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# Report on recommendations for improving seed multiplication via the use of cover crops and seed inoculation treatments

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| **KEYWORDS** | Soybean, cover crops, inoculants, bio-priming |
| **ABSTRACT (FOR DISSEMINATION)** | The main objective of the study is to assess the effects of cover crops on soybean production and seed quality and to investigate potential synergies with seed inoculation and micronutrient seed coatings in the process of seed multiplication.<br>Improving seed multiplication using cover crops and seed inoculation treatments involves various strategies aimed at enhancing seed quality and yield. Farmers and seed producers can improve seed multiplication processes by applying these practices, which will result in higher-quality seeds, better soil health, and sustainable agricultural practices. |
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# 1 Executive Summary

Global climate change further increases the situation by delaying planting, causing problems with seed germination, seedling emergence and further plant development. The issue of the availability and quality of organic seeds highlights the need for developing strategies to enhance seed multiplication. This study focuses on evaluating the impact of cover crops on soybean production and seed quality, while also exploring potential synergies with seed inoculation and micronutrient seed coatings in the seed multiplication process.

The overarching goal of experiments within ECOBREED was to enhance seed multiplication practices via testing cover crops, inoculants and micronutrient seed coatings for soybean seed multiplication in organic and low-input production. The use of inoculants is of crucial importance in organic production systems, while bio-priming contributes to their applicability and effectiveness in such conditions. Among the pre-sowing techniques, seed bio-priming has increasingly been recognised as the most simple, economical and environmentally friendly method that fits into the specifics of organic agriculture. Improving seed multiplication through the use of cover crops and seed inoculation treatments involves various strategies aimed at enhancing seed quality and yield. Farmers and seed producers can improve seed multiplication processes by applying these practices, which will result in higher-quality seeds, better soil health and sustainable agricultural practices.

# 2 Organic Soybean

Soybean [*Glycine max* (L.) Merr.] has great economic importance arising from the grown area worldwide (121.53 million ha), where about 335 million tons soybean is used for various purposes. Due to favourable nutritional composition (about 40% protein and 20% oil), soybean is used for feed and food, pharmaceutical and other industries. In addition, soybean is becoming increasingly important in human nutrition. Also, it was noted increased interest in organic soybean farming worldwide due to growing consumer demand for organic products, environmental concerns and a desire for more sustainable agricultural practices.

**Soybean has the potential to improve sustainability of agriculture production systems.**

From the agronomic aspect, it has great agronomic significance, as it enriches the soil with nitrogen, improves physical properties, which makes it a good pre-crop for almost all field and vegetable crops. Soybean has the ability to biologically fix nitrogen in symbiosis with nodules of the genus *Bradyrhizobium* and can achieve high yields with or without reduced use of mineral fertilisers. Choosing the right soybean varieties and production technology is the way to achieve economic viability while maintaining sustainable production.

**Use of inoculants and sowing of cover crops has an extremely important role in crop rotation and is an indispensable link in low-input and organic production.**

Improving seed multiplication through the use of cover crops and seed inoculation treatments involves various strategies aimed at enhancing seed quality and yield. Farmers and seed producers can improve seed multiplication processes by applying these practices, which will result in higher-quality seeds, better soil health, and sustainable agricultural practices.

**Organic soybean seed multiplication involves selecting high-quality seeds and implementing organic farming practices. Proper crop, pest and disease management, and record-keeping are essential for maintenance of organic status.**

The main objective of the study is to evaluate the effects of cover crops on soybean production and seed quality, and to investigate potential synergies with seed inoculation and micronutrient seed coatings in the seed multiplication process.

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## 2.1 Organic soybean seed multiplication

Seed production integrates breeding, cultivation practices, biotechnology and economics i.e trade. At the moment when a soybean variety is registered small quantities of seed are available. The main task of soybean seed multiplication is to produce more soybean seed from a relatively small seed stock. Soybean seed multiplication will relate to technology adoption and need to meet market demands for organic soybean seeds.

After the release of a new variety (i.e NS Ecob), limited amounts of seeds are available and it is necessary to multiply additional quantities for growing demand of all relevant markets. .

Soybean seed production includes production of:

- Pre-basic seed;
- Basic seed;
- Certified seed 1st generation;
- Certified seed 2nd generation.

Pre-basic seed is produced by breeders where a particular variety was developed, and the breeder has direct control over this process. This seed is available in a limited supply and is not meant for commercial production; rather, it serves as the raw material for subsequent seed production.

A seed production supervision system and particular requirements for soybean seeds have been devised to guarantee the purity and quality of varieties. The supervision procedure includes a number of activities, including keeping track of variety multiplication statistics, inspecting soybean seed crops in the field, and keeping an eye on seed preparation for the market.

Here are the general steps (Fig. 1) involved in organic soybean seed multiplication:

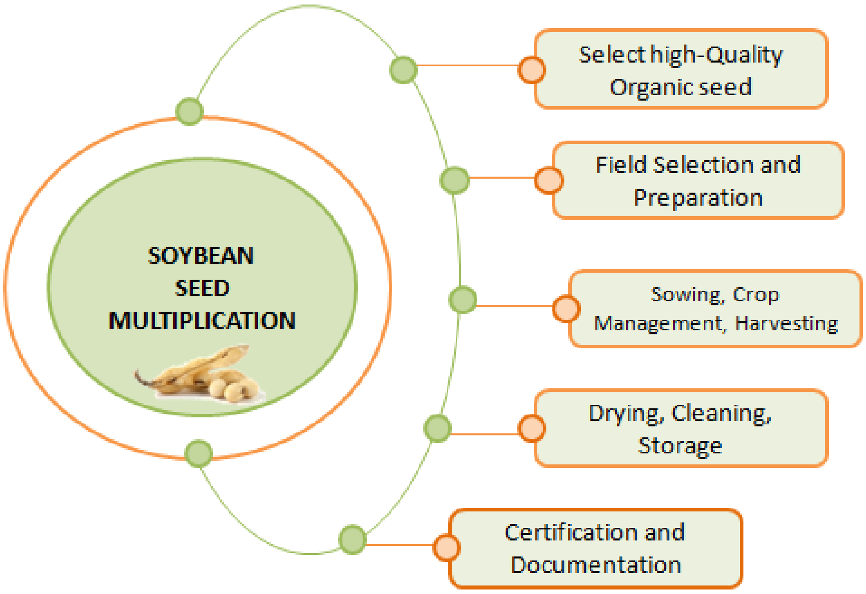

**Fig. 1:** Practices in seed multiplication.

It is essential to follow organic standards and regulations throughout the entire seed multiplication process to meet the organic quality standards of the seeds.

In the ECOBREED project two practices were evaluated to improve seed multiplication for organic and low-input systems:

1. **Use of cover crops in soybean production**
2. **Use of inoculants in soybean production**

In the ECOBREED project experiments were set up in Serbia (RS), Austria (AT) and Romania (RO) in order to examine if these two practices can improve organic soybean seed multiplication. Cover crops were selected in accordance with agro-ecological conditions and to fit crop rotation of soybean. In experiments, *Bradyrhizobium japonicum* were tested as a beneficial nitrogen-fixing bacterium, along with micronutrient coatings. In addition, the bio-priming technique as part of our research was evaluated with special emphasis on germination and vigour tests (cold test).

## 2 Cover crop potential

With the increasing popularity of sustainable systems (low-input and organic production), maintaining and preserving soil fertility and health are key priorities. Also, cover crops are increasingly being used as a tool to support agro-ecosystem services. A major challenge in low-input and organic production is to enable optimal plant nutrition, based on the efficient use of nutrients in soil (Manojlović, 2008) which is directly connected to seed multiplication and gaining of seed material with adequate

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quality. Ubavić and Bogdanović (2008) suggest selecting species with a well-developed root system that has the ability to use nutrients from less soluble compounds from deeper in the soil profile, with rapid growth and a short vegetation period and with the ability to fix atmospheric nitrogen e.g. legumes. How and why to involve cover crops in organic soybean seed multiplication can be seen through:

- *Benefits of Cover Crops*
- *Selecting the Right Cover Crop*
- *Planting, Growing and Terminating Cover Crops*

## 2.1 Cover crops in organic soybean production

### 2.1.1 Benefits of the introduction Cover Crops

Integration of cover crops can have significant ecological impacts on the farming system with numerous on-farm benefits. Cover crops are important for organic and low-input farming because they enhance the soil chemical, biological and physical properties.

The following benefits of cover crops have been identified to be directly connected with an improvement in the efficiency of seed multiplication:

- Contribution to soil quality, via improved physical and chemical soil characteristics;
- If the cover crop is a legume, then N fixation is ensured thereby increasing the yield and quality of the following crop;
- N release to the following cash crop (e.g. soybean);
- Decreased erosion and soil compaction;
- Conservation of soil moisture;
- Organic matter conservation;
- Weed control;
- Increased sustainability of production systems (low-input and organic production).

### 2.1.2 Selecting the Right Cover Crop for Organic Soybean

Choosing the most appropriate cover crop species (single or mixture) is a crucial decision. It involves considering factors like climate, purpose (soil health, weed control, etc.), crop rotation, soil conditions, growth rate, biodiversity, cost, local expertise and the option of using a single species or a mixture with complimentary benefits of each species. By carefully selecting and managing cover crops, growers can optimise the conditions for successful seed multiplication. Before choosing a cover crop, it is important to consider the cultivation system, location, soil type, input costs, as well as the timing of their establishment and destruction (Baas et al., 2015). Cover crops from the legume family fix atmospheric nitrogen which is then available for use by the next

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crop, while cover crops without the participation of legumes are mainly used to reduce soil erosion and nutrient leaching (Meisinger et al., 1991). Nitrogen fixation by legumes helps to reduce the use of nitrogen fertilisers for the next crop (Ladha et al., 2004) and is extremely important in organic production due to the inability to use external inputs.

The following winter cover crops were tested:

| Cover crop photo | Description |
|---|---|
| <br>**Fig. 2:** Rye as winter cover crop. | **Rye**<br>• Positive effect on soil structure;<br>• Good soil cover;<br>• Rye has deep root that helps in the prevention of soil compaction in annually tilled fields;<br>• Great in cover crop mixtures with legumes (winter pea, vetch, faba bean etc.);<br>• Rye has root exudates that can inhibit germination and growth of weed seeds (allelopathy) which is very important thereby supporting weed control in organic production;<br>• Life-cycle of pests is broken;<br>• Good preceding crop for soybean. |
| <br>**Fig. 3:** Pea and oat as winter cover crop. | **Pea and oat**<br>• Forage peas can be grown alone or in combination with other species, especially with small grains (oats, rye);<br>• Easy to terminate;<br>• Oats provide support to the pea and have a positive effect on weed control, while the pea provides nitrogen;<br>• Erosion control, weed suppression, improved soil health, disease management;<br>• Attract a wide range of beneficial insects and pollinators. |

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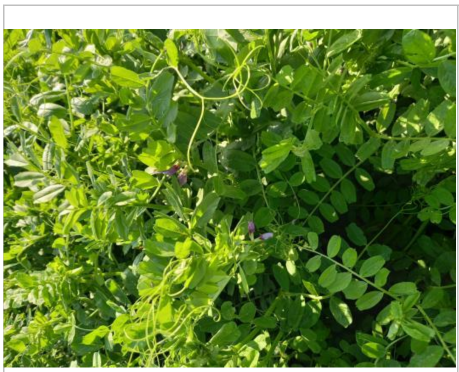

**Fig. 4:** Vetch as winter cover crop.

**Vetch**

- Nitrogen fixation;
- Erosion control, weed suppression;
- Improved soil health;
- In crop rotation vetch breaks pest and disease cycles, contributing to overall crop health and yield;
- Attract a wider range of beneficial insects and pollinators.

### 2.1.3 Planting, Growing and Terminating Cover Crops

Cover crops are typically planted in between cash crops, during fallow periods, or after the main crop is harvested. Before sowing of soybean (main crop), winter cover crops are terminated in the spring. Clark (2008) points out the beneficial effect of sowing rye and winter vetch (single crop) which enables the proper management of nitrogen in the soil, because rye thus prevents the leaching of residual nitrogen, while vetch, through nitrogen fixation, provides additional nitrogen that will be used by the next or main crop. According to Ćupina et al. (2016) the cultivation of mixed intercrops (a mixture of legumes and small grains) is recommended in order to reduce the problem of nitrogen deficit and low content of organic matter in the soil.

Some of the crucial actions of incorporating cover crops:

1. Determine which main crops will follow the cover crop and how the cover crop will fit the existing crop rotation;
2. Incorporate cover crops into your crop rotation;
3. Follow best practices in accordance with selected cover crop species;
4. Terminating winter cover crops is a key step in preparing the field for the main crop planting.

The timing and method of termination depend on various factors, including the cover crop species, climate and cropping system. In organic production mechanical termination is mostly used (Figs. 5, 6). Mechanical termination of cover crops refers to the process of terminating or killing the cover crops using machinery, typically before planting the main cash crop.

**Fig. 5 & 6:** Cover crop before and after mechanical termination.

In order to maximise the effects, agricultural producers apply mechanical measures during the termination of cover crops (Sharma et al., 2018), but also to avoid the use of chemicals e.g. glyphosate which is often used in conventional production systems for cover crop termination. These methods may include rolling, tapping, mowing, disking and reduced processing (Creamer and Dabney, 2002). Proper ploughing of the cover crop and preparation for drilling the main crop is a prerequisite for successful establishment. Due to the complex impact and length of the growing season of cover crops and meeting nitrogen needs, it is sometimes difficult to interpret the interaction between soybeans and cover crops, and it is necessary to consider all benefits and factors leading to increased soybean yields (Baas et al., 2015).

## 2.2 Material and methods

The experiments that simulate seed multiplication process were set up according to a complete block design with four replicates at two locations Čurug (organic production) and Rimski šančevi (low-input production), Serbia (2020-2022). A pure rye crop (1) and mixture of peas and oats (2) were sown as a cover crop, while the control treatment (C) was an area without cover crop (period autumn – spring). During 2022 and 2023 a trial with vetch was set up in Serbia where the same methodology was applied. After mulching of cover crop biomass and conservation tillage two soybean varieties were sown NS Mercury (00) and NS Altis (0). Soybean was harvested, moisture was measured and well as natural yield, followed with laboratory standard germination test (germination energy and germination). Based on reliable calibration models, the spectral characteristics of the examined sample are related to the content of the component of interest. Total protein and oil content of soybean, were analysed by Antaris II Thermo Scientific FT-NIR, while OMNICTM software was used for data processing and calibration. Phenolic compounds were extracted from milled (IKA®, A11 basic, Germany) and sieved (using a 0.25 mm sieve) samples. Powdered material of the control samples (seed and herbal powder) was tested in a preliminary extraction procedure (100% and 80% acetone, ethanol and methanol solutions) to obtain the highest content of total phenolics and flavonoids and the most favourable extraction agent was used in further analyses. The extraction of phenolic compounds was performed in a cooled ultrasonic bath for 1 h, centrifuged (10 min at 33600 xg) and supernatants were analysed for total phenolics and total flavonoid content. Total phenolic content was determined by the Folin-Ciocalteu method slightly modified by Mikulič-Petkovšek et al. (2012). Soil parameters were analysed at the Laboratory for Soil and Agroecology of the Institute of Field and Vegetable Crops, Novi Sad. Yield t/ha was obtained in all treatments, at each location. Near-infrared spectroscopy was used for evaluation of nutritional quality. The determination of weed species was performed on 1m$^2$ on 3 replicates on each cover crop. The green and dry mass of weeds t/ha was as well as weed determination was done in the cover crop, but also in the control plot. Seed health screening was done on soybean seed samples (25 grains) with a magnifying glass 5.0 magnification was examined. Seeds that are not infected with downy mildew were placed in sodium hypochlorite NaClO-8% suspension for 5 minutes and then rinsed in sterile water twice. Seeds were dried on sterile paper and transferred to a PDA substrate paper and PDA substrate. Seeds were left in an incubator at a temperature of 23 degrees and after 7 to 10 days an examination of seed health was carried out (microscopy and morphological determination). The data were statistically processed (StatSoft Inc., Tulsa, USA) using the analysis of variance (ANOVA) statistical method, followed by mean separation according to the Fisher's LSD test (P <0.05).

## 2.3 Results

### 2.3.1 Soybean yield and seed quality

*Soybean yield*

During 2020 and 2022 soybean yield was in the range 1.9 – 3.3 t/ha (Fig. 8). Yield and yield parameters positively reacted to the mixture (P+O) as a pre crop. In both production systems (organic and low input), the average yield for both types in 2022 was between 0.8 and 1.2 t/ha., which was affected by severe drought on both locations during July and August.

**Fig. 7:** Soybean harvest and obtaining the yield results.

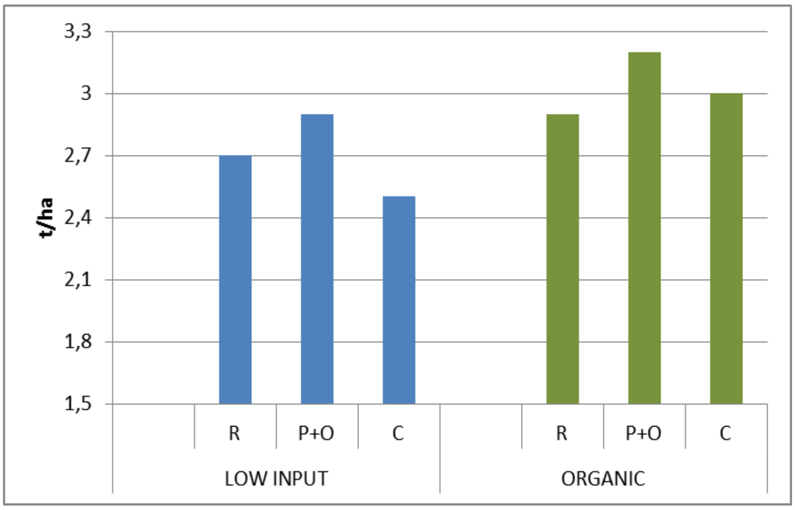

**Fig. 8:** Soybean yield (t/ha) following rye and pea/oat cover crops compared to the control treatment.

*Soybean seed quality*

After harvest is each experimental year seed germination energy and germination (%) were determined. In 2020 (Fig. 9, 10) and 2021, the germination rates of the seeds under test reached as high as 98%. However, in 2022, germination dropped to 75%, primarily due to severe drought in the summer months and excessive rain in September during the harvest.

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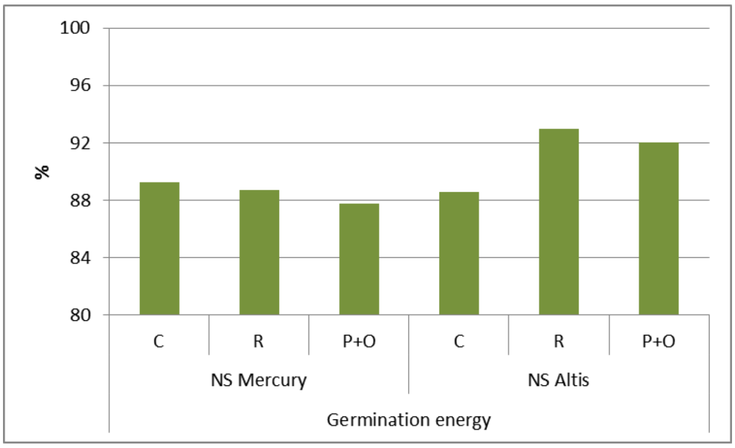

**Fig. 9:** Organic production, soybean seed germination energy in 2020.

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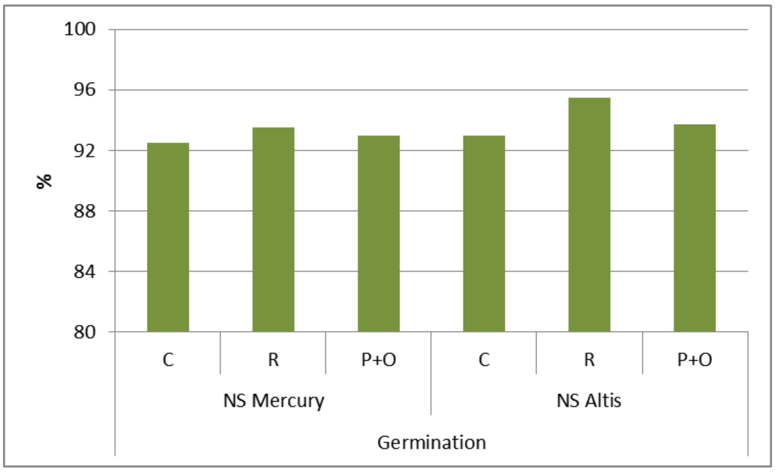

**Fig. 10:** Soybean seed germination in 2020.

Implementing appropriate cultivation practices can lead to achieving the desired quality of soybean seeds in years with proper precipitation distribution or the use of irrigation during seed multiplication.

### 2.3.2 Soybean protein, oil phenols and flavonoids

*Protein and oil content*

The relation between protein and oil content and use of cover crops was seen through obtained results. There was no direct impact of cover crops on protein and oil content, with statistical differences identified only between selected varieties. In organic production the average protein content of different genotypes was in the range of 35.6 to 44.3 % for the two varieties (Fig. 11).

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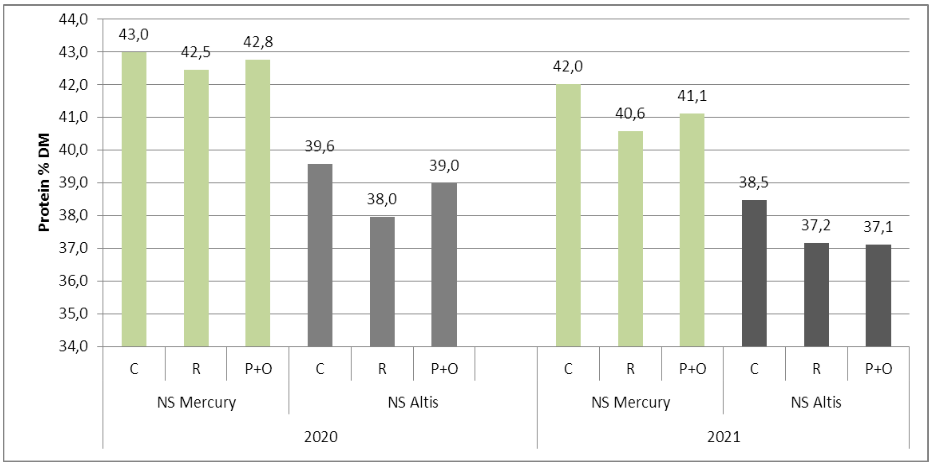

**Fig. 11:** Protein (DM) in organic production in 2020 and 2021.

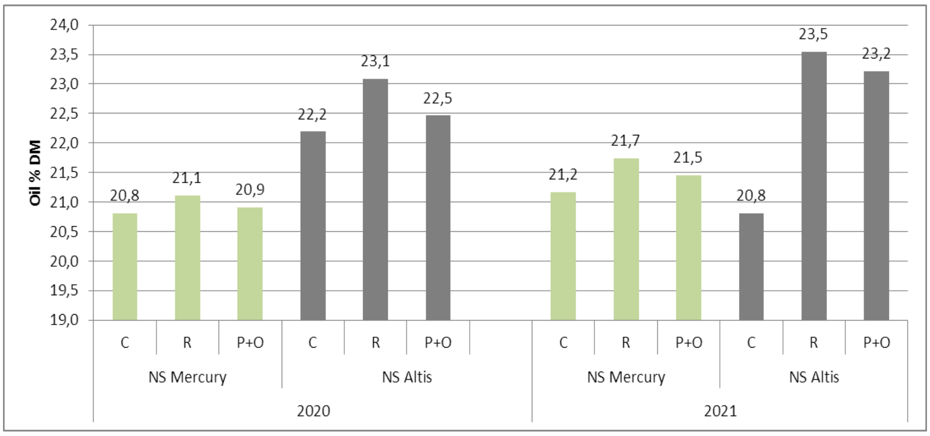

**Fig. 12:** Oil (DM) in organic production in 2020 and 2021.

For the testing of seed multiplication two varieties were selected according to the end users, one variety that has high protein content (up to 45%) NS Mercury and one that has higher oil content (22% DM) NS Altis, which was confirmed in organic certified production conditions of this trial (Fig.12).

*Total phenols and flavonoids*

Phenols and flavonoids are secondary metabolites found in various plants, including soybean which have antioxidant properties. It is mostly unknown why particular flavonoids are produced and accumulate in various plant tissues after being stimulated either internally or externally (Shah and Smith, 2020). In some plant-microbe interactions in the rhizosphere, flavonoids are particularly effective at reducing biotic and abiotic stressors (Cetinkaya et al., 2017). Within trials (2020-2022) in RS total phenols and flavonoids were determined in soybean grains. According to gathered results, statistical differences were noted only for production year (Fig.13 & 14).

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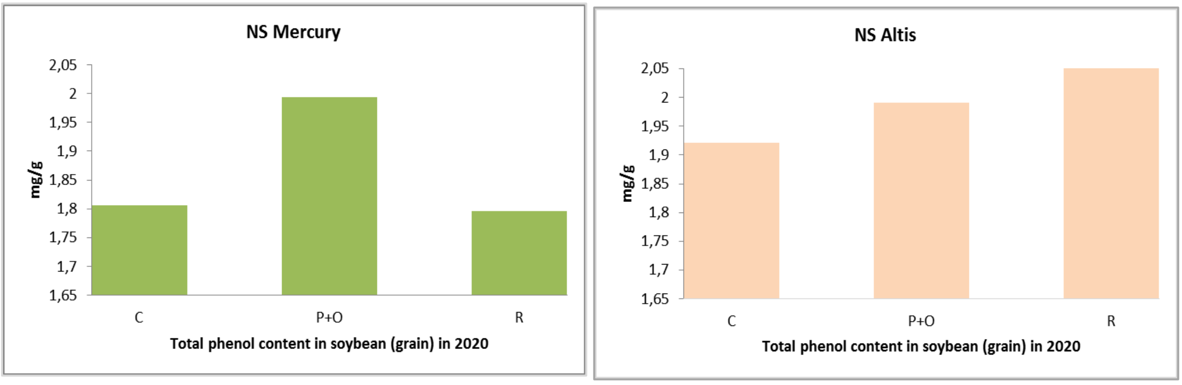

**Fig. 13 &14:** Total phenols in soybean seed in 2020.

### 2.3.3 Soybean seed health, weed screening and soil properties

*Soybean seed health screening*

The health of soybean plants was evaluated in the field following the use of cover crops. But also, screening of soybean seed health is particularly important during the seed multiplication process. The health status of seed is a key pre-requisite for gaining quality seed. The following fungal pathogens were identified: *Peronospora manshurica, Diaporthe phaseolorum*, *Alternaria* sp., *Fusarium* sp., *Cercospora* sp. and *Botrytis* sp. Statistical difference was not found during 2020 and 2021 on appearance of soybean seed causal agents (Fig. 15). In contrast, in 2022, a significant drought caused considerable challenges in the seed multiplication process, resulting in as much as a 50% infection rate among the screened seeds. The effect of cover crop, system of production and variety had no effect on seed health parameters, only production year. The connection between adverse weather conditions, particularly high temperature appeared to be negatively influencing the quality of the multiplied seeds.

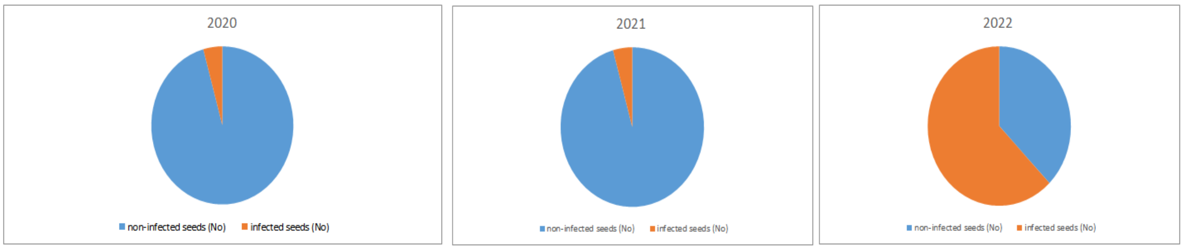

**Fig. 15:** Number of soybean infected and non-infected seeds in 2020, 2021 and 2022.

*Soybean weed screening in cover crops*

Dense canopies of cover crops shade out weeds, reducing competition from subsequent soybean plants. Some cover crops (rye, oat, buckwheat) also release allelopathic chemicals that inhibit weed growth. The numbers of weed species that appeared at the site Rimski šančevi (RS) was up to 3, while in Čurug (RS), up to 5 were recorded. Weed assessment was carried out in both the cover crop and control plots.

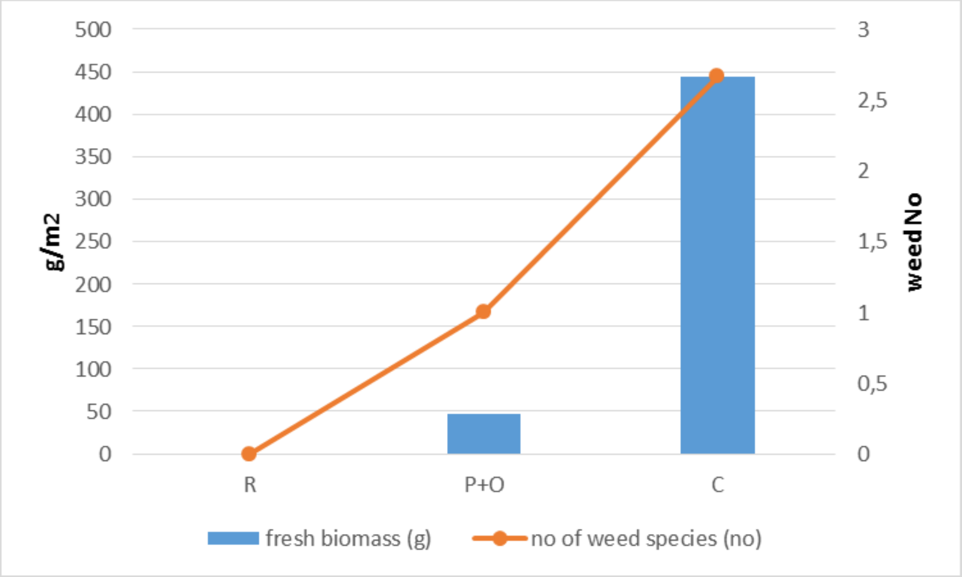

**Fig. 16:** Weed screening in low input in 2022.

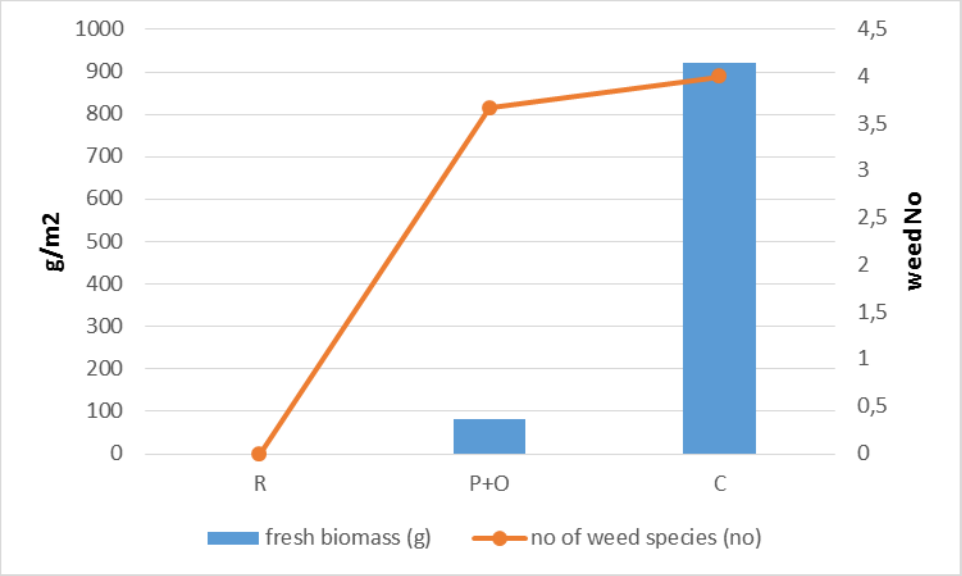

**Fig. 17**: Weed screening in organic production in 2022.

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When it comes to the cover crop with rye (R), no weed species were identified, because rye is a strong competitor. When it comes to the cover crop, the P+O crop was moderately competitive with weed species, while the highest number of weed species was recorded in the control plot. Given that weeds use nutrients from the soil, the pressure of weed plants in organic and low-input production can be reduced by cover crop sowing. Weed pressure can be very high in low-input and organic production and with the introduction of cover crops the pressure can be decreased which is important during seed multiplication process, since one of the limiting factors in organic production are weed seed-banks.

*Soil properties*

Winter cover crops can have a profound impact on both the chemical and physical properties of the soil in soybean crop rotations. They have potential to contribute to soil fertility, organic matter content, erosion prevention, weed and pest management, disease suppression, to decrease soil compaction, and increase microbial activity and soil health. Winter cover crops play a crucial role in improving soil health and overall soybean production which is vital for the improvement of seed multiplication practices. Cover crops can enhance soil fertility by capturing and recycling nutrients from deeper soil layers, preventing leaching. Favourable physical properties of the soil and aeration in the root zone can help ensure balanced growth and development of the following crops. High mechanical resistance of the soil is one of the physical properties that come to the fore in dry conditions, because the specific resistance of the soil increases markedly by reducing the moisture content. This can have a direct impact on the limited development of the root system and the further growth of plants, along with the reduced uptake of nutrients from the soil, which also results in a reduction in the productivity or yield of the plant species of interest.

Cover crops did not show significance effects on soil bulk density. In the arable layer, the lowest volumetric mass was determined for the P+O treatment (1.31 g $cm^{-3}$). Also, the decrease in soil bulk density was determined in all years on the treatment that contained legumes and small grain as a cover crop. Soil compaction, measured by Penetrologger (Fig. 18) at both locations, differed when comparing depths, years, types of cover crop and soybean variety. In all studied years, the lowest cone index was recorded in the cover crop that contained legumes. The soil at a depth of 0-20 cm on which the P+O cover crop was planted had the lowest specific soil resistance, while no differences were observed between soybean varieties. It was found that the treatment with winter peas and oats (P+O) had the least impact on soil compaction for both soybean varieties (resistance index 2.7), while the control plot (C) had the highest

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resistance index (2.9), and moisture content 19.5%. There are direct measures that can be implemented to improve the physical properties and reduce soil compaction. This leads us to the conclusion that by including cover crops in sustainable soybean production systems (low-input and organic production), specific soil resistance can be directly related to overall crop management and seed multiplication.

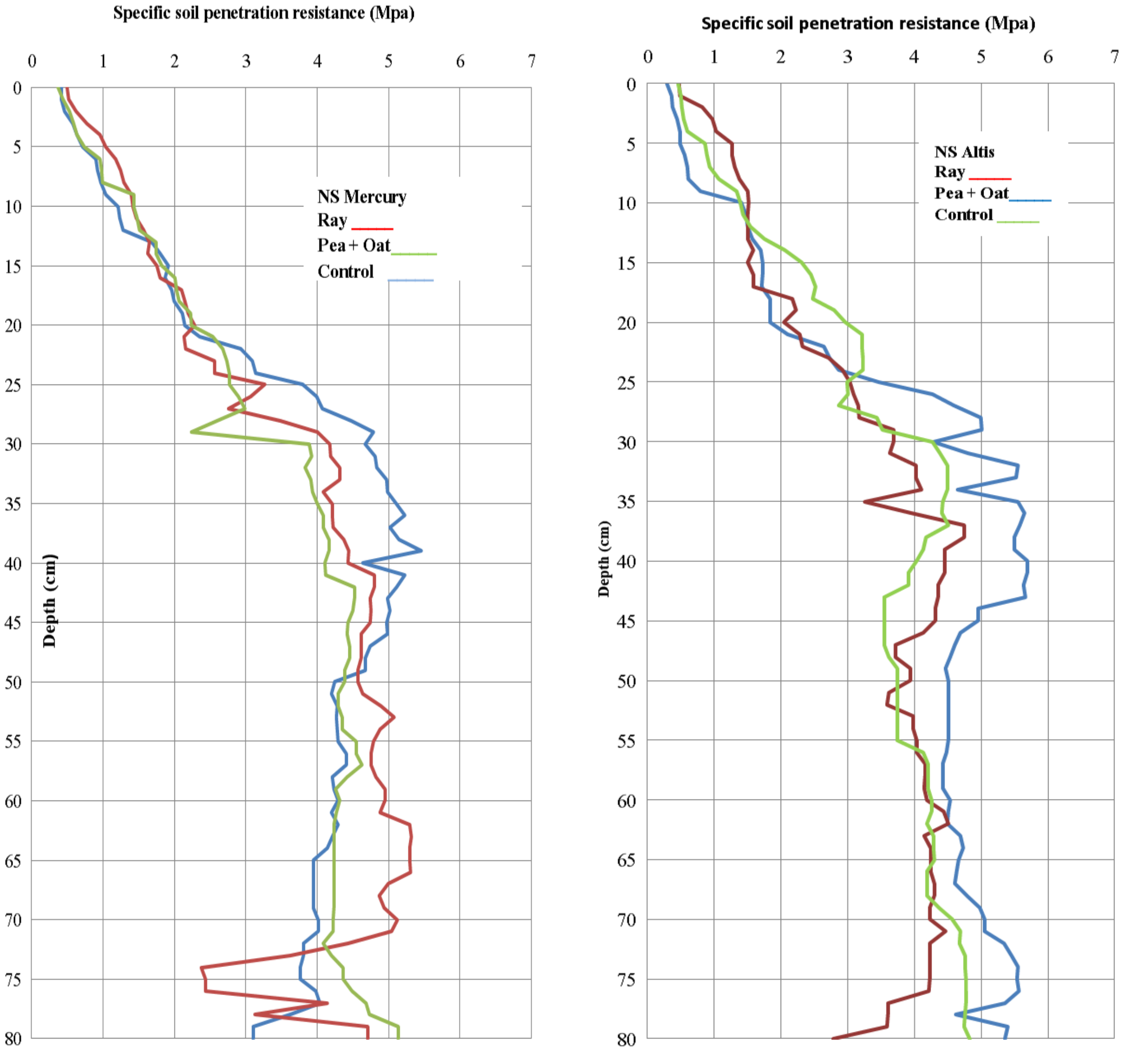

**Fig. 18:** Soil penetration resistance in low-input production.

**Agronomic practices that include the introduction of cover crops in soybean production could make a significant contribution to increasing the sustainability of agricultural production systems and to provide practical solutions that are profitable long-term investments in crop production.**

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# 3 Role of inoculants in organic soybean seed multiplication

What makes legumes unique in nature is their ability to biologically fix atmospheric nitrogen with the help of symbiosis with rhizobia. The result of this symbiosis is the formation of nodules on the roots of legumes, where, thanks to the enzyme nitrogenase, atmospheric nitrogen is reduced to ammonium ions, which further participate in the synthesis of organic compounds. The potential of symbiotic nitrogen fixation (SNF) varies depending on the plant species (variety), bacterial strain, their interaction, and numerous biotic and abiotic factors. The amounts of nitrogen fixed due to SNF in soybean range from zero to 337 kg N/ha of arable soil annually, depending on rhizobia activity (Ciampitti and Salvagiotti, 2018). The importance of legumes as a source of biological nitrogen is particularly evident in conditions of nitrogen deficiency and the impossibility of applying mineral N fertilisers, as is the case in organic production systems. In soils that are well supplied with nitrogen, mineral nitrogen fertilisers are not used in the production of legumes, while larger amounts of mineral nitrogen inhibit the process of symbiotic nitrogen fixation (Miladinović, 2012). The application of organic fertilisers in the production of legumes is also irrational from the aspect of efficiency and exploitation of the process of symbiotic nitrogen fixation because it can result in increased availability of mineral nitrogen in the soil following the mineralisation of organic matter (Marinković et al., 2014). Although there is not enough data on the nitrogen fixation potential of leguminous crops in organic production, it is known that the amounts of fixed nitrogen are lower compared to the conventional cultivation system, primarily due to the increased exposure of crops to the negative influence of biotic factors (weeds, insects, pathogenic microorganisms), as well as increased sensitivity to abiotic stress (e.g. drought) (Kebede, 2021). The low content of mineral nitrogen in organically managed soils increases the proportion of nitrogen taken up by plants from the atmosphere compared to that supplied from the soil, but the efficiency of nitrogen fixation is generally reduced. Also, there are differences depending on the type of crop, so the absolute value of nitrogen fixation is higher in forage compared to grain legumes due to differences in biomass production (Barbieri et al., 2023).

Organic farming embraces biological systems of nutrient mobilisation and solubilisation, and favours the proliferation of beneficial microbes, including rhizobia, which in turn enhances soybean production through SNF and other plant growth promoting traits (Gitonga et al. 2021). These facts indicate the necessity of applying agro-technical measures that will enable more efficient use of symbiotic nitrogen fixation in organic production, which is achieved by using inoculants based on nitrogen-fixing bacteria. By improving the cultivation technology of the most important leguminous crops, both in organic and conventional production, through the introduction and application of specially selected nitrogen-fixing bacteria for specific plant species and growing conditions, the preservation of the environment and soil fertility is ensured while achieving more stable and better yields.

Inoculation is the key to unlocking the full potential of soybean seed multiplication. By introducing beneficial rhizobia bacteria to soybean seeds, a symbiotic bond is formed that enhances nitrogen fixation, improves plant health, increases seed yield, and ensures the production of high-quality seeds.

## 3.1 Seed inoculation

Seed inoculation with the appropriate rhizobia strains is a required production technology, especially where soybean or a related species has not been grown previously (Murphy-Bokern et al. 2017). The main goal of this practice is to provoke symbiotic nitrogen fixation (SNF) to provide nitrogenous nutrients to soybeans and subsequent crops, leading to an increase in plant nutrition and plant growth, as well as yield and grain quality, with a decrease in the use of chemical fertilisers and the latter environmental impact. Intensive soil usage, as well as excessive application of mineral fertilisers and other agrochemicals, leads to a decline in soil fertility, soil erosion and ground water pollution and alter the structure and activity of soil microbial communities (Girvan et al., 2004). To minimise negative impacts of intensive agriculture, organic farming has been proposed as a sustainable agricultural practice with the main principles of the ecological cycle and biodiversity. The incorporation of organic amendments improves soil properties, including biomass, activity and diversity of soil microorganisms. Moreover, inoculation is recognised as an irreplaceable agronomic practice in legume production in organic crop systems and as alternative for chemical fertilisers in intensive agriculture management. In addition to SNF, rhizobia are able to stimulate plant growth via phosphate solubilisation, production of plant hormones and siderophores, or enzymatic activities.

The efficiency of soybean nodulating rhizobia is related to the crop genotype, geographical location, soil conditions including pH, temperature, moisture, available nitrogen, phosphorus, organic matter, etc. The persistence of rhizobial strains in the soil also depends on a native rhizobial population, the history of soybean cultivation and the use of inoculants. The inoculation strains may lose their competitiveness and effectiveness under the fluctuations of numerous abiotic and biotic factors in the outer environment. New approaches that highlight nitrogen fixation efficiency, along with competitiveness for nodulation and adaptability, must be considered as selection criteria for improving inoculant effects.

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Soil microorganisms are the main drivers of soil ecosystem functioning, including the organic matter decomposition, transformation and mineralisation, and nutrients cycling. Soil microbes are also involved, either directly or indirectly in many other processes which provide stability to the soil ecosystem. The diversity of species and functional microbial groups, and their activity are important indicators of soil health and fertility. In the concept of sustainable farming, soil microbiological aspects are extremely important; however, they are often underestimated or completely disregarded. The soil-resident microbial communities are sensitive to exogenous disturbances due to different biotic and abiotic influences including anthropogenic activities. The preservation of soil microbial diversity is crucial for a balanced agro-ecosystem, especially under increasing agricultural intensification. Different management practices, particularly in the soils with long agricultural history, might be the reason why these soils are microbiologically deficient compared to natural undisturbed soils (Głodowska and Wozniak, 2019). Conventional agriculture relies on excessive use of synthetic fertilisers and pesticides, which cause adverse impacts on soil ecosystems. However, they are also regarded as the primary factors which induce negative changes in soil microbial population, while shaping their biomass, diversity, and activity.. Due to its plant growth promoting properties and capacity to enhance nutrient availability and uptake, microbial inoculants are promising tools to mitigate the negative effect of mineral fertilisers on the environment as well as the sustainability of agriculture. Despite the benefits of plant growth, inoculation practices can potentially lead to changes in soil microbial communities, which are often neglected (Trabelsi and Mhamdi, 2013). Inoculants could promote positive modification in soil, and this potential is related to the direct effects of inoculants with specific functions, as well as indirect, cascading effects through plants and/or native microbial communities (Liu et al., 2022). Even though microbes in inoculants sometimes cannot compete efficiently with indigenous microbial populations, they stimulate root growth and modify plant metabolism at early stages and might generate long-term effects on associated microbial communities (Wang et al., 2021). However, it is believed that PGPM strains will perform better in a habitat similar to the one they were isolated from, since they are better adapted to specific environments. Within this research we have investigated the dynamics of the soil-resident microbial communities in response to rhizobial inoculation over the course of a growing season.

**Fig. 19:** Seed inoculation.

### 3.1.1 Material and methods

The effects of *Bradyrhizobium japonicum* strains on microbial soil properties, yield, yield-related traits, and grain quality were examined under low-input field conditions at the Rimski Šančevi experimental field (IFVCNS, Serbia) in three cropping seasons (2019-2021). In 2021, an additional treatment which included inoculation strains and micronutrients was tested. Two soybean varieties, NS Apolo ((I) and Rubin (II), were used in the 2019 and 2020 experiments, while only the variety NS Apolo (I) was tested in 2021. Liquid inoculum (109 CFUs/ml) was mixed with a carrier (sterile peat), and soybean seed inoculation was performed just before sowing. In 2021, the nutrient complex with the following composition was included, (% m/m): S – 5.2; Mg – 3; Mn – 1.5; Fe – 1; Zn – 1; Cu – 0.5; B – 0.3; Mo – 0.01. Soil samples for microbial analyses were collected randomly from the soybean rhizosphere at full bloom (R2) and full maturity (R8). Abundance of examined microbial groups (total number of bacteria, number of azotobacters, free-living $N_2$ fixing bacteria, ammonifiers, fungi, actinomycetes) was assessed by the indirect dilution method followed by plating of soil suspension on selective nutritive media. Dehydrogenase activity (DHA) (EC 1.1.1.) was determined spectrophotometrically by measuring the extinction of coloured triphenylformazan (TPF) at 485 nm (Casida et al., 1964). The data were statistically processed (StatSoft Inc., Tulsa, USA) using analysis of variance (ANOVA), followed by mean separation according to the Fisher's LSD test ($P < 0.05$).

### 3.1.2 Results

Whereas the mechanisms underlying plant-inoculant interactions are well studied, microbial inoculants effects on indigenous soil microbial communities, including their interactions, have received little attention. Introduction of microorganisms into the soil environment sometimes has undesirable ecological effects; therefore, it is important that their environmental effects are previously assessed. Rhizobia are reported to influence crop growth, yield, and nutrient uptake by different mechanisms which have an important role in seed multiplication. They fix nitrogen, increase the supply of other nutrients, produce plant hormones, help in promoting free-living nitrogen-fixing bacteria, enhance other beneficial bacteria or fungi, and control bacterial and fungal diseases (Trabelsi and Mhamdi, 2013). Although we understand little about the degree to which genetic and taxonomic microbial diversity affects functional ecosystem properties, it is accepted that higher-diversity ecosystems are frequently associated with soil fertility (Ambrosin et al., 2016). Impact of bacterial inoculation on agricultural systems is still unknown and varies according to the location, soil type, plant species and microorganisms introduced.

**Table 1:** Microbial abundance and activity in 2019.

| Treatment | Variety | *Azotobacter* spp. × 10 | Ammonifiers × $10^6$ | Total bacteria × $10^7$ | Free $N_2$-fixers × $10^6$ | Fungi × $10^4$ | Actinomycetes × $10^4$ | Dehydrogenase activity |
|---|---|---|---|---|---|---|---|---|
| | | CFU/g soil | | | | | | (µg TPF/g soil) |
| | | | | Full bloom stage (R2) | | | | |
| Control | NS Apolo (I) | 118 b | 55 b | 221 a | 140 a | 14 a | 8 b | 15.83 bc |
| *B. japonicum* | NS Apolo (I) | 202 a | 109 a | 323 a | 160 a | 12 a | 22 a | 33.91 a |
| Control | Rubin (II) | 142 ab | 31 b | 197 a | 135 a | 12 a | 7 b | 13.76 c |
| *B. japonicum* | Rubin (II) | 148 ab | 98 a | 311 a | 173 a | 9 a | 17 ab | 17.51 b |
| | | | | Maturity stage (R8) | | | | |
| Control | NS Apolo (I) | 99 b | 99 a | 213 a | 187 bc | 14 a | 12 a | 16.99 bc |
| *B. japonicum* | NS Apolo (I) | 125 ab | 112 a | 189 a | 257 ab | 9 a | 13 a | 32.79 a |
| Control | Rubin (II) | 144 a | 59 a | 151 a | 114 c | 11 a | 17 a | 14.71 c |
| *B. japonicum* | Rubin (II) | 151 a | 45 a | 183 a | 287 a | 11 a | 11 a | 24.75 ab |

The different letter indicates a significant difference at $P < 0.05$. CFU – colony-forming unit

Soil analyses performed during the flowering and maturity stages in 2019, indicate significant increase in abundance of *Azotobacter* spp. (71%), ammonifiers (98% and 216%), free $N_2$-fixing bacteria (152%) and actinobacteria (175% and 143%), following *B. japonicum* seed treatment. The significant beneficial effect of rhizobia inoculation on dehydrogenase activity (27-144%) was also recorded, unlike the total bacterial population and fungal abundance, which were not affected (Table 1).

**Table 2:** Microbial abundance and activity in 2020.

| Treatment | Variety | *Azotobacter* spp. × 10 | Ammoni-fiers × $10^6$ | Total bacteria × $10^7$ | Free $N_2$-fixers × $10^6$ | Fungi × $10^4$ | Actino-mycetes × $10^4$ | Dehydrogenase activity |
|---|---|---|---|---|---|---|---|---|
|  |  | CFU/g soil |  |  |  |  |  | (µg TPF/g$^{-}$ soil) |
| Full bloom stage (R2) |  |  |  |  |  |  |  |  |
| Control | NS Apolo (I) | 136 ab | 90 a | 185 b | 138 a | 14 a | 9 a | 8.47 a |
| *B. japonicum* | NS Apolo (I) | 142 ab | 87 a | 248 ab | 190 a | 14 a | 11 a | 6.27 a |
| Control | Rubin (II) | 99 b | 137 a | 353 a | 234 a | 12 a | 9 a | 8.02 a |
| *B. japonicum* | Rubin (II) | 155 a | 128 a | 283 ab | 312 a | 19 a | 12 a | 15.6 a |
| Maturity stage (R8) |  |  |  |  |  |  |  |  |
| Control | NS Apolo (I) | 126 a | 206 a | 366 a | 251 a | 52 a | 31 a | 9.20 a |
| *B. japonicum* | NS Apolo (I) | 159 a | 224 a | 369 a | 241 a | 54 a | 22 a | 13.66 a |
| Control | Rubin (II) | 65 b | 215 a | 403 a | 304 a | 37 a | 24 a | 11.48 a |
| *B. japonicum* | Rubin (II) | 118 ab | 179 a | 393 a | 276 a | 37 a | 18 a | 10.33 a |

The different letter indicates a significant difference at $P < 0.05$. CFU – colony-forming unit

In the second year (2020), the only significant change in microbial community structure and activity due to inoculation was found in the abundance of *Azotobacter* spp. (56%) (Table 2). Bacterial inoculation efficiency is associated with the beneficial features of the inoculated bacterium, as well as with the complex network of interactions occurring in the soil (Ambrosini et al., 2016). The influence of bacterial inoculants on native microbial communities depend on numerous abiotic and biotic factors, considering that indigenous populations show higher resilience under specific environmental conditions.

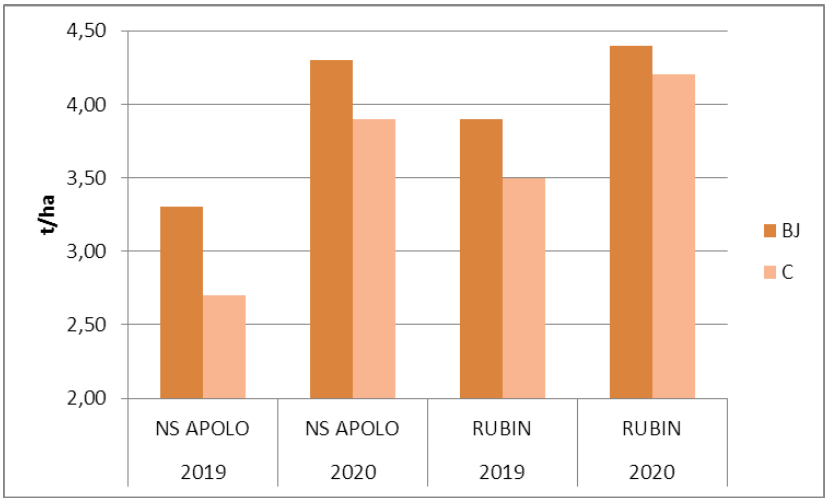

**Fig. 20:** Yield of soybean (t/ha) during 2019 and 2020 as influenced by variety and following inoculation with *Bradyrhizobium japonicum* (BJ) in comparison with a control treatment (C)).

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**Table 3.** Microbial abundance and activity in 2021.

| Treatment | *Azotobacter* spp. × 10 | Ammonifiers × $10^6$ | Total bacteria × $10^7$ | Free $N_2$-fixers × $10^6$ | Fungi × $10^4$ | Actinomycetes × $10^4$ | Dehydrogenase activity |
|---|---|---|---|---|---|---|---|
| | CFU/g soil | | | | | | (µg TPF/g soil) |
| **Full bloom stage (R2)** | | | | | | | |
| Control | 12 b | 53 b | 41 a | 37 b | 15 a | 3 a | 4.31 a |
| *B. japonicum* | 44 ab | 131 a | 87 a | 137 a | 18 a | 7 a | 6.88 a |
| *B. japonicum + nutrients (% m/m): S – 5.2; Mg – 3; Mn – 1.5; Fe – 1; Zn – 1; Cu – 0.5; B – 0.3; Mo – 0.01.)* | 86 a | 153 a | 75 a | 141 a | 12 a | 8 a | 8.71 a |
| **Maturity stage (R8)** | | | | | | | |
| Control | 34 b | 248 a | 399 a | 186 a | 26 a | 27 b | 5.74 b |
| *B. japonicum* | 33 b | 200 a | 481 a | 217 a | 26 a | 39 ab | 7.04 ab |
| *B. japonicum + nutrients (% m/m): S – 5.2; Mg – 3; Mn – 1.5; Fe – 1; Zn – 1; Cu – 0.5; B – 0.3; Mo – 0.01.)* | 91 a | 206 a | 592 a | 249 a | 74 a | 47 a | 11.04 a |

The different letter indicates a significant difference at $P < 0.05$. CFU – colony-forming unit

Both applied treatments in 2021, had a positive effect on the microbial soil properties, and the abundance of different bacterial communities were significantly enhanced compared to the control. More significant proliferation of azotobacter (617% and 167%), ammonifiers (189%), free $N_2$-fixers (281%), actinomycetes (74%) and dehydrogenase activity (92%) was obtained by applying inoculants with nutrient complex. However, total bacterial and fungal community abundance were similar independent of the treatments applied (Table 3).

Results of the three-year study showed that over the course of a growing season, a more significant impact of inoculation was recorded at the full bloom phase. The results also indicate that bacterial communities were more sensitive to changes induced by inoculation compared to fungal communities, tending to significantly increase in abundance following inoculation. Fundamental divergences between bacterial and fungal ecology may have a part in this difference (Cornell et al., 2021). The analyses of key bacterial groups related to the N-cycle (azotobacter, ammonifiers, and free $N_2$-fixers) showed significant abundance increments due to inoculation. These outcomes can also facilitate understanding of how specific microbial functional groups are impacted by rhizobia introduction. More research is needed to understand interactions among inoculant establishment, and interactions with resident soil microbial communities. Long-term experiments could provide more details about the influence of rhizobia inoculation on soil microbiome structure and soil functionality.

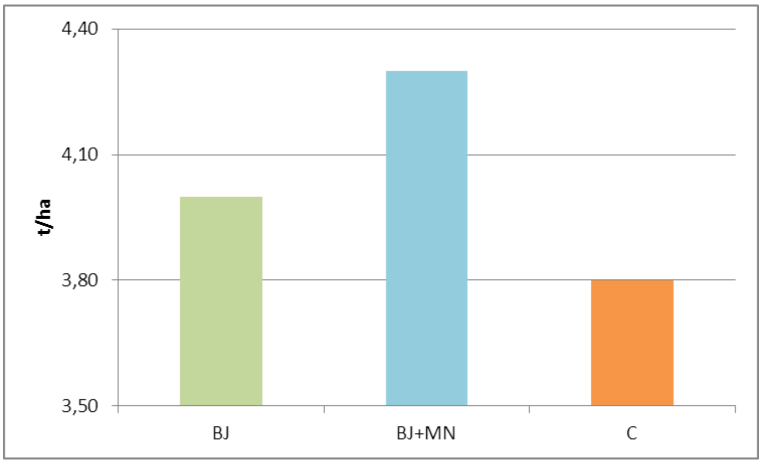

**Fig. 21:** Yield of soybean (t/ha) during 2021 folowing inoculation with *B. japonicum* (BJ), *B. japonicum* (BJ) plus Micronutrients (MN) compared with a Control (C)).

To maximise the positive effects of *Bradyrhizobium japonicum* on soybean yield (Fig. 21), it is essential for farmers to ensure that the right *Bradyrhizobium* strain is present in their soil, as the effectiveness of nitrogen fixation can vary depending on the specific strain and environmental conditions. Seed multiplication involves selecting and producing seeds from soybean plants with these desirable traits. Therefore, the quality and genetic potential of the seeds used for planting directly affect the yield of the resulting soybean crop. The integration of high-quality seeds from the seed multiplication process with the use of appropriate inoculants can enhance soybean yield. When high-quality seeds are used with effective inoculants, soybean plants are more likely to have vigorous growth, healthier root systems, and improved stress tolerance. This translates into increased yield potential. This cost-effectiveness can contribute to the economic viability of soybean farming. In summary, the relationship among seed multiplication, yield, and the use of inoculants is interconnected and crucial for successful soybean cultivation. High-quality seeds, produced through multiplication, provide the genetic foundation for optimal yield potential. When combined with effective inoculants, soybean plants are better equipped to utilise available nitrogen resources, leading to increased yields and potentially more sustainable and cost-effective farming practices.

## 3.2 Bio-priming

Organic production requires a wide selection of locally adapted organic varieties with high-quality seeds and tolerance to diverse abiotic and biotic stresses. In the context of seed multiplication within low-input and organic agriculture, the lack of organic certified seeds and insufficient testing of new or promising varieties under specific climatic conditions can hinder the successful expansion and sustainability of organic farming

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practices. Problems with soybean seed quality occur during seed production, multiplication, transport, processing, and storage. Soybean seeds are highly susceptible to adverse environmental conditions, numerous pests and diseases, seed oil and moisture content, mechanical injuries/damage, air temperature and relative air humidity (Sharma et al. 2013).

Benefits:

- Better microbial adherence/adaptation to the seeds, improved microbial colonisation of rhizosphere, and increased microbial number/diversity in the soil.
- Improved seed germination and vigor, increased plant growth and grain yield, and enhanced crop and seed quality.
- Promoted seed protection, improved plant defense against biotic stresses, and enhanced crop and soil health.
- Improved plant resilience against environmental stresses such as drought, low and high temperature, salinity, etc.
- Improved soil properties, enhanced nutrient availability, and better plant nutrition.

### 3.2.1 Material and methods

The effect of seed bio-priming with *Bradyrhizobium japonicum* strains on soybean seed quality was assessed in germination and cold tests (Hampton and TeKrony, 1995; ISTA, 2020). In short, seeds of two soybean varieties (NS Altis (0) and NS Teona (00) (IFVC selection) were sterilised, soaked in bacterial suspension ($10^9$ CFUs/g) for 5 h, rinsed and dried. In the germination test, seeds (4 × 100) were sown in sterilised moist sand and placed into a germination chamber at 25°C, with an illumination cycle of 12h of light and 12h of darkness for 8 days. In the cold test, seeds (4 × 50) were sown in a mixture of soil and sand (3:1), exposed to the low temperature of 10°C for 7 days and then transferred to a germination chamber at 25°C for 6 days.

### 3.2.2 Results

The findings of the germination test can be used to predict field emergence under optimal conditions of temperature, moisture, and light i.e. the maximum potential of a seed lot. However, the conditions for germination in the field are often less optimal or even adverse, when seeds may face certain abiotic and biotic stresses. Seed vigour tested in a cold test describes the potential of a seed lot to mitigate abiotic stresses such as low temperature. Overall, seed bio-priming with *Bradyrhizobium japonicum* improved most investigated soybean parameters when compared to non-primed seeds, both in optimal and stressful conditions. A significant effect of the seed bio-priming treatment was observed in shoot length, shoot dry weight, root dry weight, and seedling

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vigour index of normal seeds; final germination, shoot length, root length, shoot dry weight, root dry weight, and seedling vigour index of cold treated seeds. The varieties significantly altered root length and seedling vigour index in both tests, in addition to shoot length in optimal conditions. Moreover, variety × treatment had a significant effect on final germination, shoot length and seedling vigour index in the germination test; shoot length, root length, and seedling vigour index in the cold test. A different response of varieties to seed bio-priming, depending on test conditions, implies the possibility of improving seed germination and seedling growth of soybean by selecting the appropriate treatment for each variety and according to specific environmental conditions. This pre-sowing seed technique can be recommended for priming seeds prior to field sowing as an environmentally friendly strategy to improve seed germination and initial seedling growth for organic and low-input production systems.

**Table 4.** Germination and cold test.

| **Germination test** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Variety | Treatment | **FG (%)** | **AS (%)** | **SL (mm)** | **RL (mm)** | **SDW (g)** | **RDW (g)** | **SVI** |
| NS Altis (0) | Control | 85.25 b | 9.75 a | 118.83 bc | 132.88 b | 0.765 b | 0.114 c | 2145.13 b |
| | Bio-priming | 89.25 a | 8.00 a | 132.18 a | 137.88 b | 0.957 a | 0.186 a | 2410.82 a |
| NS Teona | Control | 89.25 a | 8.00 a | 118.00 c | 156.38 a | 0.884 ab | 0.128 bc | 2449.01 a |
| | Bio-priming | 88.00 ab | 8.50 a | 124.18 b | 156.25 a | 0.968 a | 0.169 ab | 2467.43 a |
| **Cold test** | | | | | | | | |
| NS Altis (0) | Control | 76.00 b | 18.75 a | 62.13 b | 67.13 d | 1.053 a | 0.049 b | 982.67 c |
| | Bio-priming | 81.25 ab | 13.25 b | 77.25 a | 92.88 b | 1.135 a | 0.077 a | 1382.42 b |
| Teona | Control | 78.00 b | 14.75 b | 57.13 b | 78.13 c | 1.061 a | 0.061 ab | 1054.30 c |
| | Bio-priming | 84.75 a | 10.75 c | 79.50 a | 110.13 a | 1.110 a | 0.078 a | 1606.81 a |

Final germination (FG), abnormal seedlings (AS), shoot length (SL), root length (RL), shoot dry weight (SDW), root dry weight (RDW), seedling vigour index (SVI).

Differences between treatments were analysed using Tukey's HSD test ($p \leq 0.05$). Different letters within a column indicate statistically significant differences of applied bacterial treatments. Both germination and cold test of soybean is a crucial step in assessing the quality of soybean seeds before sowing. It helps growers and seed producers make informed decisions about seed viability and quality, ensuring better crop establishment and production stability.

## 4 Conclusions

Inoculation and bio-priming are valuable tools for improving soybean seed multiplication. Both inoculation with *Bradyhrizobium* and cover cropping can create a synergistic effect on the multiplication of seed for low-input and organic production systems. The combination of improved nitrogen availability, reduced weed pressure, and enhanced soil health can lead to better overall soybean growth and, consequently, increased efficiency of seed multiplication. It is recommended to plan carefully crop rotation, cover crop selection, and to apply inoculation practices to optimise these benefits and to achieve sustainable and productive soybean seed multiplication in organic and low-input production systems. Additionally, local climate, soil conditions, and cultivation practices should be taken into account when implementing these practices.

**Fig. 22:** Illustration: Organic and Low-input Soybean Seed from Field to Fork.

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