasion życicy trwałej grzybami mikroskopowymi po zastosowaniu plazmy DBD (300 W, 400 W, 500 W, przez 10 s, 20 s, 30 s) na nasiona była wysoka dla większości testowanych genotypów życicy trwałej, sięgając prawie 80%. Jednak w niektórych przypadkach zastosowanie plazmy stymulowało wzrost grzybów. Ze względu na duże zróżnicowanie pomiędzy kombinacjami różnych poziomów mocy i czasów ekspozycji, niemożliwe jest określenie jednoznacznej zależności i wpływu każdego czynnika na kontaminację nasion grzybami mikroskopowymi. Ponadto, uzyskane wyniki wskazują, że genotyp życicy trwałej i poziom mocy (200 W, 300 W, 400W) plazmy DBD miały istotnie największy wpływ na stymulację aktywności glukanaz, chitynaz i peroksydazy gwajakolowej w roślinach po zaaplikowaniu na nasiona. Czas ekspozycji nasion (10 s, 20 s i 30 s) na działanie plazmy miał mniejszy wpływ na aktywność tych enzymów.

Uzyskane wyniki wskazują, że plazma DBD stosowana na poziomach mocy 200 W, 300 W, 400 W, 500 W, 600 W oraz czas ekspozycji nasion (10 s, 20 s i 30 s) istotnie zmniejszyły kolonizację życicy trwałej przez endofita, jednak nie pozwoliły na całkowitą eradykację symbionta. Dlatego też, nasiona potraktowane plazmą powinny być następnie poddane dalszej obróbce jedną z następujących metod: obróbka chemiczna (np. tebukonazolem i tiuramem), obróbka w podwyższonej temperaturze lub przechowywanie w określonych warunkach temperatury, wilgotności powietrza i okresu przechowywania. W związku z powyższym opracowano wytyczne do prototypowego projektu stacji eradykacji endofitów z wykorzystaniem plazmy DBD wraz z algorytmem postępowania.