DIVERSILIENCE - Diversifying Organic Crop Production to Increase Resilience

# D1.1 - Characterization of genetic variation for the development of diverse and resilient crops for the organic sector


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# Content

# Abstract of the development of diverse and resilient crops for the organic sector

During the past century, globalisation has been the major trend in most sectors of most societies, and this include agriculture. Agriculture has shifted from small scale production for local markets to large scale production for a global market.

In DIVERSILIENCE WP1, we have studied breeding initiatives of crops for organic production. As the state of the art of organic plant breeding differ according to crops and region, the starting point of organic plant breeding is very different depending on the crop and the logistic foundation of the breeding initiatives. In some situations, it is possible to stand on the shoulders of previous breeding initiatives such as modern varieties bred for conventional farming in the climatic zone, whereas in other situations, the breeding starts from scratch based on landraces.

Mixtures of grass and clover is often the basis of organic farming systems, and in particular in cold humid areas, the access to nitrogen from such crops is pivotal for the organic system. Hence, organic farming in Norway often suffers from lack of nitrogen, as legume crops often fails due to winter-kill. A broad collection of well-studied European lucerne material were evaluated in Norway. Based on the 3.5 years in the field it was concluded that the local varieties were better adapted to the climatic conditions, but that European material had a higher diversity of traits that could be useful in a long-term breeding program. The Norwegian breeding company Graminor has therefore collected survivor plants after 4 winters for further use.

Buckwheat is a traditional crop in several countries including Finland, but breeding of buckwheat has been limited compared to the major crops in agriculture. Plant breeding is normally focused on a single crop, but in organic farming, mixed cropping is preferred if possible to ensure nitrogen supply, weed competition and other benefits of crop diversity. However, breeding for diversity and mixed cropping is a new field of research, rarely exploited in practical breeding. To diversify crop production and maintain buckwheat production in Finland, Task 1.2 has studied the potential to select and adapt buckwheat varieties to Finnish conditions. The method of crop improvement was based on mass selection in a participatory approach. It is concluded that the optimal harvest time for buckwheat is rather flexible. Peas has a lower flexibility in optimal harvest time than

buckwheat, but in a pea-buckwheat mixture, it is possible to select a window for harvesting ripe seed of both crops. It is shown in much research, including DIVERSILIENCE WP3 that diversity and mixed cropping improve resilience. This means that a high yield can me expected in a monoculture of a well adapted variety under optimal conditions, but that mixed cropping decrease yield loss in cases of suboptimal conditions. This is also shown in the case of buckwheat-pea mixtures where the highest yielding varieties were best suited for monoculture production whereas lower yielding varieties benefited from mixed cropping with peas. Buckwheat is a cross pollinating crop with a very broad maturity and harvesting time. It seems that by harvesting early over three consecutive years, it is possible to select for early harvest and thereby gradually shift the crop to adapt local conditions.

White lupin (*Lupinus albus* L.) is grain legume crop of particular value for organic cultivation. It has high potential to produce novel foods or as a high-protein feed, owing to its protein content similar to that of soybean. A Composite Cross Population developed by CREA from a genetic base including progenies from 16 crosses between four elite sweet-seed breeding lines and four elite landraces of international origin, was submitted to an evolutionary-participatory breeding programme for cold and drought tolerance in Denmark (Agrologica) and different regions of Italy (RSR: Tuscany, Sicily, Sardinia). The resulting naturally adapted and selected populations displayed a yield increase relative to the original population after just two years of region-specific natural selection of the evolutionary population, along with a trend towards higher and rising content of alkaloids for the natural selection population, compared with elite inbred line material.

A procedure for the reliable evaluation of frost tolerance under artificial conditions was established and used to identify new breeding lines of white lupin with good frost tolerance. A good correlation between frost susceptibility in controlled conditions and winter mortality based on field data was observed. A GWAS analysis identified two SNPs that were associated with the frost survival ratio. Preliminary genomic prediction models were also developed; these exhibited prediction abilities close to 0.70. The work on frost tolerance of white lupin breeding lines identified potential new varietoes with greater frost tolerance, set up a procedure for the reliable evaluation of frost tolerant under artificial conditions, and produced key genetic information for the development of a genomic selection model for frost tolerance.

Wheat is generally an extremely well studied crop, but for organic farming, there are relevant traits that are rarely studied in conventional research.

Common bunt is a seed borne disease that has been effectively controlled by pesticides for the past century. Therefore, organic wheat production face considerable problem with this disease as organic farming abstain from use of pesticide seed treatments. In Denmark in cooperation with NARDI in Romania, wheat has been phenotypically and genotypically studied to search for genetic resources of resistance to common bunt and to develop genetic markers for selection. The research has mapped 21 RPR genes responsible for bunt resistance and have identified another 30 genes that can be used in breeding to control common bunt. Most of these genes have effect only against avirulent races of bunt, and virulence is present in Europe against most of these resistance genes. Only Bt9, the rye translocations 1RS:1AL and possible a few more genes seems to be effective against all races of bunt present in Europe. Therefore, it is in most cases necessary to pyramid several genes into wheat breeding lines to be able to control common bunt exclusively by resistance. The research also studied the effect of diversity, and it is concluded that a mixture of a high number of resistance genes in a population can reduce bunt infection even if virulence is present against the individual resistance genes in the mixture.

Common beans is an important crop for local production where diversity in colour, shape, taste and other traits differ in different places. This diversity cannot be maintained by European-wide commercial varieties leading to loss of local food cultures. An initiative in Slovenia and Bulgaria has therefore worked on selecting common bean varieties. The Task tested a collection of 25 common bean genotypes, differing on the basis of origin, and the varieties were described by 16 selected descriptors. Apart from selecting under field conditions, the material was characterised using genotyping, genomic and proteomic methods describing the genetic diversity and relevant traits. The Task identified potential accessions relevant for breeding for drought tolerance, protein concentration and resistance to three dominating plant diseases, including *Colletotrichum lindemuthianum*, *Pseudomonas savastanoi* pv. *phaseolicola*, and *Xanthomonas phaseoli* pv. *phaseoli*. The study manages to identify lines with tolerance to one, two or to all three pathogens and thereby improving the basis for further plant breeding of common bean relevant for organic plant breeding.

Cowpea has not been widely used in Europe, as it is a warm season crop. However, with increasing temperatures, cowpea could become an important crop and contribute to protein supply in Europe, given that adapted germplasm become available. A world wide collection of germplasm was studied in Italy for the potential in Southern Europe. As the climate conditions for successful cowpea production moves to the

north, an important trait is the photoperiod of cowpea, and the study therefore had this trait in focus. Growth habit was identified and grouped into idiotypes of erect, climbing and bushy types and maturity time was measured even some accessions did not mature at all under the Italian conditions.

Soybean is already established as a widely grown crop in Southern Europe, and adapted varieties are available. For both soybean and cowpea, the crops may have different purposes for either human consumption or feed production of either grain or biomass. Therefore, not a single growth habit or idiotype is optimal, as this depend on the purpose. Late maturing idiotypes with high biomass production may be suited for silage feed whereas early maturing types with lower biomass production may be better suited for grain production.

One of the side effects of the globalisation of seed production and plant breeding is the loss of local varieties and genetic diversity. To maintain local varieties, local varieties of maize was studied in Romania and characterised for yield and quality traits, and improvement was made using a participatory approach of on farm selection.

The project conclude that there is a big challenge for organic plant breeding in Europe. Many crops are in urgent need for improvement and adaptation to both biotic and abiotic stressors. It seems that the bigger the challenges, the easier the gain. Even whith a relatively short project period of only three years and growing seasons, it has been possible to achieve significant improvements in selected crops.

Different crops and different regions meets different challenges, and the relevant method for crop improvement differ according the to state of the art and available knowledge, funding and logistic. In some cases, local landraces can be maintained and improved by local farmers and in other cases genomic and proteomic research is needed to solve specific constrains for organic farming. The current research was spread over the entire range of plant breeding from farmer selection of minor crops such as buckwheat in Finland and open pollinated landraces of maize in Romania to genomic studies of the worlds most grown crops such a wheat a d soybean. In all cases, it was possible to gain significant improvement in germplasm and knowledge, facilitating organic farming. On this background, we conclude that plant breeding is a low hanging fruit to enable increased organic production in Europe. A significant improvement of organic farming is like to be achieve by further investment in organic plant breeding. We see a need for organic plant breeding at all levels of the breeding value chain to reduce the bottleneck of adapted germplasm fit for organic production.

# Introduction to the development of diverse and resilient crops for the organic sector

Plant breeding, and in particular plant breeding funded by a commercial royalty based funding system, focusses on crops and traits for large areas, whereas it is difficult to gain profit for investments made for breeding initiatives for niche production, including crops with a small production or special traits in common crops with a limited use in the wider production.

Organic farming have in general lower yield than conventional farming. Part of this is the abscence of or reduced inputs, including fertiliseres and pesticides, but some reduced yield and quality are due to lack of plant cultivars adapted to the organic farming conditions. Organic farming still covering just a few percent of European farming is a niche for which it is not profitable to fund a commercial breeding program, and in particular minor crops for organic farming are short on varieties with special traits needed in organic farming.

The DIVERCILIENCE project aims a studying the potential of diversity in organic plant production, including diversity within crops such as species mixtures (mixed cropping, WP3) and genetic diversity within a species (populations and variety mixtures, WP2). In the current workpackage, we study the potential to increase diversity between crops, with focus on development of varieties adapted to organic farming with traits to meet the special requirement of organic farming.

# Improvement of lucerne adaptation to a Nordic climate (Task 1.1)

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## Introduction to lucerne research (Task 1.1)

Lucerne (*Medicago sativa* L.) is one of the most important perennial forage legumes in temperate and Mediterranean regions of the world. It is productive and persistent and produce high quality forage. It is also very drought tolerant, and variation in winter hardiness exists. In the Nordic region, the use of lucerne is rather limited, even if there are varieties developed for the region by breeders. With warmer temperatures and the expected increase in early summer droughts, lucerne is a species that could be of increased interest in the future. It could help diversify the crop spectrum and be a complement to red and white clover which are currently the dominating forage legumes.

A dilemma that plant breeders often face, particularly when it comes to perennial crops, is how to handle trade-offs between desired traits. For example, genotypes with high growth rates and high yields are sometimes more susceptible to stress, and vice versa. Breeders will seek to develop varieties with an optimized combination of trait values. In perennial legumes there are, at least in some material, correlations among yield, stress tolerance and phenological characteristics. We used a broad population representing European (but not Nordic) elite material to investigate these associations in a Nordic cool climate with its characteristic strong annual variation in photoperiod. More specifically, we wanted to test if low persistence is associated with late autumn growth (lack of dormancy) or with early or late flowering. At the same time, we compared the behavior of this population with a small set of Norwegian varieties, to test if some of the European material could be of interest for Norwegian lucerne breeding.

## Materials and methods for lucerne research (Task 1.1)

Hundred and thirty-five half-sib families from the polycross of 10 lucerne varieties from central and southern Europe (Annicchiarico et al. 2016, in Roldán-Ruiz et al, Breeding in a World of Scarcity, Springer) were tested

under Nordic semi-organic conditions in replicate semi-dense mini-swards consisting of 25 plants per plot (2022-2024). Five Norwegian cultivars were included for comparison, and harvesting was conducted once in 2022 and two times per year in 2023 and 2024. Flowering time and autumn growth was recorded in all years. At each harvest, the number of surviving plants and dry matter yield was recorded. Phenotypic values were corrected for spatial variation using Legendre polynomials.

## Results and discussion on lucerne research (Task 1.1)

The Norwegian varieties tended to produce less biomass in the establishment year than the average of the European material but produced more than the average European material in 2023 and particularly in 2024 (Fig. 1). This may be related to the much better persistence and stronger autumn dormancy (autumn height) of the Norwegian varieties as compared to the average of the European material. Flowering time of the Norwegian varieties tended to be later in the establishment year, but earlier in the two following years, suggesting the possible presence of a stronger vernalization response in the Norwegian varieties. The European material was very variable, and for some traits this variation covered the variation of the Norwegian varieties.

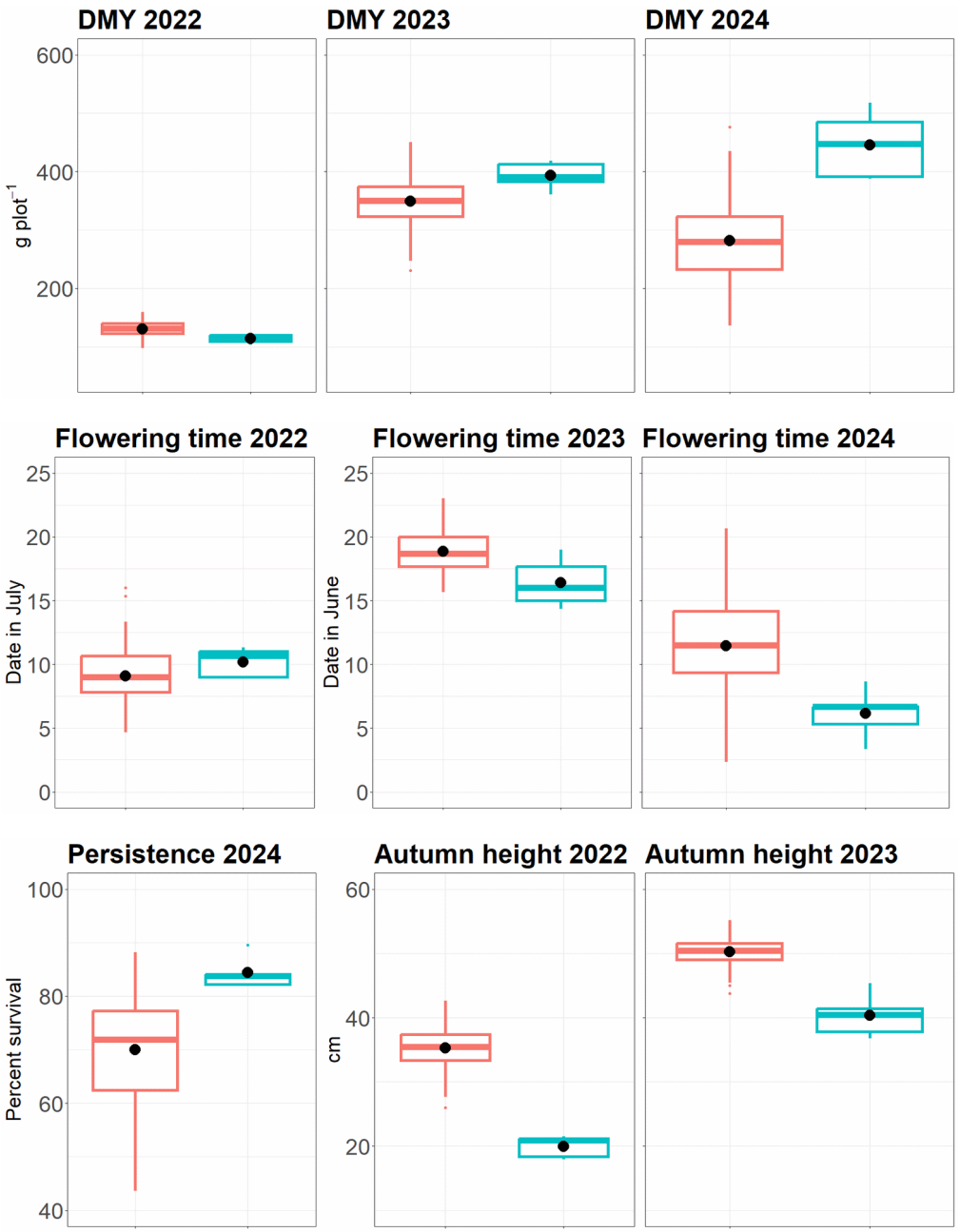
The strongest genetic correlations in the European material were found between persistence and dry matter yield in 2023 and 2024 (Table 1), suggesting that persistence was a trait that affected yield also within the European material. The association between autumn height and persistence and yield were complex, and more often positive than negative. Flowering time was weakly to moderately correlated among years, while associations between flowering time on one hand and dry matter yield, persistence and autumn height on the other, were weak and both positive and negative.

Using available high-throughput genotyping data of the maternal parents, we will estimate genetic parameters and develop genomic prediction models for the different traits. Genomic selection, based on such models, is a methodology that speeds up genetic progress in plant breeding, including forages. It is therefore an aim for most breeding companies to establish and exploit this methodology for the crops that they breed.

## Conclusions on lucerne research (Task 1.1)

The Norwegian varieties were more persistent and therefore gradually produced more annual biomass than the European material. The variation in the European material, however, was considerable. Following a winter,

Norwegian varieties flowered earlier than the European material on average, but again, the European material was variable. The Norwegian varieties had markedly less autumn growth. The mostly higher yield of Norwegian varieties in the final year suggests that this European material might not be so relevant for Norwegian lucerne breeding, although the best plants in families with comparable performance to the Norwegian varieties may harbor genetic variation that could be of value. Within the European material the associations between autumn growth, flowering time and persistence were complex.



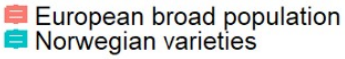



*Fig. 1. Comparison between 135 half-sib families from a polycross of a broad collection of European elite material and 5 Norwegian cultivars of lucerne. Plants were planted in semi-dense plots of 25 plants in 2022 and harvested once in 2022 and twice in both 2023 and 2024. Flowering date was recorded as the date in June when at least 10% of the plants in a plot had at least one open flower. Autumn height was measured approximately one month after the last harvest. Persistence was recorded as the percent of plants (out of the 25 in each plot) that survived up to May 2024.*

*Table 1. Genetic correlations (r) among traits of 135 half-sib families from a polycross of a broad collection of European elite material of lucerne at Ås, Norway. FT, flowering time; DMY, dry matter yield; AH, autumn height; Pers, persistence.* *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

| | FT 2022 | FT 2023 | FT 2024 | DMY 2022 | DMY 2023 | DMY 2024 | AH 2022 | AH 2023 |
|---|---|---|---|---|---|---|---|---|
| FT 2023 | 0.27** | | | | | | | |
| FT 2024 | 0.26** | 0.44** | | | | | | |
| DMY 2022 | -0.11 | 0.06 | 0.19* | | | | | |
| DMY 2023 | 0.21* | -0.18* | 0.02 | 0.30*** | | | | |
| DMY 2024 | 0.19* | -0.22* | -0.18* | -0.15 | 0.69*** | | | |
| AH 2022 | -0.02 | 0.1 | 0.07 | 0.64** | 0.14 | -0.20* | | |
| AH 2023 | 0.14 | -0.07 | -0.10 | 0.16 | 0.62*** | 0.51*** | 0.30*** | |
| Pers 2024 | 0.14 | -0.19* | -0.10 | -0.07 | 0.71*** | 0.73*** | -0.22* | 0.49*** |

# Farmer-participatory selection of buckwheat for pure and mixed cropping in Northern Europe (Task 1.2)

Luke: Marjo Keskitalo

## Materials & Methods for buckwheat research (Task 1.2)

The transformation of buckwheat into an early grower and a co-crop with peas was studied using three buckwheat strains in combination with peas, with the previous year's seed being used for the following year's sowings. Harvests were carried out at four points in time with the experimental drill, the first being at the end of August which was about a month earlier than the normal harvest. At each harvest, only the heaviest and most mature nuts were selected for the thresher and later sorted, allowing the thresher to make a mass selection. In buckwheat, the aim was to bring forward the ripening period by harvesting earlier and using the seed for the following year's sowing. For peas, it was hoped that adaptation would be improved by using seed from the mixtures for sowing the following year's mixtures. The hypotheses are strongly based on the adaptability of cross-pollinated buckwheat, which, if properly exploited, could be used by farmers to create more suitable seed themselves.

Three different strains of buckwheat were used: 'Anita' is a Belarusian variety of origin, which has been propagated in Luke since the 2000s. 'Keskinen' is a buckwheat strain that has been cultivated in Finland since the 1990s. 'Laihia' is a Finnish strain maintained by NordGen, which has been propagated at Luke since 2019 to provide sufficient seed for trials starting in 2022. For peas, farm saved seedof the variety 'Karita' was used.

Each strain was tested in 2022 and 2023 with the same set up in organic fields 1-2 km apart to prevent cross-pollination: the 'Anita' strain was tested in Lamminkylä; the 'Keskinen' strain in Ojais, Juha Maidel's field; the 'Laihia' strain was tested in Lintupaju. In the last year (2024), only the transformation of 'Anita' was tested, as the desired early harvesting of the other strains in 2023 could not be carried out due to weather conditions. The fields simply could not be threshed due to overcrowding. Thus, the seed was not exposed to the desired early harvesting.

In 2022 and 2023, three field trials were sown, with 12 experimental members and 4 one-offs, for a total of 48 test plots. For each harvest period, there was one test member of pure buckwheat, pure pea and buckwheat + pea. When grown alone, the crops were sown at the standard seed density (100%) and in mixtures the seed density was halved (50 + 50%). In 2022 and 2023, the field trial was sampled (25 x 50 cm area) before harvesting to determine the proportion of seed and other vegetation. From the harvested crop, yield (kg/ha) and seed weights were determined. Germination tests were carried out before sowing.

In 2024, the transformation of the 'Anita' strain was tested. There were 9 test members in the experiment, in four separate batches: buckwheat (early harvested seed from 2023); pea (early harvested seed from 2023); buckwheat + pea (early harvested seed from 2023); buckwheat (early harvested mixed seed from 2023); pea (early harvested mixed seed from 2023); buckwheat + pea (early harvested mixed seed from 2023); buckwheat (original 'Anita' strain); pea (original 'Karita'); buckwheat (original 'Anita') + pea (original 'Karita'). During the growing season, seedling densities were calculated, leaf area index was measured per grid (three row spacings, same row spacings each time) a total of four times. Growth samples were taken before harvest, from which length, brown and light nuts, other plant weight (leaves + stems) were determined. Yield (g/m2) and seed weights were determined from the crop.

## Results from buckwheat research (Task 1.2)

Surprisingly, the results for 2022 and 2023 show that the harvest window for buckwheat is even a month longer. For all three crops, the yields were remarkably similar, regardless of whether harvesting was done very early (19-20 August in 2022) or a month later (around 20 September in 2022). For peas, on the other hand, the two mid-week harvests tended to produce the best yields. For buckwheat, the fact that flowering and seed formation are spread over a long period, usually starting at the end of July and continuing for at least a month, is possibly a factor. The first brown nuts appear on the flower clusters about 4 to 5 weeks after the start of flowering. The formation of new emerging nuts over a long period of time appears to coincide with their stalking, as the crop did not improve as autumn progressed. In 2022, ’Anita’ produced an average of 2100 kg/ha, while the other strains produced an average of 500 and 1500 kg/ha ('Keskinen' and 'Laihia'). The average yield from the mixtures was 1000 kg/ha or slightly more (Figure 1).

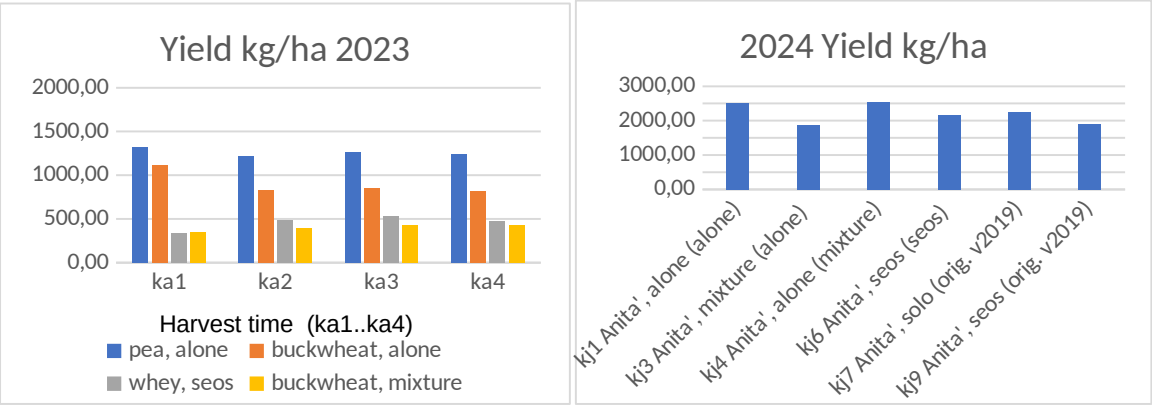



Figure 1: Yields (kg/ha) of buckwheat-pea pure and mixed (seed ratio 50:50) crops at different harvesting times (ka1-4), years (2022 and 2023) and on different stands ('Anita', 'Laihia', 'Keskinen'). Harvest dates in 2022 were 'Anita' (19.8, 25.8, 1.9, 12.9), 'Laihia' (20.8, 31.8, 12.9, 22.9) and 'Keskinen' (20.8, 31.8, 12.9, 22.9). In 2023, the harvest dates were 'Anita' (29.8, 6.9, 14.9, 21.9), 'Laihia' (29.8, 8.9; 14.9, 21.9) and 'Keskinen' (18.9, 25.9, 2.10, 9.10).

Buckwheat and pea benefited particularly in 2022 from mixed cropping, as LERs were >1 and at best close to 2.4 in all but the last harvest of the ’Anita’ strain. In 2023, LER values were lower and were >1 only at different harvest times for the 'Laihia' strain (Table 1).

Table 1: LERs of buckwheat-pea crops at different harvest times, different years and different stands. Harvest times reported in Figure 1.

| | **'Anita'** | | | **'Laihia'** | | | **'Keskinen'** | |
|---|---|---|---|---|---|---|---|---|
| **Harvest time** | **2022** | **2023** | | **2022** | **2023** | | **2022** | **2023** |
| **1** | 1,50 | 0,64 | | 1,80 | 0,66 | | 1,85 | 0,54 |
| **2** | 1,07 | 0,87 | | 1,37 | 1,20 | | 1,43 | 0,65 |
| **3** | 1,06 | 0,94 | | 1,26 | 1,05 | | 1,65 | 0,89 |
| **4** | 0,72 | 0,92 | | 1,37 | 1,31 | | 2,36 | 0,73 |

Atrial in 2024 with the 'Anita' strain showed that adapting buckwheat can have a beneficial effect on yield. Yields of buckwheat were 11-12% higher in a trial where the seed had been grown in the same field in two previous years (2022 and 2023) when compared to the original seed. On average, the best buckwheat yield was obtained from a buckwheat+pea mixture sown with buckwheat seed from the mixture. The yield of buckwheat was then 2535 kg/ha ('Anita' alone (mixture)), and almost as high

a yield was obtained from a pure crop of buckwheat (2501 kg/ha) sown with seed from an early harvested crop of buckwheat ('Anita' alone (alone)). The comparison was a buckwheat crop sown with the original 'Anita' ('Anita' alone (v2019 'Anita' original)) at 2266 kg/ha). Seed germination differences were accounted for at sowing and seedling density calculations showed similar seedling densities (Figure 2).

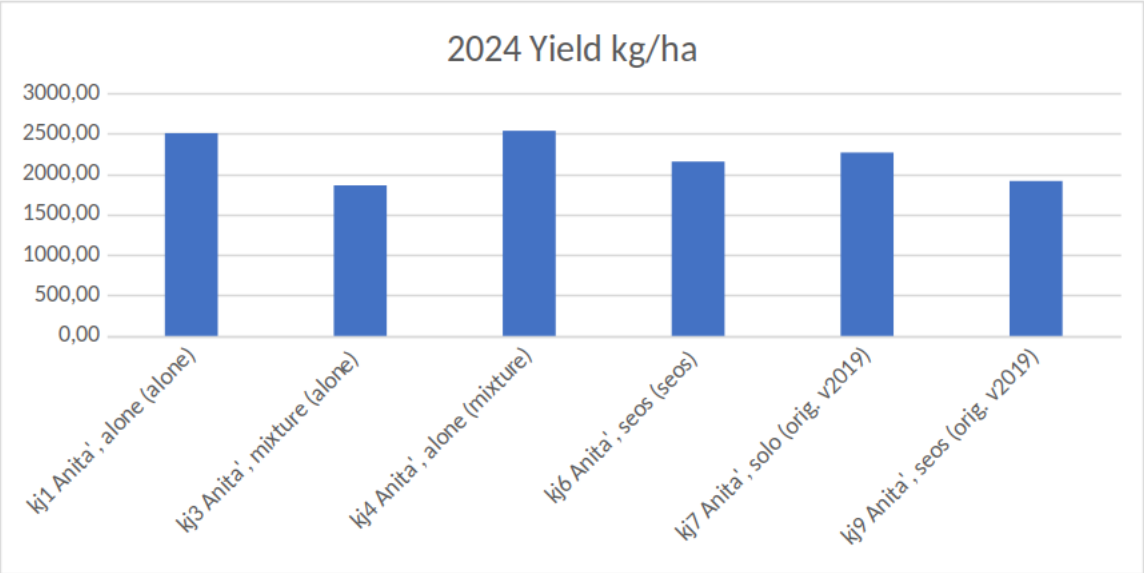



Figure 2: Yields of the 'Anita' buckwheat strain grown alone and in a mixture in 2024, testing differently produced seed. 'Kj' refers to the experimental member and only the buckwheat results are shown. The brackets indicate the system (alone or in mixture) in which seed was produced in 2022 and 2023 (all seed from harvest time1). In 2024, the 'Anita' strain was studied alone, i.e. as a pure crop, and in a mixture with peas. The comparison was made between pure stands (alone) sown with the original seeds and the mixture stands.

In the leaf area measurements, the first three measurements (8.7, 17.7 and 26.7) showed a slightly higher LAI for buckwheat sown with early harvest seed than for buckwheat sown with the original seed, while the last measurement (6.8.2024) showed a higher leaf area for buckwheat sown with the original Anita seed. This would suggest that, as expected, bringing the harvest date forward might also transfer the trait to the seeded seed, resulting in a faster development of the plant and leaf area than the original Anita strain. In addition, the early stand would in turn mature faster than the original Anita stand (Figure 3).

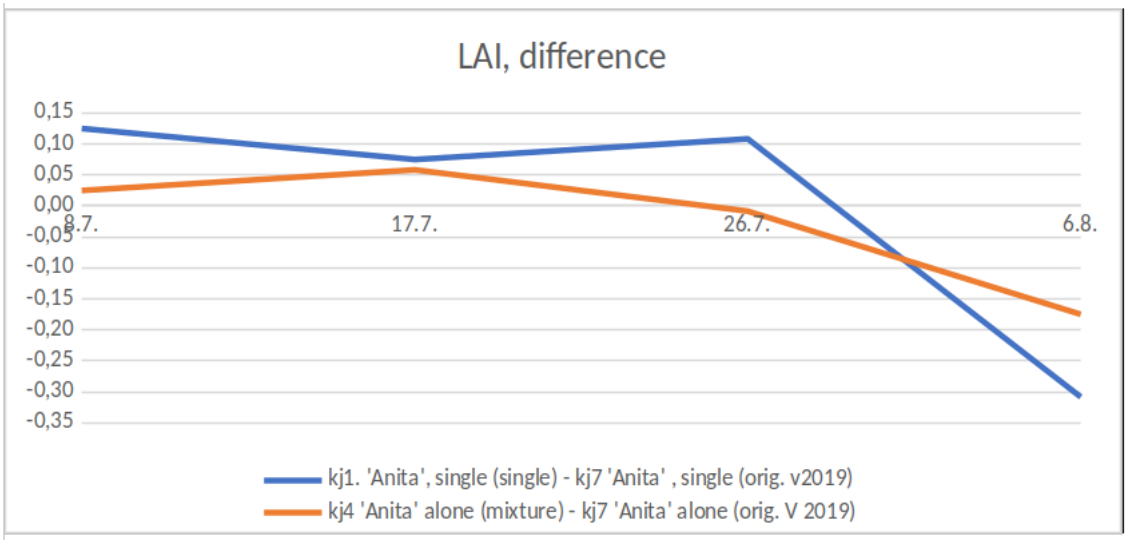



Figure 3: Difference in leaf area index between the two 'Anita' strains (kj1 and kj4) studied in 2024 and the leaf area of the original seed stock (kj7). The difference between kj1 and kj7 measured on 8 July and 26 July 2024 was 0.13 and 0.11 units, respectively, a difference of 6% and 2% from the leaf area index of the original seed-sown crop. In the last measurement (6 August 2024), the difference was about 0.30 units, which differs by 6.4% from the original crop.

## Risks regarding for buckwheat research (Task 1.2)

A study on the adaptability of buckwheat at the Natural Resources Institute would have required that the seed had been exposed to early harvesting for at least three years. Now only two years were used, with the final year being used to investigate the role of treatments. In addition, two strains were excluded from the final year of testing because early harvesting in 2023 was not successful due to weather conditions and the seed did not receive the desired treatment at that time. In this type of study, weather factors are so important that projects should be longer than three years.

## Discussion of for buckwheat research (Task 1.2)

### Importance in practice

Along with many other speciality crops, buckwheat is a crop for which there is no variety available in Finland. As a result, plant breeding will not be initiated in our country or in the neighbouring regions. Nevertheless, buckwheat has significant potential for the production of gluten-free raw materials, for the promotion of field diversity and for the balanced nutritional management of the land. Buckwheat is one of the few crops that produces nectar and pollen for pollinating insects and contributes to improving their living conditions. The demand for gluten-free raw materials is steadily increasing and although oats are an important source of raw material, other gluten-free crops are also needed in crop rotations. In addition to

being gluten-free, the plant is rich in phenolic compounds and these are known to be transferred to honey via pollinators. The phenolic compounds in buckwheat are therefore the subject of much research. The project was able to at least partially prove the hypothesis of the transformability of buckwheat, which, if used correctly, can also be exploited to the benefit of the farmer. The project created a concept of how a farmer on his farm could systematically work to improve the seed material and also produced information on the length of the harvesting window for buckwheat. Buckwheat and pea intercropping proved to be a very suitable combination of crops and the different ways of nutrient uptake/uptake support each other: Buckwheat's ability to extract phosphorus with low solubility is being studied elsewhere in Luke, while pea fixes nitrogen in its root systems. Through coexistence, it is likely that at least one of the two crops will yield a crop.

## Scientific importance of buckwheat research (Task 1.2)

The transformation of buckwheat through cross-pollination and the possibility to use the trait for the benefit of the farmer has not been studied before in Finland and there are no publications available from other parts of the world. Methods that allow the farmer to improve the seed material himself are important, as there is very little plant breeding of buckwheat in the near future. Russia has traditionally been an important country for buckwheat cultivation and plant breeding, but since the invasion of Ukraine, the opportunities for cooperation have dwindled.

## Summary and next steps for buckwheat research (Task 1.2)

- Buckwheat and peas produced the best yields per hectare when grown alone, but mixtures tended to produce relatively more yield per hectare.
- There may be differences in the suitability of buckwheat strains for intercropping with peas. The buckwheat strain that benefited most from mixed cropping had the lowest average yields when grown alone.
- The harvest window for buckwheat is longer than expected. Delaying harvesting until late autumn or after frosts does not seem necessary to maximise seed yield.
- It's a good idea to plan your pruning for a time when the weather is good. In a favourable buckwheat year, the seed yield at the end of August can be the same as the harvest three weeks later.
- It is worth trying to bring forward the ripening of buckwheat seed on your own farm by bringing forward the threshing. Early threshing will recover the first seeds to ripen. When farm saved seed is used for the next sowing and repeated over several years, the cross-pollinated buckwheat will start to adapt to the conditions on the farm.

- In the experiment, using early harvested crops for sowing seed improved the yield per hectare by about 10%, but more years of trials would be needed to confirm the result.
- The buckwheat-pea mixture controlled noxious perennial weeds at least as well as the pure buckwheat, but this was not measured separately.
- It would be possible to apply the early harvesting principle to buckwheat grown more widely in Finland, but this would require a pilot project involving cooperation with several farms.

# Research on common bunt in wheat (Task 1.3)

Angrologica: Anders Borgen

NARDI Fundulea, Romania: Matilda Ciuca

## Virulence of European common bunt Races (Task 1.3)

The project has collected fungal spores of common bunt (*Tilletia caries*) from different regions of Europe and infected a differential set of 22 wheat varieties with known resistance genes. This has led to an understanding of the virulence of European races (Borgen *et al* 2023). From infected plants with different resistance genes, spores has been collected and multiplied, and in this way a collection of races has been developed with virulence against most of the known resistance genes.

Infection in a resistant variety is not a final proof of the presence of a race being virulent to a resistance gene. Infection in a line may be caused by impurity in the line, or by the resistance gene being partly depending on environmental factors. To document presence of true virulence, spores need to be collected from resistant lines and used for re-inoculation to demonstrate increased infection rate (Flor 1946).

Virulence to diseases that spread with soil, wind or by water are normally linked to a specific geographical region, but common bunt is mainly spread via seed and may therefore spread over long distances following seed trade rather than from neighbouring fields. Therefore, the virulence of common bunt is more relevant to relate to a seed system or continent rather than a single country or climatic zone. Seed is traded freely within the EU, and it is therefore relevant to consider Europe as one zone of bunt dispersal, and North America another, since seed exchange of infected seed between the continents are limited.

Dwarf bunt on the contrary is more likely to stay stable in virulence within a smaller region as this disease is mainly a soil borne disease.

Virulence is found against most resistance genes in Europe. Bt11 and Bt12 have so far being considered safe genes in Europe, but as described below, these genes are not single genes, but combinations of several genes. The differential lines used to describe Bt11 contains a mixture of several genes, and it is still unknown if virulence is present in Europe against any of these single genes. The same is the situation with Bt12.

Several genes are called Bt8 but comes from different sources and are most likely quit different. The differential line for Bt8 suggested by Blaire Goates is Pi554120 (with PI173438 as source). Virulence against “Bt8” in Pi554120 is present in Europe. In both USA and Europe, PI178383 has been used in breeding as a source of “Bt8”, but this “Bt8” is most likely different from the Bt8 in the differential line. It is unknown if virulence is present in common bunt in Europe against “Bt8” coming from PI178383 or from the original source

‘Yayla 305’, but virulence against “Bt8” coming from PI178383 is most likely present in dwarf bunt on the island Gotland (Sweden). More research is ongoing about the understanding of Bt8 complex and possible virulence to the genes involved.

Bt9 has been properly mapped confirming that it is a single gene. There are indications of infection in lines having Bt9, but the efficiency of Bt9 is partly climate dependent and it still needs to be confirmed that it is true virulence under field conditions, and not just random infection in off type plants.

Most other genes can be infected by bunt spores from Europe, including Bt1-7, Bt10, Bt13-15 and BtZ. We have not found any virulence to BtP, but little is known about this gene, based on the results from other genes, it may be worth investigating if BtP is really just a single gene or if it could be another example of a combination of other genes.

## Development of genetic markers for resistance to common bunt (T1.3)

Wheat varieties and breeding lines from organic breeders in Europe, with unknown resistance has been screed for infection using a set of different purified virulence races. Based on the reaction to the different races, the active resistance gene(s) within each variety and breeding line has been identified, and the lines has been genotyped using 25k single nucleotide polymorphism (SNP). With statistical methods such as GWAS and fine-mapping, genetic markers for each resistance gene has been identified.

### Bt1

Bt1 has been mapped to chromosome 2B, and initial results has been published (Christensen and Borgen 2023D). Afterwards, the mapping has

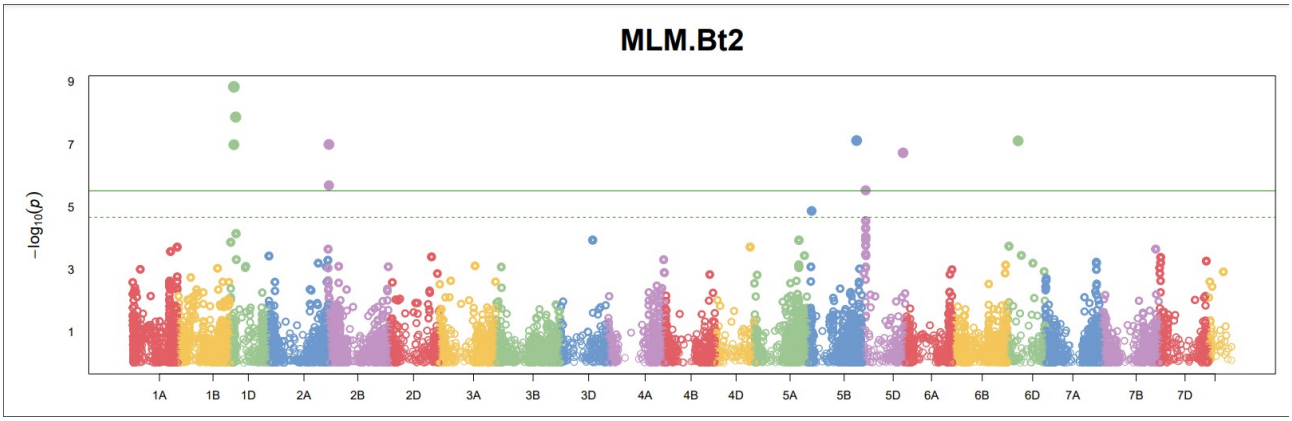
been improved and a reduced interval of markers can be used for MAS (unpublished).

*Table 1 Markers that can be used to map Bt1*

| | |
|---|---|
| AX-158609666 | A |
| Excalibur_c48404_59 | C |
| wsnp_Ex_c15646_23969140 | A |
| BS00065302_51 | G |
| AX-94890379 | G |
| BS00083998_51 | G |
| Ra_c105904_187 | C |
| Ra_c105904_1191 | G |
| AX-158610188 | A |
| AX-94808568 | G |
| AX-158562114 | C |
| Kukri_c49784_86 | A |

## Bt2

Mapping Bt2 has been difficult and we finally have an idea why. In the screening, four different genes have been identified giving identical infection patterns in phenotyping with the used virulence races and two/three of them (Bt2 differential + Bussard (Bt_Bussard+ Bt_magnifik_5D)) even behave identically across all 44 virulences races (Borgen *et al* 2023). This resulted in lack of power in the GWAS and the signals are still not impressive, but at least consistent between Blink, FarmCPU and MLM. Observe the lack of signal at 7A, where we have BtH from an earlier analysis.



The four genes with virulence pattern identical to Bt2 are:

1. The real Bt2 found in Hussar and present in the differential PI554097 on Chromosome 1D
2. Then there is BtHereward/BtH/BtQ_7A. This gene is found alone in Skagen and Inthaler and some breeding lines.
3. One gene at 2B preliminary named Bt_Bussard_2B. This gene appears to be responsible for the Bt2-like resistnce in varieties such as ’Dream’, ’Paroli’ and ’Complet’.
4. One gene at 5D preliminary named Bt_Magnifik_5D. This gene is found alone in a few breeding lines descending from ’Skotte’ or’Quebon’, but it is not possible to say anything definite about the phenotyping pattern.

Fine mapping of the four genes is a work in progress and the quality is still low. Due to lack of parents/offspring triplets it is not possible to identify recombination events precisely and simple haplotype comparison is not as robust.

Virulence against Bt2 is present in ~50% of all bunt races in Europe (Borgen *et al* 2023). This can be explained by the fact that the three new genes found to give the same virulence pattern as the original Bt2 is unintended present in many European varieties.

**Bt3**

Bt3 has been mapped to 1A, and is further described below in chapter “Dimenit and Bt11 Differential Genes”

**Bt4 and Bt6**

Bt4 and Bt6 are behaving identically with all virulence races so far in historic trials and in this project (Borgen *et al* 2023). Both genes are at 1B and are either the same gene or tightly linked. Many historic and well characterized Bt4 containing lines were genotyped. We have a good mapping of Bt6 and studying haplotypes in an extended interval around the Bt6 interval in the Bt4 lines gave a reasonable mapping. The Bt4 and Bt6 intervals are overlapping and Bt4/Bt6 lines have identical haplotypes in the Bt6 interval.

For breeding and for the differential set there is no value in keeping both Bt4 and Bt6, and no harm.

**Bt5**

We have mapped the Bt5 gene to an interval on 1B (163,225,664 – 283,930,031 bp) (unpublished) and markers are available for MAS. Due to lack of marker polymorphism across the interval, we get many false positives. In our panel 30%. There is much better marker polymorphism just outside the interval and we can get the false positive rate down to 5% by adding some markers outside the interval, but at the cost of an increased false negative rate, from 5 to 10 %.

Our panel had very few lines where we had the parents as well, and it was therefore not possible to detect recombination events. We genotyped 90 lines described in the old papers this year and many of them have Bt2 in addition to Bt5. This helped improve the mapping very much.

**Bt7**

Bt7 has been mapped to 2D and published (Christensen and Borgen 2023A).

**Bt8**

Bt8 was originally discovered in ‘Yayla 305’ (PI 178210) by Waud and Metzger (1970) and used as differential until the Goates (1996, 2012) update, where ‘M72-1250’ (PI 554120) replaced it. ‘M72-1250’ (PI 554120) inherits Bt8 from ‘7845’ (PI 173438), which was shown to have it by a test cross to ‘Yayla 305’ (PI 178210). Neither of the two sources of Bt8 have been genetically mapped in either of these varieties and it is not firmly established that ‘M72-1250’ (PI 554120) is monogenic and its resistance is identical to Bt8 in ‘Yayla 305’ (PI 178210). Also the ‘Yayla 305’ (PI 178210) Bt8 gene is possibly not monogenic. The Turkish landrace ‘6256’ (PI 178383) was shown to carry Bt8 and has served as the major source of Bt8 in especially US wheat breeding, but also in Sweden.

As a first step towards making a Starke II based Bt8 NIL, we have in a previous CoreOrganic project COBRA crossed Starke II and PI 554120. From this population we have genotyped and phenotyped 13 RILs. Two of these RILs are nearly NILs (98.6% identical), one with Bt8 and one without, and we have used them to get a few candidate intervals. All but one on 4B could be dismissed by including a few more RILs and the parents.

The phenotyping results give a clear indication that PI 554120 has two genes. We have no good mapping of the second gene.

From testing the 4B marker block in the entire panel, we conclude that it looks promising to be the dominating gene Bt8 from PI554120. There are approximately 200 Bt8 lines in the field in 2024. Approximately half descending from PI 554120 and half from Magnifik (and therefore from Pi178383 via Stava), which is another potential assigned carrier of Bt8.

We have no solid, or even semi solid, information on the original Bt8 described in 'Yayla 305' yet. The genebank accession of Yayla 305 seems heterogeneous which complicates the search for Bt8 in this original source. The work will be continued in a new project GRAINGOOD funded by the national DK program (Organic-RDD-10).

**Bt9**

Bt9 has been mapped to 2B (Steffan et al 2017) and a refined mapping has been don and published (Christensen and Borgen 2023B).

Later research demonstrated that two of the markers published were wrong. They are not at 6D and have been removed. The interval has not changed but the new markers for MAS are:

*Table 2 New Markers for mapping Bt9*

| | |
|---|---|
| Kukri_rep_c107605_164 | T |
| wsnp_CAP8_rep_c4586_2232878 | C |
| wsnp_CAP7_c1735_859875 | G |
| wsnp_CAP7_c1735_859744 | T |

**Bt10 and BtZ**

Bt10 and BtZ are two genes with a lot in common and may even be identical. Phenotypically they are identical, and they are both present at Chromosome 6D. However, the mapping is not 100% identical.

Bt10 has been mapped to 6D (Laroche *et al* 2000, Menzies *et al* 2006) and a refined mapping was developed in DIVERSILIENCE and published (Christensen and Borgen 2023C). BtZ has been also been mapped to 6D has also been published (Chrisenten and Borgen 2023E). Later work has

been made getting a slightly different result. In a previous CoreOrganic project COBRA, a small RIL population has been made from the cross Starke II x Inna. Lines are called NIL-Z because they are the first step towards making a Starke II based BtZ NIL.

*Table 3 Markers to potentially detect BtZ*

| | |
|---|---|
| RAC875_rep_c118305_446 | T |
| BS00065960_51 | C |
| Kukri_c73802_205 | A |

Orange markers are flanking the QTL interval potentially including BtZ. Green marker may be used for MAS, but monomorphic in this population

There are two RefSeq High Confidence genes in that interval

*Table 4 RefSeq High Confidence genes*

| Chromosome | Phys Pos Min | Phys Pos Max | Gene | Function |
|---|---|---|---|---|
| Chr6D | 4363458 | 4366232 | TraesCS6D03G0022800 | Receptor-like protein kinase |
| Chr6D | 4533591 | 4537917 | TraesCS6D03G0023800 | F-box family protein |

Bt10 and BtZ behaves identically with all virulence races used in the field trial(Borgen *et al* 2023) and in all previous trials made, and they map to near identical intervals at 6D. The original publication of BtZ (Chrisenten and Borgen 2023E), the interval overlapped with the Bt10 interval, but the new one presented here does not. We are convinced that the new Bt10 interval is inaccurate and that BtZ = Bt10 = TraesCS6D03G0022800. Phenotyping 75 Saatsucht Donau lines from a Bt10 x BtZ cross we found 1-2 lines with a single or a few infected heads. It remains to be investigated whether this is true segregation or an error.

BtZ is supposed to be present in Zarya as a *Thinopyrum intermedium* introgression, inherited from AG.IN via PPG-599 and Lutescens.126-65.

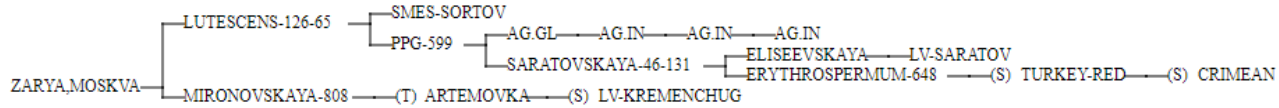



*Figure 1 Phylogenetic tree*

It seems a bit unlikely that such an introgression should contain Bt10 and end up at 6D. To investigate that, Mironovskaya 808 and PPG-599 will be phenotyped. Lutescens-126-65 and Smes-Sortov are unfortunately not available in any gene banks.

## Bt11 and Dimenit Genes

Lunzer *et al* 2023 mapped a number of loci/genes in the Bt11 differential line ‘M82-2123’ (PI 554119) and in ‘Dimenit’ (PI 166910) in the ECOBREED project(HORIZON-2020). Four biparental mapping populations were used: M82 2123 x Mulan, Rainer x Dimenit, Dimenit x Rainer and Dimenit x Lukullus. Three loci at 4BS, 4BL and 6DL were mapped in M82-2123 and a fourth locus at 2A was segregating in that population. In the Rainer x Dimenit and Lukullus x Dimenit populations two loci at 4BS and 6DL were mapped. Dimenit was the donor. It turned out that Dimenit was not homogenous and in the Dimenit x Rainer population, and three (or four) additional loci at 1A, 1B and 7B were found. It is uncertain whether the 4BL locus was identified in this population as the 4B QTL spanned the entire chromosome.

All phenotyping and genotyping data from the ECOBREED project were made public and we did a detailed analysis of the data and found that our Bt3 markers (see above) detected the gene at 1A, and the Bt6 markers detected the gene at 1B. The Bt7 markers were also validated. Bt7 is not very effective against the virulence used in the ECOBREED study and was therefore not detected, but still this gene was segregating in the population.

During the initial population validation, we discovered that the Dimenit selection with the extra genes was identical to the one we have later genotyped. We also discovered lines having the wrong parents and lack of inbreeding/selection, especially in the Dimenit x Lukullus population.

The detailed analysis identified recombination events that could be used to refine the intervals from the QTL mapping, and in most cases gave much smaller intervals. However this was not possible for the 4BL and 7B loci as these were masked by the other loci in most lines and not very effective against the used inocula.

The 6D locus gives immunity to both inocula used by Lunzer *et al* (2023), but interestingly, the 4BS locus was highly resistant to the BOKU “house keeping” inoculum, but the “aggressive” inoculum used in 2022 was able to give infections in the 12-24 % range.

Unfortunately we have not managed to get lines from the mapping populations with each gene isolated, into the field trial of 2024, except for a few exceptions; U11.15 with the 6D locus and U11.50 with the 7B locus. From the 2022 trial, we know that breeding lines from Agrologica with the 6D locus alone is fully resistant to all eight virulence races in our core set.

Getting lines with 4BS and 4BL alone in the 2024 trial was a high priority, but we did not succeed in finding such lines.

A big question is whether the 6D QTL in Dimenit may be identical to the Bt9 gene. We have this evidence so far:

1) Abdallah *et al*. (1984) found Bt7, Bt9 and Bt11 in Dimenit
2) Bt9 and Dimenit 6D both are at 6DL
3) The haplotypes of Bt9/Bt11 lines are very different around the mapped intervals
4) There is no overlapping of Dennis’s intervals for Bt9 and Dimenit 6D so far

Based on our analysis, we preliminarily suggest replacing Bt11 by two new assigned Bt gene numbers and also exchange the line in the differential set by the separate QTL carriers. Nevertheless, a bit of validation work has to be done still.

### Bt12 and other PI 119333 Genes

Bt12 was mapped by Müllner *et al* (2020) to 7DS in the interval 6.5 – 10.8 Mbp in RefSeq 1.0 positions. Chromosome 4B was found to contribute some resistance too. The 7DS interval was found not to be 100% linked to Bt12, meaning that it is a signal only and actually an exclusion interval.

The ECOBREED project kindly gave us access to genotypic and phenotypic data, which we have analysed.

We phenotyped 37 lines from the mapping populations with 7-9 virulence races and we found that two genes at 7D and two at 4B could explain the infection patterns.

To get a mapping at 4B we analysed markers across the chromosome and found 18 recombination events, giving 19 intervals. Interval 1 at 1,306,080 – 15,855,852 bp was found to be harbour some resistance gene(s). Intervals 2-14 were not related to the presence of resistance and interval

15-19 at 648,869,446 – 670,633,612 bp (end of chromosome) again provided resistance.

In this table, we see the infection patterns for the four genes and for their combinations. It was very difficult to figure this out based on the patterns from the 37 lines only and there may be errors in it. There may also be inaccuracies and errors in the phenotyping and the table should not be taken as the final word.

*Table 5 Infection pattern from Bt4, Bt12 and their combinations*

| | Vr0 | Vr2 | Vr-3 | Vr-5 | Vr10 | Vr-13 | Vr-Z | Vr-DOT | Vr-Iran |
|---|---|---|---|---|---|---|---|---|---|
| Bt4B_1 | | | Bt4B_1 | | Bt4B_1 | | | | |
| Bt4B_2 | | Bt4B_2 | | Bt4B_2 | Bt4B_2 | | | | |
| Bt12A | Bt12A | | Bt12A | Bt12A | | | | | |
| Bt12B | Bt12B | | | Bt12B | Bt12B | | | | |
| Bt4B_1+Bt4B_2 | | | | | Bt4B_1+Bt4B_2 | | | | |
| Bt12A+Bt12B | Bt12A+Bt12B | | | | | | | | |
| Bt4B_1+Bt12A<br>Bt4B_2+Bt12A | | | Bt4B_1+Bt12A | | | | | | |
| Bt4B_1+Bt12B | Bt4B_1+Bt12B | | | | Bt4B_1+Bt12B | | | | |
| Bt4B_2+Bt12B | | | | Bt4B_2+Bt12B | Bt4B_2+Bt12B | | | | |
| Bt4B_1+Bt4B_2+Bt12A | | | | | | | | | |
| Bt4B_1+Bt4B_2+Bt12B | | | | | Bt4B_1+Bt4B_2+Bt12B | | | | |

We see that the perceived strength of “Bt12” comes from the good combining ability of the four genes in PI 119333.

## Bt13

Bt13 has been mapped to 7D and published (Christensen and Borgen 2023F). There is so far no indications that Bt13 is identical to other Bt-genes found at 7D.

## Bt14

Differential set line “Doubbie” - a durum- carrying resistance Bt14 has been crossed into hexaploidy wheat by Farrer in Australia (Watson, 1958 ) and germplasm from this cross, called accession ‘186’ (PI 172201), is

available from the American genebank. This accession is heterogenous, six head selections have been made and they have been genotyped and phenotyped this year. The lines turned out not to be homogenous and phenotyping results are not conclusive. It appears that three genes are segregating. Further line creation, phenotyping and genotyping is in progress to be done in the newly fuded GRAINGOOD project.

**Bt15**

Bt15 is found in durum wheat Carleton. But only little is know about this gene.

**BtP**

BtP is present in the line PI 173437. Little is known about this gene, but no virulence has so far been found in Europe. We have made crosses with a susceptible line to be analysed in the newly fuded GRAINGOOD project.

**New Genes identified in the DIVERCILIENCE project**

In the search of known genes, many “new” genes have been revealed. Too many that we know very little about, but a few deserve to be mentioned.

**Bt_Mariann_XX**

A RIL population from the cross (Magnifik x Tataros) x (Spelt x Sebesta Blue) is called Mariann. Phenotyping of 33 lines with the core virulence set in our nursery identified a unique infection pattern in three RILs. The underlining gene is called Bt_Mariann_XX and we have no mapping for it yet. Two lines showed a Bt2’ish pattern, probably from Bt_Magnifik_5D.

Bt_Mariann_XX can explain the full resistance in the remaining lines together with Bt_Magnifik_5D, but “Bt8” from PI 178383 via Magnifik can also be present. 100 more lines are in the pipelineto be analysed in the newly fuded GRAINGOOD project.

**Bt_Blizzard_7A (QBt.ifa-7AL)**

Müllner et al (2020B) mapped four loci in ‘Blizzard’ and ‘Bonneville’.

We’ve phenotyped 24 lines from the mapping population selected to carry single genes and we found infection patterns consistent with Bt3, Bt6, Bt7, and in addition this pattern for the 7A locus.

No lines with the 7D locus alone was found, but a number of lines show complete resistance that cannot be explained by the other genes.

## Researches for wheat resistance to common bunt at NARDI Fundulea, Romania (T1.3)

Starting from the necessity to obtain resistant bunt cultivars, from the complexity of wheat genome, from the testing difficulty for common bunt resistant genotypes selection and from the molecular markers utility, this work proposed to look for new resistance resources and the stack of common bunt resistance genes to ensure durable resistance. Regarding this goal, in this project, we have worked on three approaches: (1) old resistant materials; (2) synthetic amphiploids and (3) lines that carry 1RS:1AL translocation from F000628G34-1 breeding line.

(1) **Old resistant materials**: 14 winter wheat lines (developed by crosses with different known sources of Bt genes and unknown resistance sources) were tested under artificial inoculation in Romania-Fundulea (mix of local races) and Denmark (tested 8 different races of common bunt). The results showed that in both fields from Denmark and Romania only four lines were free of teliospores: FDL94895GM1-21 (Bt12 from Dr. R. J. Metzger), FDL95601GM37 (Bt13 from Dr. R.J.Metzger), F00628G34-1 (1RS:1AL translocation) and F96915G1-1 (WGRC23). The resistance source, in WGRC23, probably comes from Triticum monococcum accessions PI 266844 or/and PI 355520. These sources have kept the resistance over time and across fungal races covering a broad range virulence occurring in Europe.

(2) **Synthetic amphiploids**: 27 synthetic amphiploids (SHWs) were tested under artificial inoculation with mix of Tilletia spp. local races, in Romania at NARDI Fundulea. The phenotypic observation, from two years (2022-2023 and 2023-2024), showed only two SHWs free of infection (0%) for both years: E22 and E34. The SHW_E22 was obtained from Aegilops. tauschii ssp. tauschii Anotim (2417) accession and Triticum durum- Condur parents. The SHW_E34 had different parents, Ae. tauschii ssp. tauschii (2550) and Grandur.

In 2024 we developed populations using SHW_E22 and winter wheat cultivars Pitar, Otilia and Consecvent (1RS.1AL translocation). Future studies will prove if the resistant SHW lines carry new/unknown or known Bt gene/genes on D-genome. Synthetic amphiploids can constitute a new source of bunt resistance contributing to the improvement of wheat germplasm.

(3) **Rye translocation (1RS:1AL)** from F000628G34-1 breeding line: 78 DH lines (obtained from Izvor x F00628G34-1 cross) were tested under

artificial inoculation in Romania. The molecular marker assay with TSM592 marker showed that 50 DH lines carry 1RS:1AL translocation and phenotypic observations showed that 32 lines from these were free of teliospores and the maximum level of infection was 8% while the DH lines without rye translocation showed the minimum infection level at 5% and the maximum at 100%. The rye translocation (1RS.1AL) contributes to resistance to common bunt. Future researches, regarding this subject, look for the best molecular marker that can make difference between 0% and 1% infection in wheat lines with 1RS.1AL translocation.

At present there are two cultivars FDL Abund and FDL Consecvent that carry this rye translocation with good results under artificial inoculation tested in Germany (2024), where FDL Abund was free of teliospors and FDL Consecvent showed 1,7% infection. On the other hand, in Austria the same cultivars showed a higher percent of infection, FDL Abund showed 6.5% and FDL Consecvent-9,5%.

In our study, not all lines/cultivars with the 1RS translocation are resistant to common bunt, the decisive role could be played by the rye source used in obtaining the lines with this translocation.

# Breeding wheat varieties and populations for organic farming (Task 1.3)

Agrologica: Anders Borgen

OHM can be composed by mixing of all offspring from crosses (original composite cross populations), but the mixture will be similar or slightly better than the average of the parents. It is therefore crucial to use only well adapted parents. If suboptimal parents are included, pure lines needs to be selected, and the OHM be composed only by selected offspring (complex variety mixture).

Some traits are difficult but not impossible to improve further after the OHM have been composed. Baking quality can be improved by single seed NIR sorting (eg. BoMill Insight® technology), colour sorting or by gravity sorting. However, at this point this can only increase gross grain protein content and seed hardiness, but is unlikely to improve gluten index significantly. To compose mixtures with optimal gluten index above the average of the parents, we have in DIVERCILIENCE developed SNP markers for gluten subunits to be used for MAS in order to compose mixtures with a diversity of HMWG subunits within the mixture.

Weed competition is best improved by visual assessment and selection for early vigour, and plant height before heading stage with due respect of the risk of lodging.

Wheat lines assessed for bunt resistance (see above) has also been assessed for other traits, including resistance to rust, mildew, lodging earliness and relevant traits. Using the genotyping results, genetic markers have been developed for 38 genes other than bunt resistance, including gluten sub-units, resistance genes for rust and nematodes, Rht, Vrn, Ppd, a.o.

Cereal Cyst Nematodes (CCN, *Heterodera avenae*) is in Denmark mainly a problem in spring wheat and no varieties on the EU Catalogue are resistant. The market for special varieties with this trait is limited as farmers tend to use non susceptible crops rather than resistanct varieties in case of nematode infestation of a field. Therefore CCN resistance should be included in all spring wheat as a preventive rather than a curative measure. Marker Assisted Selection (MAS) is the best way for selection, as field phenotyping is difficult and expensive. We have therefore developed SNP markers the Cre1 resistance gene for this selection.

Leaf diseases such as mildew and in particular rust diseases can be devastating in organic farming. These diseases can be selected in the field based on leaf symptoms, but the experience indicates that if resistance is based on one or few vertical resistance genes, the varieties or OHM can turn susceptible within 1-3 years aong with conventional varieties carrying the same resistance genes. Therefore, horizontal (adult plant) resistance genes or multiple vertical resistance genes must be included in organic varieties to maintain durability of the resistance.

Funding organic plant breeding is difficult as the market is too small to fund the breeding. The OHM developed in DIVERCILIENCE and parallel projects is therefore distributed via the member organisation Landsorten, based on home saved seed production to reduce cost for the seed production. Two OHMs have been officially registered, ‘Mariagertoba®‘ and ‘Popkorn’, whereas other OHMs are produced in smaller amount without registration. By not selling seed, the activities of Landsorten is legal, and small seed lots can be distributed under the Article 3 exemption or research, trial and breeding purposes as a base for multiplication of home saved seed. Landsorten supports in this way a production of ~1800ha of organic bred varieties and OHMs in Denmark, England, Belgium and The Netherlands.

# Development of winter-hardy lines of white lupin (T1.4)

CREA, Italy: Paolo Annicchiarico,

## Background

White lupin (*Lupinus albus* L.) is a cold-season pulse crop of particular interest for its outstanding seed protein content close to 40% dry matter. It can play a potential role in reducing European imports of high-protein feedstuff while enhancing soil fertility and agricultural sustainability. It is typically sown in autumn in mild-winter regions and in late winter or early spring in cold-prone regions of Europe, but the changing climate is expected to expand autumn sowing northwards. This shift would enable a longer crop cycle with earlier maturity, reducing the risk of terminal drought and increasing yield potential. However, the trend towards milder winters may emphasize the destructive effect of sudden frosts due to insufficient plant cold acclimation. In this context, frost tolerance remains a key breeding target, but its selection in field conditions is challenging due to the wide and increasing climatic variation across years. Selection under controlled conditions is crucial to enable applicability and uniformity. As part of the Diversilience project, we conducted two experiments aimed to the genetic improvement of white lupin cold tolerance: (i) a methodological study, to identify the optimal temperature for high-throughput phenotypic selection and assess the consistency of plant mortality responses across controlled and field conditions; and (ii) a subsequent large-scale evaluation of breeding lines, to investigate the genetic architecture of frost tolerance and, in case of polygenic control, develop a preliminary genomic selection model (which, in that case, is more convenient than selecting based on marker-assisted selection for a few markers).

Additional research work involved the selection of evolutionary populations in different regions and the study of the alkaloid content and its evolution in these populations. The results relative to the development and the experimental assessment of the populations are reported in the Deliverable 2.1.

## Material and methods

The methodological study included 11 genotypes with a wide range of winter mortality based on earlier field trials in northern Italy, which underwent four freezing treatments: -7, -9, -11, and -13 °C. Each of the four replicates per genotype included 10 pregerminated seeds transplanted into a peat substrate, grown for 10 days at 22.5 °C, hardened for 15 days at 4 °C, cooled for 3 hours at -3 °C, treated for 4 hours according to the treatment level, recovered for 6 days at 4 °C, and regrown for 15 days at 15/20 °C. We evaluated the mortality ratio at the conclusion of the regrowth period, as well as biomass damage, using a visual score based on the degree of necrosis after both recovery and regrowth, and mortality. For each accession, we computed the lethal temperature corresponding to 50% of mortality ($LT_{50}$) using a binomial GLM with a probit link function, and we compared genotypes by ANOVA.

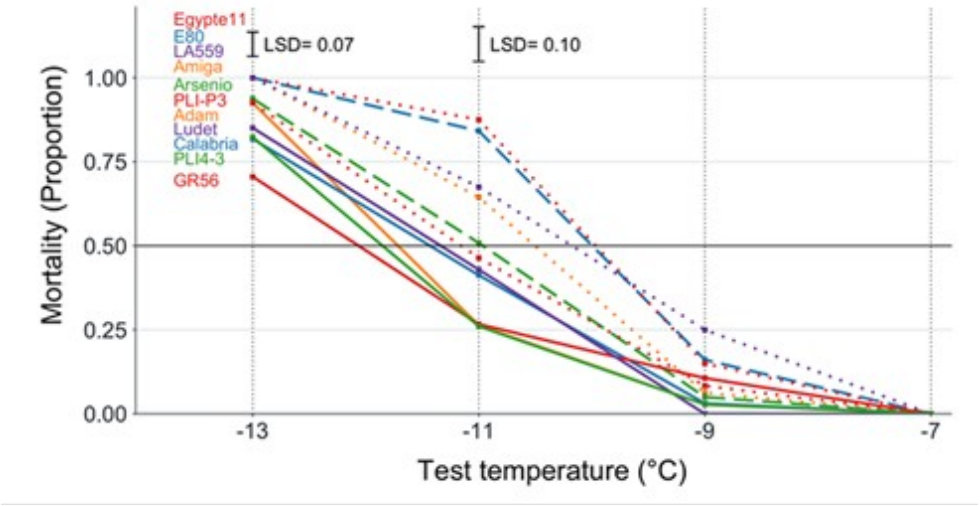
A further study examined the frost tolerance of 144 white lupin inbred lines from a reference population obtained from 16 crosses between four sweet-seed elite cultivars and four landraces. The genotypes were evaluated at -11 °C using an α-lattice design with 4 replications, 12 incomplete blocks, and 15 plants per replication, following the protocol previously described. The broad sense heritabilities of mortality ratio and biomass damage based on the visual score were calculated, as well as their association with nearly 33000 SNP markers obtained via ApekI-based genotyping-by-sequencing, through a Genome Wide Association Study (GWAS) utilising the BLINK model. A genomic prediction study compared three prediction models (rrBLUP, Bayesian LASSO, and Bayes B) based on their prediction accuracy values (i.e., correlations between observed and genomically predicted genotype values in cross validations) for mortality ratios.

## Results

In the methodological study, plant mortality did not occur at -7 °C, whereas there was significant variation ($P < 0.01$) at -11 °C and -13 °C freezing temperatures. The largest variability, ranging from 0.26 to 0.88, occurred at -11 °C (**Figure 1**), indicating that this temperature has the highest screening value. High correlations ($P < 0.001$) were found between genotype mortality at -11 °C and biomass injury score for the same temperature ($r = 0.97$) and LT50 ($r = 0.94$), as well as between the last two traits ($r = 0.91$). These findings show that screenings at one single optimal freezing temperature (-11 °C), based on mortality ratio and visually scored damage, can produce very similar results to screenings at

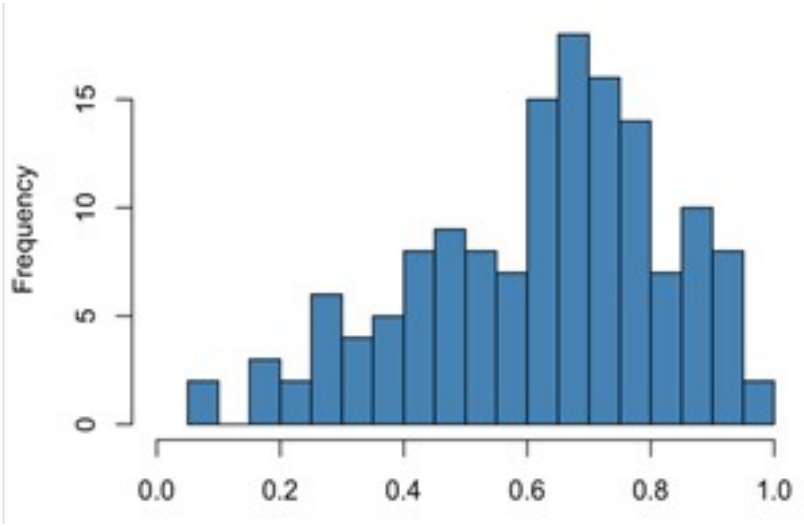
multiple freezing temperatures (four in our study) to compute the $LT_{50}$, making the evaluation of large genotype numbers more suitable. Furthermore, we observed a good correlation between frost susceptibility in controlled conditions and winter mortality based on field data. The five genotypes belonging to the low winter mortality class exhibited the lowest plant mortality at -11 °C, while three of the four genotypes from the high winter mortality class also had very high frost mortality **(Figure 1**). These results encourage the adoption of controlled environments for frost tolerance screenings. The reported methodological study has already been published (Franguelli et al. 2024).

**FIGURE 1 - Plant mortality at four freezing temperatures of 11 white lupin genotypes classified into three winter hardiness classes (solid line, low winter mortality; broken line, intermediate winter mortality; dotted line, high winter mortality). Least significant difference values at P < 0.05 reported only in the presence of overall genotype differences at P < 0.05.**



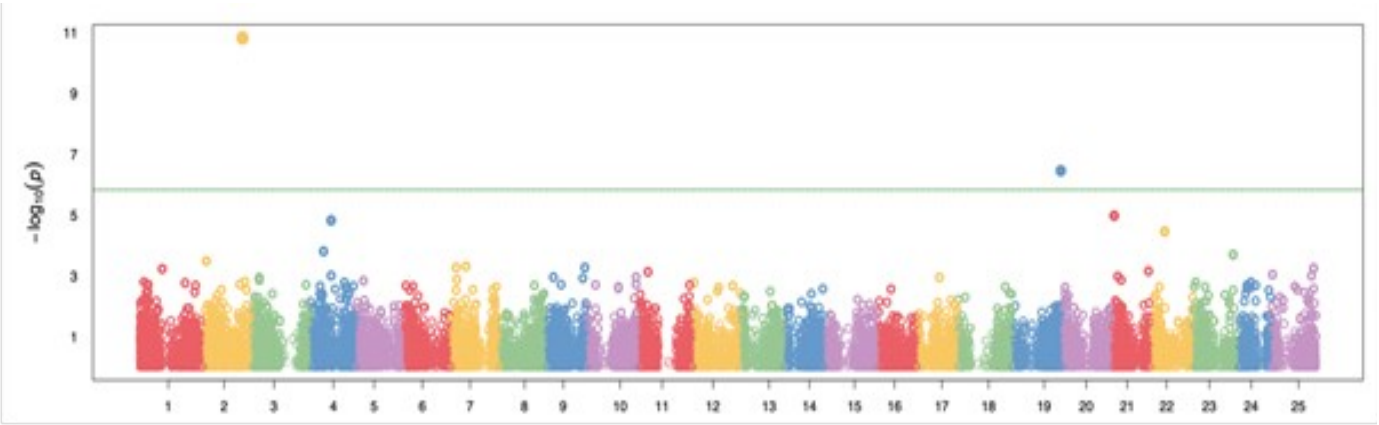

The large-scale evaluation study focused on 144 white lupin inbred lines representing a genetically broad breeding population (originated from 16 crosses of four international landrace genotypes by four sweet-seed international cultivars or breeding lines, with progenies selected in early generations for low alkaloid content). These genotypes, evaluated at -11°C freezing temperature, revealed significant and large variation for plant mortality proportion, which ranged from 0.07 to 1.00 (with 43% of the lines having a mortality ratio between 0.60 and 0.80: **Figure 2**).

**FIGURE 2 – Distribution of plant mortality proportion values for 144 white lupin lines evaluated at -11°C freezing temperature**




The broad-sense heritability of lupin intrinsic frost tolerance was fairly high, namely, 0.82 for the plant mortality ratio, and 0.77 for the visually assessed damage. The GWAS identified a significant association between two SNPs on chromosomes 2 and 19 and the frost mortality ratio (**Figure 3**), while no SNP was significantly associated with visually scored damage. However, various peaks appeared in the Manhattan plots without reaching the significant threshold for both traits, indicating a polygenic architecture of the frost tolerance trait. This genetic architecture encouraged the development of a genome-enabled selection model, which could properly account for the effect of several different major and minor genes.

**FIGURE 3 - Manhattan plot showing the association scores of 33,014 SNPs with intrinsic frost tolerance, measured as plant mortality ratio following a treatment at -11 °C. The line represents Bonferroni's threshold at *P* < 0.01**




We conducted a preliminary genomic prediction study focusing on the mortality ratio trait, envisaging three prediction models (rrBLUP, Bayesian

LASSO, and Bayes B). These models exhibited similar and high prediction ability, close to 0.70. Such a high value would definitely support the use of genome-based prediction of frost tolerance applied to a large number of genotyped lines, in order to perform the phenotypic selection just on the most predictively tolerant lines. The results from this study will be complemented by a similar, large scale evaluation performed on a different genetic base (a collection of landrace genotypes) within the H2020 project BELIS, with the generation of a comprehensive genomic selection model that will be evaluated within BELIS on inbred lines produced by breeding programs of France and the Netherlands.

In conclusion, CREA work within this task was able to (a) identify new breeding lines with greater frost tolerance, (b) set up a procedure for the reliable evaluation of frost tolerant under artificial conditions, and (c) produced key genetic information contributing to develop a genomic selection model for frost tolerance.

## Variation in cold tolerance in white lupin (Task 1.4)

RSR, Italy: Matteo Petitti

From a genetic base including progenies from 16 crosses between four elite sweet-seed breeding lines and four elite landraces of international origin, CREA developed an original evolutionary population by (a) the initial selection for low alkaloid content of F3 progeny plants, (b) pooling nearly 1800 sweet-seed seeds sorted out in equal amounts from plants of the 16 crosses and letting the material evolve under natural selection in autumn-sown, densely-planted plots for two cropping years in Lodi. From this material, RSR and Agrologica planned the selection of region-specific evolutionary populations for Sardinia, Sicily, Tuscany, and Denmark under autumn sowing across two cropping years. In Italy, only the selection in Sardinia could be completed (due to too calcareous soils in Tuscany, excessive frosts in Denmark, and eccessive drought in Sicily). Participative stratified selections with farmers, technicians and researchers, where conducted in the organic farm of the Cooperative San Nicolò Gerrei, in San Nicolò Gerrei (Sardinia) in 2022 and 2024. These field days served also as a dissemination opportunity about the project and the new opportunities offered by Organic Heterogenous Material. In the last project year, RSR and CREA carried out a comparative trial and participative evaluation of

the naturally adapted and selected lupin populations, in collaboration with AGRIS (the agency for agricultural, agro-industry and forestry research and innovation of the Region of Sardinia) which hosted the experiment at «San Michele» experimental farm in Ussana (Sardinia). The experiment is described in detail in D2.1.

# Genetic variation, drought tolerance and disease resistance in common bean (Task 1.5)

Maritsa VCRI, Bulgaria: Nasya Tomlekova

KIS, Slovenia: Barbara Pipan and Vladimir Meglic

After the exchange of materials between KIS and MVCRI and the arrangement of SMTA documents, we prepared a collection of 25 common bean genotypes (Table 1). The selected genotypes differ from each other on the basis of results on origin, resistance traits and phenotypic traits. Before sowing, we photographed 5 seeds per bean genotype and evaluated them on the basis of 16 properties or descriptors for the morphological characterization of bean seeds (length, width and thickness of the seed, size, shape, ratio between length and width, ratio between width and thickness, mass 100 of seeds, seed colour, number of colours on the seed, primary and secondary colour of the seed, distribution of the secondary colour on the seed, brilliance and veining of the seeds).

Table 1: List of common bean genotypes in vegetation at KIS

| Field label | Genotype name | Remarks (origin/tolerance) | Growth type |
|---|---|---|---|
| KIS_1 | KIS Amand | New variety at KIS | dwarf |
| KIS_2 | ref:417x316 | KIS breeding line | dwarf |
| KIS_3 | ref:359x417 | KIS breeding line | dwarf |
| KIS_4 | Topolovec | Slovenian commercial variety | dwarf |
| KIS_5 | Stortion di trento | European commercial variety | indeterminate |
| KIS_6 | Jeruzalemski | Slovenian commercial variety | indeterminate |
| KIS_7 | Cipro | Slovenian commercial variety (garden) | indeterminate |
| KIS_8 | 302/2/babc | KIS breeding line | indeterminate |
| KIS_9 | 227/2acac | KIS breeding line | indeterminate |
| KIS_10 | 239/5cbbc | KIS breeding line | indeterminate |
| MVCRI_1 | Trakiya/Tpakus | Biotic stress | dwarf |
| MVCRI_2 | DG 9-11-1 | Biotic stress | indeterminate |
| MVCRI_3 | DG 12-11-18 | Biotic stress | indeterminate |
| MVCRI_4 | DG 13-12-22 | Biotic stress | indeterminate |
| MVCRI_5 | DG 17-38-16 | Biotic stress | indeterminate |
| MVCRI_6 | DG 17-38-38 | Biotic stress | indeterminate |
| MVCRI_7 | No.11 | Biotic stress | dwarf |
| MVCRI_8 | Local, Bejanovo | Water stress | dwarf |
| MVCRI_9 | Local, Sadovets | Water stress | dwarf |
| MVCRI_10 | Local, Belitsa | Water stress | indeterminate |
| MVCRI_11 | Local, Belitsa | Water stress | dwarf |
| MVCRI_12 | Local, Sv.Petka | Water stress | indeterminate |
| MVCRI_13 | Local, Sv.Petka | Water stress | dwarf |
| MVCRI_14 | Line, Plovdiv, MVCRI | Control, high productivity, high tolerance to water stress | dwarf |
| MVCRI_15 | Line, Plovdiv, MVCRI | Control, high productivity, high tolerance to water stress | dwarf |

Sowings took place in the second half of May in 2022. For each genotype, 16 bean seeds were sown in the case that it was of indeterminate growth type (8 seeds per drying plant) and 20 seeds for the dwarf genotypes. Before the plants grew, we placed the poles next to those with indeterminate growth. Due to high temperatures and low rainfall, the seed

germinated from 12 to 30 days, with most seeds germinating within 16 days. In cases where the crows pulled out or damaged the established plants or the seeds did not germinate and we still had seeds available, we re-sowed them. Towards the end of June, when all the planted seeds had germinated and the plants had developed true leaves, we removed the weeds and covered the surface with straw, thus reducing the evaporation of water from the soil and preventing the growth of weeds (Figure 1). The plants were grown in accordance with organic production guidelines.

Figure 1: materials, grown within Task 1.5 in Slovenia (left and right side)

During the vegetation period, the plants were visually evaluated on the basis of 68 characteristics/descriptors (compiled common bean descriptors from different descriptor lists), of which 6 descriptors were linked to the leaf, 3 descriptors for hypocotyl pigmentation, 10 descriptors for flowering phases and flower colour, 19 descriptors for pod characterization (technologically and physiologically mature pods) and 14 descriptors for plant characterization and growth period (number of days to emergence, number of days to technological maturity of pods, number of days to the first harvest of physiologically mature pods, growth type, stem diameter and plant height). Before the stage of technological maturity of the pods, the plants were assessed visually and in terms of their suitability for breeding and intercropping. We also evaluated the plants with 15 descriptors related to the characterization of resistance/tolerance/susceptibility to diseases in common beans. In 11 genotypes, we determined infection with bean common mosaic virus (BCMV) or bean common mosaic and necrosis virus (BCMNV). Most of the plants had physiologically mature pods by the end of August in 2022, which we picked and dried. Then we recorded post-harvest descriptors for

all harvested materials. Materials were exposed to -20C° for 72 hours as a preventive measure to avoid bruchid appearance (from potentially infected pods). The common set of 25 common bean genotypes from Bulgaria (MVCRI) and Slovenia (KIS) were phenotyped under ecological, Central European growing conditions in vegetation 2022. Out of 25 genotypes, four of them were selected as the most suitable ones for intercropping experiment in Slovenia for vegetation 2023. The selected genotypes are:

- Dwarf growth type:
  - Ref: 417x316 – KIS_2 (Slovenia)
  - MVCRI_7 (Bulgaria)

- Indeterminate growth type:
  - Stortino di trento – KIS_5 (Slovenia)
  - DG 17-38-16 – MVCRI_5 (Bulgaria)

These selected genotypes from Task 1.5 were delivered for further field experiment under Task 3.3.

# Genetic variation (Task 1.5.1)

The study on genetic diversity includes 20 common bean local accessions from various regions, varieties and breeding lines from Bulgaria. The selected accessions differ in terms of their origin, resilience traits, and phenotypic characteristics (Table 1).

**Table 1.** Common bean collection studied by molecular marker techniques.

| **Origin** | **Accessions** |
|---|---|
| Local accessions - the names correspond to the regions of collecting | Bezhanovo, Sadovets, Belitza 1, Belitza 2, Sveta Petka 1, Sveta Petka 2 |
| Varieties | Skitia, Blyan, Ustrem, Beslet, Trakia |
| Breeding lines | DG-9-11-1, DG-9-11-2, DG-9-12-11-18, DG-12-11-20, DG-13-12-22, DG-13-12-32, DG-17-38-16, DG-17-38-38, DG-17-38-46 |

The collection is analyzed using SSR, ISSR, and iPBS markers. Gradient PCR optimization is performed for 23 SSR, 38 ISSR, and 32 iPBS reactions. Of the 38 ISSR primers, 20 (55%) amplify over five fragments, while 17 of 32 iPBS primers (50%) do the same. The most efficient primers are selected after initial testing with two accessions.

To assess genetic diversity, 7 SSR, 16 ISSR, and 21 iPBS reactions are conducted. Eight SSR markers are linked to abiotic stress tolerance, including drought tolerance (BMd 162). BMd 143 fails to amplify and is excluded. Four SSR reactions are polymorphic, and three are monomorphic (Table 2).

ISSR markers effectively evaluate genetic diversity, with primers ISSR 2, 3, 3_1, 5, 6, 9, 11, UBC 807, and UBC 809 producing sufficient fragments (Figure 1, Table 3).

The iPBS marker technique proves effective, generating sufficient fragments. Highly polymorphic primers include iPBS 2219, 2222, 2224, 2228, 2230, 2252, 2277, 2298, and 2394 (Figure 1, Table 3). Primers with low fragment amplification are excluded.

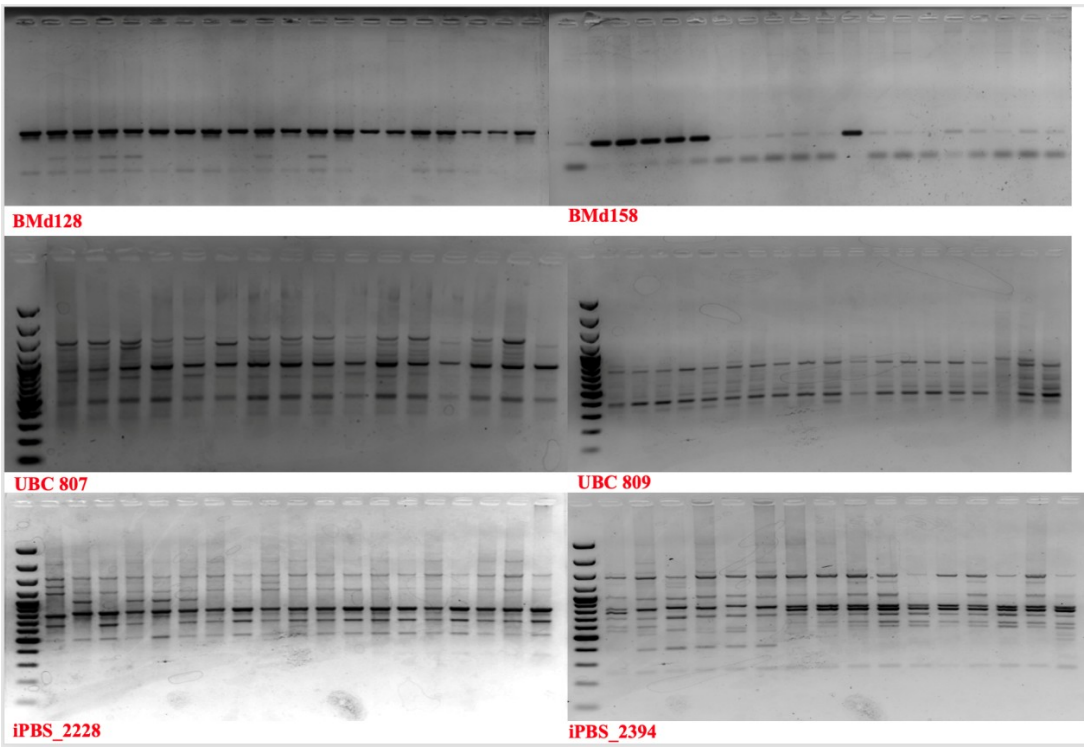



**Figure 1.** Polymorphic profiles of common bean using SSR (BMd128, BMd158), ISSR (UBC 807, UBC 809), and iPBS (iPBS_2228, iPBS_2394) markers.

The highest genetic distance is observed between “Skitia” and “DG-9-11-1” based on SPSS Statistics 26 data. Five other accessions, including

“Sadovets,” “Belitsa 1,” “Belitsa 2,” and “Sveta Petka 1,” show significant genetic distance from “DG-17-38-16” and “DG-17-38-38.”

ISSR and iPBS polymorphic profiles indicate that Bezhanevo, Sadovets, Belitsa 2, Sveta Petka 1, Sveta Petka 2, and DG-12-11-20 are the most genetically distant. iPBS also identifies Belitsa 1 and DG-17-38-46.

The study includes seven advanced mutant lines from two initial varieties, selected for valuable agronomic traits.

## Genetic Diversity Analysis Using Sequencing Technologies

The genetic diversity of three selected advanced M7 mutant common bean lines (possessing enhanced productivity, drought tolerance, and pathogen resistance No.19: M 564-191-1-1-5, No.22: M 564-193-8-2, No.26: M 564-193-9-1-1) along with their initial line, "Evros," is assessed using the Illumina NovaSeq 6000 at Iwate Biotechnology Research Center, Japan. After filtering low-quality reads and adapters, high-quality reads align to the common bean reference genome.

A total of 3,084,830 polymorphisms are detected, including 2,635,011 SNPs and 449,819 INDELs. After filtering, 7,720 polymorphisms remain: 6,900 SNPs and 820 INDELs.

### Oxford Nanopore Sequencing at MVCRI.

MVCRI adopts Oxford Nanopore Technology (ONT) to sequence the initial variety "Evros" using the PromethION 2 Solo system. This generates 3,247,211 high-quality reads with an average length of 4,933.4 bases, assembling the genome into 5,489 contigs. The data align with the common bean reference genome to identify polymorphisms and analyze genome structure.

Comparing "Evros" to the reference genome reveals genetic differences linked to drought tolerance and disease resistance. Further analysis focuses on annotating related genes. A study by Gokul et al. (2020) resequenced common bean varieties, identifying over 40 million variants, highlighting the role of SNPs and INDELs in trait mapping. Expected is that ONT's long reads enhance the resolution of complex genomic regions, supporting breeding programs and advancing common bean genetic research.

**Table 2.** Polymorphic profiles with SSR reactions

| SSR Markers/ Locus/ Repeated motif | Expected Fragment by Blair et al., 2012 (bp) | Amplified Fragment in our study (~bp) | Polymorphic Profiles |
|---|---|---|---|
| BMd 128 [1]**EC911538** $(AGA)_4$ | 203 | 300, 400, 650, 700 | **Profile 1:** Sadovets, Belitsa 1, Belitsa 2, Sveta Petka 1, Beslet, DG-9-11-1 |
| | | 300, 650, 700 | **Profile 2:** Bezhanovo, Blyan, Trakia, DG-13-12-22, DG-13-12-32, DG-12-11-18 |
| | | 300, 700 | **Profile 3:** Skitia, Ustrem, Sveta Petka 2, DG-17-38-16, DG-12-11-20, DG-17-38-38 |
| | | 650, 700 | **Profile 4:** DG-17-38-46, DG-9-11-2 |
| BMd 143 [2]**CX129672** $(AGA)_4$ | 203 | 203 | **Profile 1:** All study genotypes, except DG-12-11-20 |
| | | Absent | **Profile 2:** DG-12-11-20 |
| BMd156 [3]**EG594347** $(CTCA)_4$ | 207 | 150, 207 | **Profile 1:** DG-9-11-1, DG-17-38-16, DG-13-12-22, DG-13-12-32, DG-17-38-38, DG-11-2, DG-17-38-46, Bezhanovo, Blyan |
| | | 150, 220 | **Profile 2:** DG-12-11-18, DG-12-11-20, Sadovets, Belitsa 1, Belitsa 2, Sveta Petka 1, Sveta Petka 2, Ustrem, Trakia |
| | | 150, 207, 220 | **Profile 3:** Skitia |
| BMd158 [4]**EG594328** $(TCT)_4$ | 186 | 186, 320 | **Profile 1:** All study accessions, expected DG-9-11-1, DG-12-11-20, Sadovets, Belitsa 1 |
| | | 320 | **Profile 2:** DG-9-11-1, Sadovets, Belitsa 1 |
| | | 186 | **Profile 3:** DG-12-11-20 |
| BMd134 [5]**EC911333** $(AG)_8$ | 232 | | **All accessions** |
| BMd155 [6] **EG594347** $(TA)_5$ | 200 | | **All accessions** |
| BMd162 [7]**EC997033** $(GAGAAA)_2$ | 189 | | **All accessions** |

**Notes**:

[1]mRNA transcript obtained from bean roots under water deficit (Rodriguez-Uribe et al., 2010). https://www.ncbi.nlm.nih.gov/nuccore/EC911538

[2] mRNA transcript obtained from bean roots under water deficit (Rodriguez-Uribe et al., 2010)
https://www.ncbi.nlm.nih.gov/nuccore/CX129672.1/

[3]Stress response induced by phosphorus deficiency (Tian et al., 2007)

https://www.ncbi.nlm.nih.gov/nuccore/EG594347.1/

[4]Linked to a gene for sensitivity to phosphorus deficiency, not directly related to the drought response (Tian et al., 2007).
https://www.ncbi.nlm.nih.gov/nuccore/EG594328.1/

[5]mRNA related drought stress in common bean (Rodriguez-Uribe et al., 2010; Hoyos-Villegas et al., 2017).
https://www.ncbi.nlm.nih.gov/nuccore/EG594328.1/

[6]Stress response induced by phosphorus deficiency (Tian et al., 2007)
https://www.ncbi.nlm.nih.gov/nuccore/EG594347

[7] Drought stress-related transcript similar to putative heat shock protein (HSP
https://www.ncbi.nlm.nih.gov/nuccore/EC997033.1/

**Table 3.** Polymorphic profiles with ISSR and iPBS reactions

| **ISSRs** | **Polymorphic profiles / accessions** |
|---|---|
| ISSR 2 | 1st: Belitsa 2, Sveta Petka 1, Sveta Petka 2<br>2nd: Trakia, DG-9-11-1, DG-9-11-2, DG-9-12-11-18<br>3rd: DG-17-38-38; DG-17-38-46; DG-13-38-16, DG-12-11-20<br>4th: Blyan, DG-13-12-22 |
| ISSR 3 | 1st: Sveta Petka 2, Skitia, Blyan, Ustrem, Trakia, DG-9-11-1, DG-9-12-11-18, DG-17-38-38, DG-13-12-22, DG-13-38-16<br>2nd: Belitsa 2<br>3rd: Sveta Petka 1<br>4th: Beslet, DG-9-11-2 |
| ISSR 3_1 | 1st: Sveta Petka 2, Skitia, Blyan, Trakia, DG-9-11-1, DG-9-12-11-18.<br>2nd: Beslet, DG-9-11-2, DG-17-38-38.<br>3rd: Ustrem, DG-13-12-22, DG-13-38-16 |
| ISSR 5 | 1st Belitsa 2<br>2nd : Sveta Petka 1<br>3rd : Sveta Petka 2<br>4th : Skitia<br>5th: DG-9-12-11-18<br>6th: DG-12-11-20<br>7th: Blyan, Ustrem, Beslet, DG-9-11-2, DG-17-38-38, DG-13-12-22, DG-13-38-16<br>8th: Trakia, DG-9-11-1 |

| | |
|---|---|
| ISSR 6 | 1st: Belitsa 2, Sveta Petka 1, Sveta Petka 2<br>2nd: Blyan, Beslet, DG-17-38-38, DG-13-38-16<br>3rd Trakia, DG-9-11-1, DG-9-11-2, DG-9-12-11-18, DГ-13-12-22, Scythia<br>4th: Ustrem<br>5th: DG-12-11-20 |
| ISSR 9 | 1st: Belitsa 2, Sveta Petka 1<br>2nd: Sveta Petka 2, Skitia<br>3rd: Blyan, Ustrem, Beslet, Trakia, DG-9-11-1, DG-9-11-2, DG-9-12-11-18, DG-17-38-38, DG-13-12-22, DG-13-38-16<br>4th: DG-12-11-20 |
| ISSR 11 | 1st: Belitsa 2, Sveta Petka 1, Sveta Petka 2, Skitia<br>2nd: Blyan<br>3th: Ustrem, Beslet, Trakia, DG-9-11-1, DG-9-11-2, DG-9-12-11-18, DG-17-38-38, DG-17-38-46, DG-13-12-22, DG-13-38-16, DG-12-11-20 |
| UBC 807 | 1st: Belitsa 2, Sveta Petka 1, Sveta Petka 2, Skitia, Blyan, Ustrem<br>2nd: Beslet, Trakia, DG-9-11-1, DG-9-11-2, DG-9-12-11-18, DG-17-38-38, DG-13-12-22, DG-13-38-16<br>3th: DG-17-38-46, DG-12-11-20 |
| UBC 809 | 1st: Bezhanovo, Sadovets, Trakia<br>2nd: Belitsa 1, Belitsa 2, DG-9-11-1, DG-9-11-2, DG-9-12-11-18, DG-17-38-46<br>3rd: Sveta Petka 1, Sveta Petka 2, Scythia, Blyan, Ustrem<br>4th: Beslet, DG-17-38-38 |
| **iPBSs** | **Polymorphic profiles / accessions** |
| iPBS 2219 | 1st: Bezhanovo, Sadovets, Belitsa 2, Trakia, DG-9-11-1, DG-9-12-11-18, DG-17-38-38, DG-13-12-22, DG-13-38-16<br>2nd: Skitia, Blyan, Ustrem |
| iPBS 2222 | 1st: Bezhanovo<br>2nd: Belitsa 1<br>3rd: Sadovets 1, Belitsa 2, Sveta Petka 1, Sveta Petka 2<br>4th: Skitia, Blyan, Ustrem, Beslet, Trakia, DG-9-11-2, DG-9-12-11-18, DG-17-38-38, DG-17-38-46, DG-13-12-22, DG-13-38-16 |
| iPBS 2224 | 1st: Skitia, Blyan, Ustrem<br>2nd: Belitsa 2, Sveta Petka 2<br>3rd: Beslet<br>4th: DG-9-12-11-18, DG-17-38-38, DG-13-12-22, DG-13-38-16<br>5th: DG-9-11-1, DG-9-11-2, DG-17-38-46, DG-12-11-20 |
| iPBS 2228 | 1st: Belitsa 2, Sveta Petka 2<br>2nd: Skitia, Blyan, Beslet, Trakia, DG-9-11-1, DG-9-11-2, DG-9-12-11-18, DG-17-38-46, DG-13-12-22, DG-13-38-16<br>3rd: Belitsa 1, DG-12-11-20<br>4th: Bezhanevo<br>5th: Sadovets 1, Sveta Petka 1 |
| iPBS 2230 | 1st: Bezhanevo<br>2nd: Belitsa 1, Sveta Petka 1<br>3rd: Skitia, Ustrem, DG-9-11-1<br>4th: Sadovets 1, Belitsa 2, Blyan, Beslet, Trakia, DG-9-11-2, DG-9-12-11-18, DG-17-38-38, DG-17-38-46, DG-13-12-22, DG-13-38-16 |
| iPBS 2252 | 1st: Bezhanovo<br>2nd: Belitsa 2, Sveta Petka 2<br>3rd: Skitia, Blyan<br>4th: Beslet, Trakia, DG-9-11-1, DG-9-11-2, DG-17-38-38 |
| iPBS 2277 | 1st: Belitsa 2, Sveta Petka 1, Sveta Petka 2<br>2nd: Skitia, Blyan |

| | |
|---|---|
| | 3rd: Ustrem, Trakia, DG-9-11-1, DG-9-11-2, DG-9-12-11-18, DG-13-12-22, DG-12-11-20<br>4th: Beslet, DG-17-38-46<br>5th: DG-13-38-16 |
| iPBS 2298 | 1st: Bezhanevo<br>2nd: Belitsa 2, Sveta Petka 1, Sveta Petka 2<br>3rd: Skitia<br>4th: Blyan<br>5th: Sadovets, Belitsa 1<br>6th: DG-9-11-1, DG-9-11-2, DG-17-38-46<br>7th: DG-9-12-11-18, DG-17-38-38, DG-13-12-22 |
| iPBS 2394 | 1st: Bezhanevo<br>2nd: Sadovets 1<br>3rd: Belitsa 1<br>4th: Belitsa 2, Sveta Petka 1, Sveta Petka 2<br>5th: Trakia, DG-9-11-1, DG-9-12-11-18<br>6th: Skitia, Blyan, Ustrem, Beslet, DG-9-11-2, DG-17-38-38<br>7th: DG-17-38-46 |

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# Selection of Drought-Tolerant Common Bean Accessions in Organic Production Systems (Task 1.5.2)

Method of **treatment with $PEG_{6000}$** to simulate drought stress was previously published (See the link https://www.agrojournal.org/28/03-10.html). The plants were grown with a liquid of ½ MS (Murashige and Skoog) nutrient solution without $PEG_{6000}$ as control, and treated with the same liquid containing 20% $PEG_{6000}$ (equivalent to – 1.2 MPa).

The selection of tolerant forms was carried out **in greenhouse and in field** in three stages.

**Proline levels and water content** were measured during the pre-screening of 22 accessions in greenhouse pots, from which 7 accessions exhibiting high drought tolerance were selected for field evaluation.

In the second stage, the functional activity of the photosynthetic apparatus (PSA) in the same 22 accessions was assessed by analyzing chlorophyll fluorescence parameters after $PEG_{6000}$ treatment. The parameters measured included the maximum quantum yield of photosystem II (Fv/Fm), the ratio of maximum fluorescence to initial fluorescence (Fm/Fo), the ratio of variable fluorescence to initial fluorescence (Fv/Fo), and the time to reach maximum fluorescence (Tfm). The data on PSA activity corroborated the pre-screening results based on proline concentrations in the drought-tolerant accessions. Chlorophyll fluorescence parameters were used to calculate the ratios Fv/Fo (potential photochemical efficiency) and Fv/Fm (maximum quantum efficiency of PSII), both of which serve as indicators of PSII efficiency in primary photochemical reactions.

In the third stage, two local accessions and four breeding lines with the highest proline concentrations and photosynthetic performance, compared to the control plants, were sown in the field. Data were collected and will be validated in a second year of evaluation for drought tolerance. In all accessions, drought tolerance was initially assessed in pots using $PEG_{6000}$ treatment, and in most cases, these results were confirmed under field conditions.

The field drought stress treatment was applied using a drip irrigation system, as previously described (see link: http://agrarninauki.au-plovdiv.bg/2021/issue-30/7-30/). Productivity parameters, including leaf and stem mass, pod and seed weight, number of pods and seeds per plant, and weight of 100 seeds, were recorded for both treated and untreated plants. Based on relative water content and proline accumulation under induced drought stress in the 2023 field experiment, 17 genotypes were identified as sensitive or tolerant, including: (№19),

(№22), DG-17-38-38, DG-17-38-16, DG-12-11-18, Sveta Petka (6), Sveta Petka (5), Blyan, Trakia, Skitia, DG 9-11-2, DG 13-12-22, M197-3, M198-1, M207-3, M2012-4, and Mastilen (Table 4).

The experiment included a control group (optimal irrigation) and a treated group (drought stress). Drought stress lasted for 15 days, after which stressed plants were watered weekly, while control plants received water twice a week. Biometric parameters, such as leaf and stem weight, and pod number and weight per plant, were key indicators of drought tolerance. Significant reductions in these traits under drought stress indicated sensitivity to water scarcity.

Under optimal irrigation, leaf mass ranged from 29.50 to 43.50 g, while under drought conditions it dropped to 19.34 to 35.50 g. Stem mass ranged from 40.55 to 58.66 g under normal irrigation, decreasing to 23.33 to 45.25 g under drought. Tolerant variants produced the heaviest pods (115.56–140 g under normal irrigation), while sensitive variants showed a >20% decrease in pod weight under reduced irrigation (Table 4).

Yield components (Table 4) varied under normal irrigation, with pod numbers ranging from 28.45 to 34.33 and seeds per pod from 3.33 to 4.52. ‘Skitia’ had the highest total seed count (126.25), followed by №19 (125.84). Nine variants, including DG-12-11-18, 'Blyan', and 'Mastilen' 11b, had seed counts between 117.37 and 124.28. Except for 'Sveta Petka' (6) (74.93 g), 100-seed weight ranged from 40.62 to 44.03 g, with five samples falling below 35 g (Table 4).

Reduced irrigation significantly reduced productivity, with pod numbers decreasing by 53.54–64.87% in sensitive variants and 33.7–66.3% in seed count. The mass of 100 seeds decreased by 5.2–10 g in all samples, with the highest losses in DG 9-11-2, DG-17-38-16, 'Sveta Petka' (6), №22, and DG 13-12-22 (9.6–11.74 g), while tolerant variants like Skitia and Blyan showed a reduction of 5–7 g (Table 4).

Drought-stressed plants showed delayed budding and flowering (5–8 days later) compared to well-watered plants. Flower drop was significant across all variants, and pod-setting occurred about 5 days later, with many fruits smaller and seeds underdeveloped. Botanical maturity in drought-stressed plants occurred 10 days earlier, likely due to consecutive 7-day drought cycles combined with high temperatures and low humidity. Premature leaf abscission was also observed.

Reduced irrigation during bud formation, flowering, and fruit/seed development significantly impacted all yield components. Pod weight decreased by over 20% in sensitive variants (№22, Sveta Petka 6, DG 9-11-2, DG 13-12-22). However, variants like №19, DG-17-38-38, DG-12-11-18, Sv. Petka (5), M 197-3, M 198-1, M 2012-4, Skitia, and Blyan showed good performance and are suitable for breeding programs focused on drought resistance.

Following the 2023 field experiment, the genotypes selected for further study in 2024 include Mastilen, №19, M197-3, M198-1, M207-3, M2012-4, DG17-38-38, DG12-11-18, and Sveta Petka (6). Plant height ranged from

26.65 to 30.60 cm in Mastilen, №19, M197-3, M198-1, M207-3, and M2012-4, while DG17-38-38 and DG12-11-18 reached 37 cm, and Sveta Petka reached 42 cm (Table 5, Figure 2). Flowering began at 50–55 days for most genotypes and 60 days for Sveta Petka. At eight weeks, the main stem reached its maximum height: 59.38–63.38 cm for all variants except DG17-38-38 and DG12-11-18 (82.85–87.50 cm, Figure 2).

Fresh leaf weight ranged from 37.50 g to 58.55 g at eight weeks, except for Sveta Petka (73.50 g). Stem mass ranged from 80.25 g to 88.55 g for DG-17-38-38 and DG-12-11-18, and 37.50 g to 58.55 g for Mastilen, №19, M197-3, M198-1, M207-3, and M2012-4. Yield component analysis (Table 6) showed that the average number of pods per plant ranged from 31.00 (Mastilen) to 36.55 (№19), while the average number of seeds per pod varied from 3.90 (Mastilen) to 4.22 (DG-12-11-18). The highest number of seeds per plant was recorded in Sveta Petka (157.01), followed by №19 (146.93) and DG 17-38-38 (144.00). 100-seed weight ranged from 36.00 g to 41.75 g, except for Sveta Petka, which had significantly larger seeds (76.55 g).

Drought stress is a critical factor impacting plant development. To better understand plant responses to water deficit, we initiated a study to identify drought-responsive proteins and their associated genes in Bulgarian common bean accessions.

## Conclusion on Drought-Tolerant Common Bean Accessions

All the studied genotypes exhibited consistency in key morphological traits and showed high productivity. The genotypes with the best yield components were No. 19, DG17-38-38, M2012-4, M197-3, and Sveta Petka (6). These selected accessions have strong potential for inclusion in breeding programs focused on developing new common bean varieties with enhanced agronomic traits.


**Figure 2**. Experimental Setup for Drought Stress (**A, B**); Common bean plants at: **C**) 14 days; **D**) 4 weeks; **E**) 5 weeks; **F**) 6 weeks Plants during different drought periods (**G-I**); Common bean plants with different habit (**J, K**); Common bean plant with fully developed fruits (**L**); **M**) Common bean plant with fully developed fruits; N) Fruit coloration in the studied variants.

**Table 4.** Effect of Water Stress on Leaf, Stem, Pod Mass and Yield Components

| Accession | Leaf Massg | Stem Massg | Pods, g | Leaf Mass, g | Stem Massg | Pods, g | Pods Reduced/ Normal Regime, % | Number of pods per plant | Number of Seeds per Pod | Number of Seeds per Plant | 100-Seed Weight, g | Number of Pods per Plant | Number of Seeds per Pod | Number of Seeds per Plant | 100 Seed Weight, g |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Normal Irrigation Regime (NIR)** | | | | **Reduced Irrigation Regime (RIR)** | | | | **NIR** | **NIR** | **NIR** | **NIR** | **RIR** | **RIR** | **RIR** | **RIR** |
| (№19) | 30.67 | 49.67 | 140.00 | 24.33 | 35.27 | 120.75 | 86.25 | 34.33 | 4.52 | 125.84 | 40.62 | 23.05 | 3.02 | 84.72 | 35.42 |
| (№22) | 29.50 | 45.50 | 97.28 | 19.34 | 23.33 | 75.55 | 77.65 | 28.75 | 3.88 | 115.55 | 30.62 | 18.65 | 2.89 | 52.57 | 20.08 |
| DG-17-38-38 | 37.50 | 55.50 | 120.85 | 30.25 | 42.50 | 100.25 | 82.95 | 34.20 | 3.39 | 120.94 | 38.72 | 24.00 | 2.82 | 78.96 | 32.05 |
| DG-17-38-16 | 36.50 | 56.25 | 112.30 | 29.66 | 40.50 | 90.55 | 80.63 | 30.50 | 3.75 | 114.38 | 32.10 | 20.78 | 3.04 | 59.25 | 20.36 |
| DG-12-11-18 | 38.50 | 58.66 | 115.56 | 28.66 | 42.66 | 100.66 | 87.11 | 30.16 | 4.02 | 121.25 | 38.55 | 21.66 | 3.32 | 78.55 | 30.03 |
| Sveta Petka (6) | 34.50 | 50.25 | 119.25 | 25.05 | 40.50 | 95.25 | 79.87 | 28.45 | 3.75 | 90.75 | 29.75 | 20.56 | 3.00 | 51.10 | 19.75 |
| Sveta Petka (5) | 69.50 | 70.50 | 175.33 | 53.46 | 58.66 | 145.00 | 82.84 | 33.25 | 3.53 | 117.37 | 74.93 | 18.25 | 2.80 | 74.68 | 69.10 |
| Blyan | 38.50 | 55.66 | 115.90 | 22.25 | 45.50 | 105.25 | 90.81 | 29.33 | 4.22 | 123.77 | 33.71 | 20.40 | 2.30 | 76.12 | 29.30 |
| Trakia | 34.25 | 45.24 | 95.75 | 26.55 | 35.33 | 85.25 | 89.03 | 30.16 | 3.74 | 112.80 | 35.14 | 20.55 | 2.01 | 49.35 | 28.56 |
| Skitia | 36. | 40. | 115. | 26.5 | 28. | 104. | 89.85 | 31.72 | 3.98 | 126.2 | 31.72 | 21.35 | 1.81 | 70.88 | 25.88 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 50 | 55 | 75 | 0 | 55 | 00 | | | | 5 | | | | | |
| DG 9-11-2 | 35.25 | 46.50 | 99.00 | 25.66 | 37.66 | 70.00 | 77.77 | 29.25 | 3.57 | 104.43 | 28.54 | 15.66 | 1.20 | 35.19 | 16.23 |
| DG 13-12-22 | 39.50 | 48.25 | 108.75 | 27.25 | 36.00 | 85.25 | 78.39 | 28.50 | 3.34 | 95.10 | 38.94 | 17.80 | 2.13 | 37.91 | 29.34 |
| M 197-3 | 43.00 | 47.50 | 130.00 | 34.00 | 41.50 | 115.25 | 98.65 | 32.80 | 3.85 | 124.28 | 39.80 | 24.90 | 1.89 | 76.84 | 32.55 |
| M -198-1 | 42.50 | 48.33 | 132.55 | 32.33 | 30.33 | 118.50 | 89.04 | 32.40 | 3.71 | 120.20 | 41.18 | 22.85 | 1.92 | 78.56 | 35.67 |
| M-207-3 | 45.00 | 52.55 | 130.50 | 34.50 | 45.25 | 115.67 | 88.64 | 30.33 | 4.26 | 120.55 | 44.03 | 23.50 | 1.89 | 74.00 | 38.69 |
| M 2012-4 | 43.50 | 49.50 | 132.50 | 35.50 | 40.50 | 118.45 | 89.40 | 34.00 | 3.62 | 123.08 | 41.55 | 23.45 | 1.85 | 74.80 | 36.35 |
| Mastilen 11b | 35.50 | 41.50 | 110.75 | 28.67 | 31.66 | 96.75 | 87.36 | 30.66 | 3.84 | 117.73 | 39.60 | 23.66 | 2.05 | 68.50 | 31.90 |

| **Accessions** | **Number of Pods per Plant** | **Number of Seeds per Pod** | **Number of Seeds per Plant** | **Weight of 100 Seeds, g** | **Leaf Mass, g** | **Stem Mass, g** | **Pods, g** |
|---|---|---|---|---|---|---|---|
| Mastilen 11b | 31.00 | 3.90 | 120.90 | 36.00 | 37.50 | 62.55 | 105.25 |
| DG-17-38-38 | 36.00 | 4.00 | 144.00 | 40.50 | 45.52 | 88.55 | 145.33 |
| DG-12-11-18 | 32.26 | 4.22 | 136.14 | 38.55 | 47.50 | 80.25 | 130.55 |
| (No.19) | 36.55 | 4.20 | 146.93 | 41.75 | 53.67 | 71.33 | 145.85 |
| Sveta Petka (6) | 39.75 | 3.95 | 157.01 | 76.55 | 52.85 | 69.55 | 133.25 |
| M197-3 | 34.50 | 4.05 | 139.73 | 39.50 | 57.66 | 70.33 | 135.55 |
| M198-1 | 33.80 | 3.80 | 128.44 | 40.20 | 53.00 | 80.55 | 135.55 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| M207-3 | 32.55 | 3.85 | 125.32 | 40.50 | 58.55 | 85.50 | 140.55 |
| M2012-4 | 35.20 | 4.02 | 141.50 | 41.20 | 73.50 | 100.50 | 180.50 |

**Table 5.** Yield component and Fresh weight of leaves, stems and pods

**Table 6.** Periodic growth and statistical parameters of the plant height trait (cm)

| | Plant age (weeks) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | | 4 | | 6 | | 8 | | 10 | |
| | Range | Means ±SE | Range | Means ±SE | Range | Means±SE | Range | Means±SE | Range | Means ±SE |
| Mastilen 11b | 9.70-13.90 | 11.8± 0.80 | 23.2-30.10 | 26.65 ±0.85 | 36.30 - 44.60 | 40.45 ±1.2 | 52.80-63.50 | 58.15±1.42 | 52.20-60.90 | 56.55 ±1.36 |
| DG17-3838 | 15.25-19.30 | 17.27 ±0.4 | 33.55-38.80 | 36.18 ±0.45 | 54.95 - 60.50 | 57.73 ±0.59 | 85.55-89.45 | 87.5 ±0.42 | 72.30-83.40 | 77.85 ±1.08 |
| DG12-1118 | 16.60-19.45 | 18.03 ±0.31 | 35.45-39.45 | 37.45 ±0.37 | 56.25 - 62.80 | 59.25 ±0.9 | 80.25-85.45 | 82.85±0.52 | 69.50-80.50 | 75.0± 1.52 |
| No.19 | 10.20-14.3 | 12.3± 1.01 | 25.50-32.10 | 28.95 ±0,77 | 38.20 - 46.20 | 42±1.45 | 54.60-65.30 | 58.95±1.25 | 54.50-62.80 | 58.65 ±1,48 |
| M 197-3 | 10.55-13.55 | 12.05 ±0.29 | 25.7-33.50 | 29.60 ±0.81 | 40.20 - 45.50 | 42.85 ±0.53 | 54.2-64,55 | 59.38±0.91 | 50.50-64.20 | 57.35 ±1.23 |
| M 198-1 | 11.20-13.80 | 12.05 ±0.27 | 26.80-33.70 | 30.25 ±0.71 | 32.40 - 47.50 | 39.95 ±1.62 | 56-40-63.20 | 59.8 ±0.94 | 49.80-56.80 | 53.30 ±0.68 |
| M 207-3 | 11.90-14.66 | 13.28 ±0.39 | 26.90-32.45 | 29.68 ±1.18 | 34.69 - 48.55 | 41.67 ±1.35 | 58.50-65.30 | 61.0 ±1.41 | 50.45-60.56 | 55.5± 1.36 |
| M 2012-4 | 12.20-16.80 | 14.5± 0.59 | 28.80-32.40 | 30.6± 0.63 | 44.35 - 55.80 | 50.08 ±0.87 | 60.25-66.50 | 63.38±0.75 | 58.20-68.20 | 63.20 ±0.94 |
| Sveta Petka | 17.80-23.55 | 20.68 ±0.67 | 38.75-45.25 | 42.0± 0.76 | 68.80 - 78.55 | 73.68 ±1.06 | 100.55 - 130.25 | 115.4±1.93 | 120.45-129.80 | 125.12±1.05 |

## 2D-DIGE Gel-Based Proteomics and Western Blot Analyses of Drought Tolerant Genotypes

Subsequently after drought stress treatment and untreated control plants the total leaf protein extracted, separated to two dimensions, up- and down-regulated peptides selected by Image Master 2D Platinum 7.0, and analysed on Mass Spectrometry Identification of Selected Spots. A change in protein expression was summarized by Hierarchical Cluster Analysis (HCA) with MatLab software according to standard algorithms. From the 22 pre-screened genotypes, two mutant lines (26, and 19) with the highest proline levels were selected as drought-tolerant based on field evaluation, including assessments of productivity and photosynthetic performance (PSA). Additionally, a drought-sensitive mutant line (22) was chosen as the control. Line 22, on one side, and Lines 26 and 19, on the other, exhibited the highest separation in the PC1 analysis and displayed the most significant phenotypic differences after drought stress. They also represented the extremes in productivity, with Line 26 having the highest and Line 22 the lowest, as confirmed by PSA results. In the next stage of evaluation, these two breeding lines with the most distinct responses to water deficit were selected for proteome and Western blot analysis that further highlighted their contrasting drought tolerance and sensitivity. We found a relation between rates of photosynthesis and accumulation of ribulose-1,5-bisphosphate carboxylase in the tolerant accession, thus securing the plant productivity in drought conditions. In parallel, in result of Western blot analysis we found that the accumulation of molecular chaperones such as dehydrins and Small Heat Shock Proteins (sHSPs) is required to ensure plant survival and productivity during drought stress. The identified genes responsible for the reaction of common bean plants, which in the future will allow their manipulation or simply the selection of favorable accessions. This study was published (See the link https://www.mdpi.com/2073-4395/13/4/1022).

## LFQ LC-MS/MS Proteomic Studies on Bulgarian Common Bean Accessions Subjected to Water Deficit

Through the described lab screening and field drought tests, four tolerant and one sensitive (M22) genotype were selected for isotope-free quantitative protein analysis involved extraction from leaves using the phenol protocol (Faurobert, 2007), reduction/alkylation, digestion, and processing. Peptides were analyzed using LFQ LC-MS/MS on a Q Exactive Plus mass spectrometer with an EASY-Spray column (15 cm, 75 µm ID, PepMap C18, 3 µm particles, 100 Å pore size) connected to an Ultimate 3000 RSLnano system. Data were processed in Proteome Discoverer 2.2 and MaxQuant to identify significantly regulated proteins ($p<0.05$) in *P. vulgaris* genotypes.

In genotype **DG-17-38-38**, a total of 10 proteins were identified: 5 proteins were expressed in both control and treated plants; 5 were unique to plants treated by water deficit. A total of 38 proteins in breeding line

**DG-17-38-16** were detected, with 12 common to both treated and untreated plants, while 26 were exclusive to untreated plants. In **DG-12-11-18**, 14 proteins were identified: 2 in both treated and untreated plants, 7 only in untreated, and 5 in treated.

In **‘Sveta Petka’**, 5 proteins were found in both untreated and treated samples, 17 only in untreated, and 3 in treated.

Fifteen drought tolerance response unique proteins were identified, with their associated genes listed in the UniProt database: petA, rps 7-A/B, PEP-BETA, bpgip4, THI-1, ATPA, accD, GATA, ABAGT, rpoC1, petD, ndhA, PFK, PSI-N, and eif4E (Table 7).

In the selected sensitive genotype (M22), **130 proteins** were identified, with 42 found in both treated and control plants, 38 exclusive to untreated plants, and 50 exclusive to treated plants, differing from those in tolerant genotypes. The sensitive genotypes exhibit a greater number of proteins due to their heightened physiological response to environmental factors. Sensitive plants activate multiple stress pathways to reduce its effects, expressing proteins such as chaperones and antioxidants, while simultaneously triggering additional protective mechanisms and proteins for dehydration tolerance. By contrast, **tolerant genotypes** have **more efficient adaptive mechanisms** that require a smaller number of proteins. They regulate stress with minimal energy expenditure, maintaining homeostasis through a smaller number of key proteins. Most of the identified proteins in this study are involved in proline biosynthesis, photosynthesis, primary metabolism, cellular homeostasis, stress signaling, and recovery processes.

## Investigated were protein concentrations in mature common bean seeds

Protein concentration (in µg/µL) was measured spectrophotometrically following the protein extraction protocol by Laemmli (1970). The highest protein concentration in the seeds was found in the breeding line DG-17-38-38 (8.71 µg/µl), while the lowest was in the local sample Belitsa 2 (2.50 µg/µl).

**Figure 3.** Protein concentration in common bean seeds

**Table 7.** Identified unique proteins in response to drought stress in all studied common bean accessions.

| **Protein** | **Gene** | **Function** |
|---|---|---|
| Cytochrome f | *petA* | Crucial for photosynthetic electron transport, energy production, osmotic regulation, and activation of drought stress responses (Silva et al., 2024). |
| Small ribosomal subunit protein uS7cz/ uS7cy | *rps7-B / rps7-A* | Ensures ribosome assembly, translational fidelity, and stress-responsive protein synthesis, maintaining cellular homeostasis under drought stress (Yadav et al., 2024) |
| Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit beta | *PEP-BETA* | Regulates energy and carbon metabolism, aiding drought stress adaptation (Wang et al., 2016). |
| 50S ribosomal protein L17, chloroplastic | *bpgip 4* | Maintains chloroplast function, supports protein synthesis, and facilitates stress responses under water-limited conditions (Pishchik et al., 2024). |
| Thiamine thiazole synthase, chloroplastic | *THI-1* | Supports thiamine production, enhances energy metabolism, and regulates oxidative stress for drought adaptation (Yin et al., 2022). |
| ATP synthase subunit alpha, mitochondrial | *ATPA* | ATP production, which supports energy-dependent processes, metabolic adaptations, and stress responses during drought conditions. Its role in maintaining cellular functions and promoting plant resilience underscores its importance in managing drought stress effectively (Ibrahim et al., |

| | | |
|---|---|---|
| | | 2023). |
| Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic | *accD* | Regulates lipid metabolism, energy storage, and membrane integrity, supporting drought adaptation (Wu et al., 2024). |
| Glutamyl-tRNA(Gln) amidotransferase subunit A, chloroplastic/mitochondrial | *GATA* | Facilitates glutamine synthesis, nitrogen assimilation, and metabolic adaptation for drought tolerance (Jiang et al., 2019). |
| Glycosyltransferase | *ABAGT* | Modifies cell structures, synthesizes protective compounds, and regulates metabolic pathways for drought resistance (Li et al., 2017; Liu et al., 2021). |
| DNA-directed RNA polymerase subunit beta' | *rpoC1* | Regulates gene expression crucial for stress adaptation, ensuring resilience under drought conditions (Ahmad et al., 2017). |
| Cytochrome b6-f complex subunit 4 | *petD* | Supports energy production, ROS management, and overall drought adaptability in plants (Li et al., 2021; Degen et al., 2024). |
| NAD(P)H-quinone oxidoreductase subunit 1, chloroplastic | *ndhA* | Manages oxidative stress, regulates photosynthesis, and enhances metabolic flexibility under drought (Li and Wang, 2023). |
| ATP-dependent 6-phosphofructokinase | *PFK* | Regulates energy production, metabolic flexibility, and stress-adaptive biochemical pathways (Mehari et al., 2022). |
| Photosystem I-N subunit | *PSI-N* | Stabilizes photosynthesis, supports electron transport, and protects against oxidative stress under drought (Hu et al., 2023; Zeng et al., 2024). |
| Eukaryotic translation initiation factor 4E-1 | *eif4E* | Regulates protein synthesis, facilitates translation of stress-related genes, and integrates signaling pathways for drought adaptation (Sukarieh et al., 2009; Marondedze et al., 2020). |

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# Integrating phytopathological and molecular approaches for disease resistance in bulgarian and slovenian common bean (*Phaseolus vulgaris*) breeding (Task 1.5.3)

Common bean (*Phaseolus vulgaris*) is essential for global nutrition, but its production is threatened by diseases like anthracnose, halo blight, and common bacterial blight, leading to significant yield losses (Singh and Schwartz, 2010). Disease resistance breeding is a key strategy for maintaining production, especially in organic systems (Miklas et al., 2006). Evaluating resistance in Bulgarian bean lines requires combining phytopathological methods and molecular techniques such as PCR and marker-assisted selection to identify resistance genes and quantitative trait loci (Ferreira et al., 2013). Additionally, local genetic resources may harbor alleles for drought tolerance and abiotic stress resilience (Guzmán et al., 2017).

Resistance to bacterial blight (XPP) and halo blight (PSP) was assessed under field and greenhouse conditions. Inoculations were performed at the R6 and R8 stages using a bacterial suspension with an optical density (OD) of 50-55. Disease severity was evaluated 14 days after inoculation using a 9-point scale, and the resistance was categorized based on the mean disease index (MDI) (Figure 4).

For resistance to *Colletotrichum lindemuthianum* (CL), plants were inoculated with a spore suspension of $10^6$ spores/mL at the primordial leaf stage. Disease reactions were recorded 10 days later using the same 9-point scale (Figure 5).

Genomic DNA was extracted from young leaves using the CTAB method, with quality and quantity assessed using a NanoDrop spectrophotometer and gel electrophoresis. SCAR markers were

amplified in a 20 µL PCR reaction, and the products were visualized on a 1% agarose gel with a 100 bp DNA ladder for size estimation (Table 8).

### 1. Germplasm from Dobrudja Agricultural Institute, General Toshevo

The study included 9 breeding lines and 5 varieties of dry beans registered in Bulgaria (Table 9). Among the breeding lines, DG 17-38-16 and DG 17-38-38 are resistant to race 6 of *Colletotrichum lindemuthianum* (CL), while DG 13-12-22 is resistant to race 8. DG 9-11-1, DG 13-12-22, DG 17-38-38, and DG 17-38-46 are resistant to race 320. Most lines are also resistant to both races of *Pseudomonas savastanoi* pv. *phaseolicola* (PSP). For *Xanthomonas phaseoli* pv. *phaseoli* (XPP), DG 9-11-2, DG 17-38-16, DG 17-38-38, and DG 17-38-46 are resistant. The line with the highest resistance index (R-index, ratio of resistant to susceptible reactions) across the studied races and strains is DG 17-38-38, with an R-index of 83%.

The *Trakia* variety is resistant to three CL races, including race 81, while the *Beslet* variety has the highest R-index at 75%.

### 2. Local Common Bean Accessions

Table 10 presents the reaction of six local bean accessions from Bulgaria to the causative agents of anthracnose, halo blight, and common bacterial blight. The accession *Belitsa 2* is highly resistant to anthracnose (CL) races 8, 81, and 320. All accessions are resistant to both races of *Pseudomonas savastanoi* pv. *phaseolicola* (PSP) but susceptible to both strains of *Xanthomonas phaseoli* pv. *phaseoli* (XPP). The highest R-index is observed in *Sveta Petka 1*, which is resistant to CL races 8 and 320 as well as both races of PSP.

### 3. Slovenian Common Bean Accessions

Four of the studied accessions are resistant to the five races of *CL* and the two races of *PSP* (Table 11). Samples JERUZALEMSKI and 230/2/BABC are resistant to the two races of PSP, but susceptible to the five races of anthracnose.

### 4. Mutant Lines with the Parent Variety Mastilen 11b

The parent variety *Mastilen 11b* is susceptible to all four races of anthracnose and both races of halo blight (Table 12). With the exception of M 2012-4, the remaining mutant lines exhibit the same reaction to the studied pathogens as the parent variety. M 2012-4 shows moderate resistance to CL race 81. This mutation is significant, as most bean varieties registered in the country are susceptible to this race (Table 9).

## 5. Mutant Lines with the Initial Variety IP 564 (Evros)

The variety *IP 564* is resistant to CL races 6 and 81 and exhibits moderate resistance to both races of *Pseudomonas savastanoi* pv. *phaseolicola* (PSP). However, it is sensitive to both strains of *Xanthomonas phaseoli* pv. *phaseoli* (XPP). Six mutant lines show varying degrees of resistance to the three pathogens, with an R-index of 100% (Table 13). Given that *IP 564* is sensitive to both strains of XPP, it can be concluded that the mutations in these lines have resulted in increased resistance to bacterial blight. Additionally, these lines also show an enhanced level of resistance to both races of PSP.

## 6. Resistance Analysis of *P. vulgaris* Breeding Lines, Bulgarian Varieties, and Local Accessions to Major Pathogens Using Phytopathological and Molecular Methods

### 6.1. Resistance to *C. lindemuthianum* (CL)

Resistance to CL is predominantly race-specific. As of 2017, 17 loci controlling resistance in common beans were noted in the LIST OF GENES - *Phaseolus vulgaris* L. (http://bic.css.msu.edu/Genetics.cfm). The *Co-2* gene, initially reported as Are, is localized in the *Pv11* group of the integrated genetic map of *Phaseolus vulgaris* and is near two race-specific genes (*Ur-3* and *Ur-11*) controlling resistance to bean rust.

The SCAR marker SQ4 included in the study is closely linked to genes *Co-2* and *Ur-11*. According to literature data, the length of the amplified fragment is 1440 bp. The Co-2 gene is part of the Pv11 group of the genetic map of *P. vulgaris*. This genetic region is critical because it contains genes responsible for race-specific resistance to bean rust, a disease caused by the fungal pathogen *Uromyces appendiculatus*. The gene Co-2 is located near two other genes, Ur-3 and Ur-11, which specifically confer resistance to different races of the rust pathogen. The SCAR marker SQ4 is mentioned, which is a sequence-characterized amplified region marker used in the study. The marker is closely linked to both the Co-2 and Ur-11 genes, which are key in conferring resistance to bean rust.

The study uses PCR to amplify a specific fragment, which serves as a molecular indicator of the presence of certain genes. According to the literature, the expected length of this amplified fragment is **1440 base pairs (bp).** This fragment size acts as a marker for the presence of the linked genes **Co-2** and **Ur-11**.Total of the 25 samples studied, the expected fragment was amplified only in DG 13-12-32 and Sveta Petka 1 (Table 14). The data in the table show that line DG 13-12-32 is sensitive to race 8, overcoming resistance to *Co-2*, but the sensitive reaction to races 6, 81 and 320 rejects the hypothesis of the presence of *Co-2* since this gene provides resistance to these races. The presence of an amplified fragment in Sveta Petka 1 also contradicts the phytopathological analysis. The reaction of the sample to race 8 rejects the hypothesis of the presence of *Co-2*. Since marker SQ4 is linked to

genes *Co-2* and *Ur-11*, it can be assumed that both samples possess the *Ur-11* gene controlling resistance to bean rust.

Both DG 13-12-32 and Sveta Petka 1 show the presence of the 1440 bp amplified fragment, which indicates that these lines carry *Ur-11* (a race-specific gene for resistance to bean rust) (Table 14). However, the *Co-2* gene does not seem to be present in either line based on the reaction to different races of the pathogen. *Ur-11* is, therefore, the more likely candidate for the resistance observed in these two samples, which fits with the data indicating that *SQ4* marker is more closely linked to *Ur-11* than to *Co-2*.

## 6.2. Resistance to *P. s. pv. phaseolicola* (PSP)

Current research focusses on the **resistance of** common **beans** (*P. vulgaris*) to **halo blight**, a disease caused by the bacterium *Pseudomonas syringae pv. phaseolicola* (PSP). The resistance in beans to halo blight is primarily **race-specific**, meaning that different races of the pathogen can be resisted by different genetic mechanisms in the beans. Resistance in beans to the causative agent of halo blight is predominantly race-specific. At this time, 6 loci have been identified, marked as *Pse*-genes. The *Pse-1* gene is localized in Pv10 of the integrated genetic map and controls resistance to races 1, 5, 7 and 9. The *Pse-3* gene is localized in Pv02 and controls resistance to races 3 and 4. In the present study, SW13 for *Pse-3* (expected fragment - 690 bp) and SH11 for *Pse-1* (expected fragment - 800 bp) were used as markers.

The data for the phytopathological evaluation of 25 bean genotypes to races 1 and 6 of PSP show that all samples have a resistant reaction to the studied races (Table 15). The molecular analysis confirms the phytopathological assessment (Table 15, Figure 6). The results obtained from both analyses indicate the presence of both genes in the studied materials. Since at this stage, no gene for resistance to race 6 of the pathogens has been identified, it can be assumed that the resistance in the samples also has a race-non-specific nature.

In conclusion, the results from the molecular analysis (via PCR amplification of the markers SW13 and SH11) confirm the findings from the phytopathological evaluation. The molecular analysis reveals the presence of both resistance genes (*Pse-1* and *Pse-3*) in the studied genotypes, supporting the conclusion that these genotypes have the genetic makeup necessary to resist multiple races of the pathogen. Specifically, the presence of *Pse-1* and *Pse-3* genes was confirmed, both of which are race-specific for certain races of the pathogen. However, the resistance to race 6, for which no specific resistance gene has been identified, hints that additional, non-race-specific mechanisms may be at play, contributing to the overall resistance seen in these beans.

The study underscores the complexity of resistance to plant diseases, where race-specific resistance (such as that conferred by *Pse-1* and *Pse-*

*3*) can be combined with non-race-specific mechanisms, providing broader protection against multiple strains of the pathogen.

### 6.3. Resistance to *X. p. pv. phaseoli* (XPP)

Resistance to bacterial blight is predominantly quantitative. At present, only one dominant gene for resistance to both pathogens has been reported—*Xap-1*, which co-segregates with SAP6 QTL in Pv10. The study included two molecular markers: the SAP6 QTL marker *Xap-1* gene (expected fragment—820 bp) and the SU91 QTL marker (expected fragment—700 bp) (Figure 6).

The ***SU91* gene** refers to a **quantitative trait locus (QTL)** associated with resistance to **bacterial blight** in beans, specifically against the ***Xanthomonas*** pathogens responsible for the disease. While the exact molecular details of **SU91** may not be as widely documented in literature as other well-known resistance genes, it plays a role in the **quantitative resistance** observed in certain bean genotypes, making it an important marker in breeding programs for bacterial blight resistance. Unlike single dominant genes for disease resistance, **SU91** is likely part of **a quantitative resistance system**, meaning its effects are not as strong or simple to trace as those of a single gene but rather depend on the interaction of several genes contributing small effects. The ***SU91* gene** is often studied alongside other **QTLs** and **resistance genes**, such as ***SAP6***, as part of an effort to understand the genetic foundation of bacterial blight resistance. While ***SAP6*** may confer stronger resistance (such as through co-segregation with ***Xap-1***, a dominant resistance gene), ***SU91*** is considered another contributor to the resistance, and its presence helps indicate a broader genetic base for controlling the disease. In the study you referenced earlier, **both the SAP6 and SU91 markers** were used to screen bean lines for their resistance to ***Xanthomonas***. The results showed that some **susceptible genotypes** had amplification for both ***SAP6*** and ***SU91***, indicating that resistance was **quantitative** and involved multiple loci.

The data from the phytopathological analysis show that genotypes DG 9-11-2, DG 17-38-16, DG 17-38-38, DG 17-38-46, M26, M22, Beslet and GTB Skitia are resistant to both strains of XPP (Table 16). In DG 9-11-2, M26 and M22, expected amplification was observed for both markers (Figure 6). In the remaining resistant samples, amplification was observed only for SAP6. It is important to note that lines DG 17-38-16 and DG 17-38-38 are the progeny of the cross Beslet x GTB Skitia, in which amplification was observed for marker SAP6.

Beslet and GTB Skitia are the first Bulgarian varieties with high resistance to more than 50 strains of the pathogen. This shows that the SCAR marker for *SAP6* is suitable for the indirect identification of resistance in generations of crosses with their participation. In some susceptible genotypes, amplification was observed for both *SAP6* and *SU91* genes. The presence of amplification in susceptible genotypes is

probably due to the quantitative nature of the inheritance of resistance. It is important to note that resistance to bacterial blight is also strain-specific. For example, line DG 9-11-1 exhibits varying degrees of resistance to the strains used in the phytopathological analysis.

Among these, DG 9-11-2, M26, and M22 showed the expected 820 bp fragment for both markers (SAP6 and Xap-1), suggesting the presence of both resistance genes.

Beslet and GTB Skitia are identified as the first Bulgarian varieties with high resistance to more than 50 strains of the bacterial blight pathogen.

These varieties were used as parents in a cross to produce progenies like DG 17-38-16 and DG 17-38-38, which show resistance through the SAP6 marker.

**SU91** plays an important role in providing **partial resistance** that can be **stably inherited** across generations. This resistance, when combined with other resistance loci like **SAP6**, provides a more **durable and comprehensive solution** to managing bacterial blight in bean breeding programs. This indicates that SAP6 could be a reliable marker for selecting resistance in the progeny of these varieties.

The fact that these varieties confer resistance to a wide range of bacterial strains is significant for breeding resistance.

## Conclusion on phytopathological and molecular approaches for disease resistance in common bean

On the basis of the results presented in this study, the most suitable common bean accessions for organic production were identified based on their resistance to three major pathogens: *Colletotrichum lindemuthianum* (CL), *Pseudomonas savastanoi* pv. *phaseolicola* (PSP), and *Xanthomonas phaseoli* pv. *phaseoli* (XPP).

- **Accessions resistant to all three pathogens:**

o **DG 17-38-38** (R-index 83%) demonstrated high resistance across multiple races of CL, PSP, and XPP, making it the most promising line for organic production.

o **Beslet** (R-index 75%) exhibited a high level of resistance to all three pathogens, confirming its potential suitability.

o **DG 17-38-46** (R-index 75%) showed consistent resistance across the studied diseases.

o **M 564-190-3-7-1, M 564-190-3-7-5, M 564-193-8, and M 564-193-11** (R-index 100%) from the mutant lines of IP 564 demonstrated the highest level of resistance and should be considered for further organic breeding programs.

o

- **Accessions resistant to two pathogens:**

  - **Trakia, GTB Skitia, DG 9-11-2, and DG 17-38-16** exhibited good resistance to CL and PSP but moderate resistance to XPP.

  - **Belitsa 2 and Sveta Petka 1** from local Bulgarian accessions showed resistance to CL and PSP but were susceptible to XPP.

  - **CIPRO** (Slovenian accession) showed resistance to PSP and moderate resistance to CL.

  -

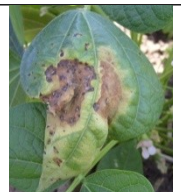
- **Accessions resistant to only one pathogen:**

  - Several accessions, including some breeding lines and local varieties, displayed resistance to only one pathogen but remained susceptible to the other two, limiting their potential for organic production.

Overall, **DG 17-38-38, Beslet, DG 17-38-46, and selected mutant lines of IP 564 (M 564-190-3-7-1, M 564-190-3-7-5, M 564-193-8, and M 564-193-11)** are the most suitable for organic production due to their high and consistent resistance to all three pathogens. These accessions should be prioritized for further breeding programs aimed at developing disease-resistant common bean varieties for sustainable agriculture.

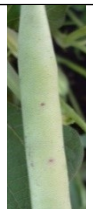






| 1 | 3 | 5 | 7 | 9 |
|---|---|---|---|---|

Scale for recording leaf reaction to *P. s. pv. Phaseolicola*





| 1 | 3 | 5 | 7 | 9 |
|---|---|---|---|---|

Scale for recording pods reaction to *P. s.* pv. *phaseolicola* и *X. p.* pv. *Phaseoli*





| 1 | 3 | 5 | 7 | 9 |
|---|---|---|---|---|

Scale for recording leaf reaction to *X. p.* pv. *Phaseoli*

**Figure 4.** Nine-point scales for assessing leaf and pod reaction to halo and bacterial blight

**Figure 5.** Test for for hallo blight, anthracnose

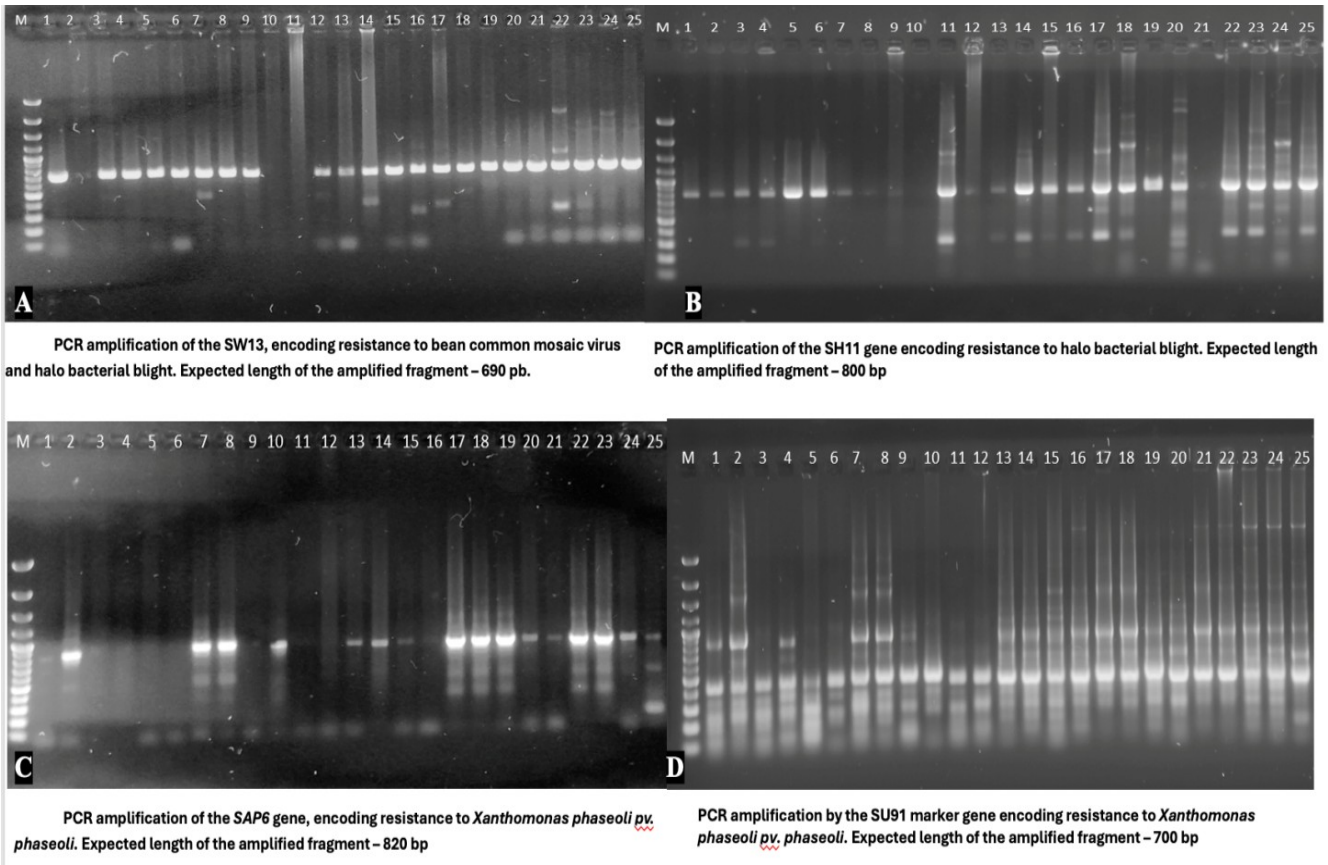



**Figure 6.** SCAR reactions: A) SW13, Halo bacterial blight; B) SH11, Halo bacterial blight; C) SAP5, Common bean blight (*Xanthomonas phaseoli pv. phaseoli*; D) SU91, *X. phaseoli pv. phaseoli*

**Table 8.** SCAR markers used for identifying resistance to pathogens in common bean

| SCAR Name | Marker of Origin | Pathogen | Size (bp) /orientation | Tagged Locus | LG | Reference |
|---|---|---|---|---|---|---|
| SAP6 | AP6 | Common Bacterial Blight (CBB) | 820 bp | Major QTL (GN#1 sel 27) | 10 | Miklas et al., 2000 |
| SU91 | U9 | CBB | 700 bp | Major QTL (XAN 159) | 8 | Pedraza et al., 1997 |
| SW13 | W13 | Bean Common Mosaic Virus (BCMV) & HBB | 690 bp | I Pse-3 | 2 | Haley et al., 1994 Melotto et al., 1996 Fourie et al., 2004 |
| SH11 | H11 | HBB | 800 cis | Pse-1 | 10 | Miklas et al., 2007 |
| SQ4 | OQ4 AGT GCG CTG A | Anthracnose & Rust | 1440 | Co-2, Ur-11 | 11 | Awale et al., 2008 Young and Kelly, 1998 |

**Table 9.** Reaction of breeding lines and bean varieties registered in Bulgaria to the causative agents of anthracnose, halo blight and bacterial blight.

| Accession | *Colletotricum lindemuthianum* *(CL)* | | | | *Pseudomonas savastanoi pv. phaseolicola* *(PSP)* | | | | *Xanthomonas phaseoli pv. phaseoli* *(XPP)* | | | | R Index (%)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Race 6 | Race 8 | Race 81 | Race 320 | Race 1 Leaf | Race 1 Pods | Race 6 Pods | Race 6 Pods | XB96221 | XB96221 | XB99132 | XB99132 | |
| DG 9-11-1 | S* | S | VS | MR | R | R | R | R | MR | S | VS | VS | 50 |
| DG 9-11-2 | MS | VS | VS | VS | R | R | R | R | MR | R | R | R | 67 |
| DG 12-11-18 | VS | VS | VS | VS | MR | I | MR | MS | S | MR | VS | VS | 33 |
| DG 9-11-20 | S | S | VS | S | R | R | R | R | S | S | S | S | 33 |
| DG 13-12-22 | VS | R | VS | R | R | R | R | R | S | S | VS | S | 50 |
| DG 13-12-32 | S | MS | VS | VS | R | R | R | R | S | S | S | S | 33 |
| DG 17-38-16 | R | S | VS | S | R | MR | R | R | R | I | R | R | 75 |
| DG 17-38-38 | R | S | VS | MR | I | I | I | I | MR | R | R | R | 83 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DG 17-38-46 | S | S | VS | MR | I | R | R | R | R | R | R | R | 75 |
| Trakia | VS | I | R | I | MR | MR | MR | MS | S | VS | VS | VS | 50 |
| GTB Blyan | VS | R | VS | I | R | MR | R | R | VS | VS | VS | VS | 50 |
| Beslet | R | S | VS | S | R | R | R | R | R | R | R | R | 75 |
| GTB Skitia | MS | MR | S | MS | R | R | R | R | R | R | R | R | 67 |
| GTB Ustrem | VS | S | VS | S | R | MR | R | R | S | S | S | S | 33 |

*Resistant – I-MR (score 1.0-5.0); Susceptible – MS-S (score over 5.0)

**R=(number I-MR/ total number I-VS)*100

**Table 10.** Reaction of common bean local accessions, breeding lines and varieties registered in Bulgaria to the causative agents of anthracnose (*CL*), halo blight (*PSP*), and common bacterial blight (*XPP*).

| Accession | *CL* | | | | *PSP* | | | | *XPP* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Race 6 | Race 8 | Race 81 | Race 320 | Race 1 Leaf | Race 1 Pods | Race 6 Pods | Race 6 Pods | XB96221 | XB96221 | XB99132 | XB99132 | |
| Bezhanovo | MR* | VS | S | VS | R | R | I | I | S | S | S | S | 42 |
| Sadovets | VS | S | S | VS | I | I | I | I | S | S | S | S | 33 |
| Belitsa 1 | VS | VS | MR | VS | MR | MR | R | R | S | S | S | S | 42 |
| Belitsa 2 | VS | I | I | I | MR | MR | S | S | S | S | S | S | 42 |
| Sveta Petka 1 | VS | I | S | I | I | I | MR | MR | S | S | S | S | 50 |
| Sveta Petka 2 | VS | VS | S | I | MR | MR | MR | MR | S | S | S | S | 42 |

*Resistant – I-MR (score 1.0-5.0); Susceptible – MS-S (score over 5.0)

**R=( number I-MR/ total number I-VS)*100

**Table 11.** Reaction of 10 common bean accessions from Slovenia to the causative agents of anthracnose and halo blight.

| Accessions | *CL* | | | | | *PSP* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Race 1 | | Race 6 | | |
| | Race 2 | Race 6 | Race 8 | Race 81 | Race 320 | Leaf | Pod | Leaf | Pod | |
| KIS AMAND | VS* | VS | VS | VS | VS | S | S | S | S | 0 |
| KIS EVZEBIJ | VS | VS | VS | VS | VS | S | S | S | S | 0 |
| KIS MARCELIJAN | I | MR | R | I | R | MR | MR | MR | MR | 100 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| TOPOLOVEC | VS | VS | VS | VS | S | S | S | S | S | 0 |
| STORTION DI TRENTO | VS | VS | VS | VS | VS | S | S | S | S | 0 |
| JERUZALEMSKI | VS | VS | VS | R | VS | MR | MR | MR | MR | 44 |
| CIPRO | R | VS | MR | S | S | R | R | R | R | 67 |
| 230/2/BABC | VS | VS | R | VS | S | R | R | R | R | 44 |
| 227/2ACAC | I | MR | S | MR | R | I | I | I | I | 100 |
| 239/5CBBC | R | MR | MR | MR | I | I | I | R | R | 100 |

*Resistant – I-MR (score 1.0-5.0); Susceptible – MS-S (score over 5.0)

**R=(number I-MR/ total number I-VS)*100

**Table 12.** Reaction of Mastilen 11b and mutant lines to the causative agents of anthracnose, halo and common bacterial blight.

| Genotype | *CL* | | | | *PSP* | | | | *XPP* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Race 2 | Race 6 | Race 81 | Race 320 | Race 1 Leaf | Race 1 Pods | Race 6 Pods | Race 6 Pods | XB96221 leaf | XB96221 Pod | XB99132 Leaf | XB99132 pod |
| Mastilen 11b | VS* | VS | S | S | R | R | R | R | S | S | S | S |
| M 197-3 | VS | VS | VS | S | I | I | I | I | S | S | S | S |
| M 198-1 | VS | VS | S | VS | I | I | I | I | VS | VS | VS | VS |
| M 207-3 | VS | VS | S | VS | I | I | I | I | S | S | S | S |
| M 2012-4 | VS | VS | MR | VS | R | R | R | R | VS | VS | VS | VS |

*Resistant – I-MR (score 1.0-5.0); Susceptible – MS-S (score over 5.0)
** R=(number I-MR/ total number I-VS)*100

**Table 13.** Reaction of line *IP 564* and mutant lines to the causative agents of anthracnose (*CL*), halo blight (*PSP*), and bacterial blight (*XPP*).

| Genotype | *CL* | | | | *PSP* | | | | *XPP* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Race 8 | Race 81 | Race 320 | Race 1 Leaf | Race 1 Pods | Race 6 Pods | Race 6 Pods | XB96221 leaf | XB96221 Pod | XB99132 Leaf | XB99132 pod | |
| IP 564 | R* | | R | | MR | MR | MR | MR | S | S | S | S | 60 |
| M 564-23-1 | I | | VS | R | I | I | MR | MR | S | S | S | S | 55 |
| M 564-177-1 | I | | S | VR | R | R | R | R | S | S | S | S | 55 |
| M 564-177-2 | I | | S | - | I | I | R | R | S | S | S | S | 50 |
| M564-110-1-2 | I | S | R | R | R | MR | R | R | R | MS | MR | MR | 83 |
| M564-190-1-1-1 | I | I | R | I | R | R | R | R | MR | MR | R | MR | 100 |
| M564-190-3-7-2 | I | R | I | I | I | I | I | I | S | S | S | S | 67 |
| M564-190-3-7-1 | I | I | I | I | R | R | R | R | R | R | R | R | 100 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M564-190-3-7-2 | I | R | I | I | I | I | I | I | S | S | S | S | 67 |
| M 564-190-3-7-5 | R | | R | R | R | R | R | R | R | R | R | R | 100 |
| M 564-190-3-5A | I | | R | I | I | I | I | I | I | I | I | I | 100 |
| M 564-191-1-1-5 | I | | R | I | I | I | I | I | S | S | S | S | 64 |
| M564-191-1-1-3 | I | I | I | I | R | R | I | I | S | S | S | S | 67 |
| M564-191-1-1-2 | I | R | S | I | R | MR | R | MR | R | S | R | S | 75 |
| M564-193-8-2 | R | | VS | R | R | R | R | R | I | I | I | I | 91 |
| M564-193-9-1-1 | I | VS | VS | I | R | R | R | MS | R | MS | MR | MS | 58 |
| M 564-193-11 | I | | I | I | I | R | R | R | R | R | R | R | 100 |
| M 564-193-12 | I | | S | I | I | I | I | I | R | R | R | I | 91 |
| M 564-193-13 | I | | MR | I | I | I | I | I | MR | MR | S | S | 82 |
| M 564-193-8 | R | | I | R | R | R | R | R | R | R | R | R | 100 |
| M564-193-9-1-1 | I | VS | VS | I | R | R | R | MS | R | MS | MR | MS | 58 |
| M564-193-9-1-2 | I | I | R | I | R | R | R | MS | R | S | MR | S | 75 |
| M564-193-8-1A | I | I | I | I | R | R | I | I | S | S | S | S | 67 |
| M 564-194-3 | R | | I | I | I | I | I | I | MR | MR | S | S | 82 |

*Resistant – I-MR (score 1.0-5.0); Susceptible – MS-S (score over 5.0)

** R=(number I-MR/ total number I-VS)*100

**Table 14.** Phytopathological and molecular analysis of common bean genotypes for resistance to *C. lindemuthianum*.

| **Variety/ breeding line** | ***Colletotricum lindemuthianum*** | | | | ***SCAR reactions*** |
|---|---|---|---|---|---|
| | Race 6 | Race 8 | Race 81 | Race 320 | ***SQ4 marker (C. lindemuthianum)*** ***Co-2, Ur-11 genes*** |
| **DG 9-11-1** | S | S | VS | MR | NO |
| **DG 9-11-2** | S | VS | VS | VS | NO |
| **DG 12-11-18** | VS | VS | VS | VS | NO |
| **DG 12-11-20** | S | S | VS | S | NO |
| **DG 13-12-32** | S | MS | VS | VS | **1440 bp** |
| **DG 13-12-22** | VS | R | VS | R | NO |
| **DG 17-38-16** | R | VS | VS | VS | NO |
| **DG 17-38-38** | R | S | VS | MR | NO |
| **DG 17-38-46** | S | S | VS | MR | NO |
| **M4** | I | S | R | R | NO |
| **M19** | I | | R | I | NO |
| **M22** | R | | VS | R | NO |
| **M26** | I | VS | VS | I | NO |
| **Belitsa 1** | VS | VS | MR | VS | NO |
| **Belitsa 2** | VS | I | I | I | NO |
| **GTB Blyan** | VS | R | VS | I | NO |

| | | | | | |
|---|---|---|---|---|---|
| **Beslet** | R | VS | VS | VS | NO |
| **Bejanevo** | MR | VS | S | VS | NO |
| **Sveta Petka 1** | VS | I | S | I | **1440 bp** |
| **Sveta Petka 2** | VS | VS | S | I | NO |
| **GTB Skitia** | S | S | VS | S | NO |
| **Sadovets** | VS | S | S | VS | NO |
| **Trakya** | VS | I | R | I | NO |
| **GTB Ustrem** | VS | S | VS | S | NO |

**Table 15.** Phytopathological and molecular analysis of bean genotypes for resistance to *P. s. pv. phaseolicola*.

| Variety / Line | *Pseudomonas savastanoi* pv. *phaseolicola* | | | | | |
|---|---|---|---|---|---|---|
| | | | | | SCAR reaction SW13 marker I Pse-3 gene (expected fragment - 690 bp) | SCAR reaction SH11 marker *Pse-1gene (expected fragment - 800 bp)* |
| DG 9-11-1 | R | R | R | R | 690 bp | 800 bp |
| DG 9-11-2 | R | R | R | R | 690 bp | 800 bp |
| DG 12-11-18 | MR | I | MR | MS | 690 bp | 800 bp |
| DG 9-11-20 | R | R | R | R | 690 bp | 300, 800 |
| DG 13-12-32 | N/A | N/A | N/A | N/A | 690 bp | 800 bp |
| DG 13-12-22 | R | R | R | R | 690 bp | 800 bp |
| DG 17-38-16 | R | MR | R | R | 450, 690 bp | 800 bp |
| DG 17-38-38 | I | I | I | I | 690 bp | 800 bp |
| DG 17-38-46 | I | R | R | R | 690 bp | 800 bp |
| M4 | R | MR | R | R | NO | NO |
| M12 | R | R | R | R | NO | 200, 800, 1500 |
| M19 | I | I | I | I | 450, 690 bp | 800 |
| M22 | R | R | R | R | 690 bp | 200, 800 |
| M26 | R | R | R | MS | 400,69 | 200, 800 |
| Belitsa 1 | MR | MR | R | R | 690 bp | 300, 800 bp |
| Belitsa 2 | MR | MR | S | S | 350, 690 bp | 300, 800 bp |
| GTB Blyan | R | MR | R | R | 400, 690 bp | 300, 500, 800, 1500 bp |

| | | | | | | |
|---|---|---|---|---|---|---|
| Beslet | R | R | R | R | 690 bp | 300, 500, 800, 1500, 2500 bp |
| Bezhanovo | R | R | I | I | 690 bp | 800, 810 bp |
| Sveta Petka 1 | I | I | MR | MR | 690 bp | 250, 300, 500, 800, 1200, 3000+ |
| Sveta Petka 2 | MR | MR | MR | MR | 690 bp | NO |
| GTB Skitia | R | R | R | R | 300, 690, 900, 2000 bp | 300, 800 bp |
| Sadovets | I | I | I | I | 300, 690 bp | 300, 500, 800, 1000, 1500 bp |
| Trakia | MR | MR | MR | MS | 690, 1800 bp | 300, 500, 800, 1500 bp |
| GTB Ustrem | R | MR | R | R | 690 bp | 300, 800 bp |

**Table 16.** Phytopathological and molecular analysis of common bean genotypes for resistance to *X. p. pv. phaseoli*.

| Variety / Line | *Xanthomonas phaseoli* pv. *phaseoli* | | | | | |
|---|---|---|---|---|---|---|
| | | | | | SCAR reaction SAP6 QTL marker Xap-1 gene (expected fragment - 820 bp) | SCAR reaction SU91 QTL marker (expected fragment - 700 bp) |
| DG 9-11-1 | MR | S | VS | VS | 820 bp | 450, 900 bp |
| DG 9-11-2 | MR | R | R | R | 500, 820 bp | 400, 700, 900, 1800 bp |
| DG 12-11-18 | S | MR | VS | VS | NO | 450 bp |
| DG 9-11-20 | S | S | S | S | NO | 450, 700, 900 bp |
| DG 13-12-32 | N/A | N/A | N/A | N/A | NO | 450, 1000 bp |
| DG 13-12-22 | S | S | VS | S | NO | 450, 1000 bp |
| DG 17-38-16 | R | I | R | R | 500, 600, 820, 1050 bp | 450, 900, 1800 bp |
| DG 17-38-38 | MR | R | R | R | 500, 600, 820, 1050 bp | 450, 900, 1800 bp |
| DG 17-38-46 | R | R | R | R | NO | 900 bp |
| M4 | R | MS | MR | MR | 820 bp | 400 |
| M12 | R | R | R | R | NO | 450 |
| M19 | S | S | S | S | NO | 400 |
| M22 | I | I | I | I | 820 bp | 400... 700 |

| | | | | | | |
|---|---|---|---|---|---|---|
| M26 | R | MS | MR | MS | 820 bp | 400… 700 |
| Belitsa 1 | S | S | S | S | 820 bp | 450, 900, 1200, 1800 bp |
| Belitsa 2 | S | S | S | S | 820 bp | 450, 900, 1800 bp |
| GTB Blyan | VS | VS | VS | VS | 400, 500, 820 bp | 450, 900 bp |
| Beslet | R | R | R | R | 400, 500, 820 bp | 450, 900 bp |
| Bezhanovo | S | S | S | S | 400, 500, 820 bp | 450, 900 bp |
| Sveta Petka 1 | S | S | S | S | 820 bp | 450, 700, 900 bp |
| Sveta Petka 2 | S | S | S | S | 820 bp | 450, 900 bp |
| GTB Skitia | R | R | R | R | 400, 500, 820 bp | 450, 900 bp |
| Sadovets | S | S | S | S | 400, 500, 820 bp | 450, 900 bp |
| Trakia | S | VS | VS | VS | 820 bp | 450, 700, 900 bp |
| GTB Ustrem | S | S | S | S | 300, 500, 820 bp | 450, 700, 900 bp |

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# Adaptation to Southern Europe and plant type characterization for intercropping of cowpea and soybean accessions (Task 1.6)

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## Background of cowpea and soybean accessions (Task 1.6)

Soybean (*Glycine max* [L.] Merr.) is a major warm-season grain legume cultivated in Southern Europe because of its high grain protein content (39–42% dry weight), oil content (19–22% dry weight) and yield (Andrijanić *et al*., 2023).

Climate trend from 1981 to 2015 evidenced a relevant evolution towards drier conditions (increase of frequency and severity of drought events) over Central Europe in spring, the Mediterranean area in summer, and Eastern Europe in autumn (Spinoni *et al*., 2018). Climate models predicted for the period 2071–2100 a further rise of temperatures, reduction of precipitations and increase in the frequency of drought events in spring and summer everywhere in Europe (EEA, 2019). Warmer temperatures will allow the expansion of soybean cultivation area by 31–38% due to more favourable growing conditions towards Central Europe, but will increase the risk of crop failure due to drought and heat in Southern and Eastern Europe (Nendel *et al*., 2023). Therefore, protein production under changing climate in Europe requires on one side breeding soybean cultivars with greater drought and heat tolerance, and on the other side the cultivation of other grain legumes more adapted to drier environments.

Cowpea (*Vigna unguiculata* [L.] Walp.) is one of those legumes; it is a warm-season crop cultivated worldwide for grain and fodder production because it has high yield potential under dry and hot conditions. In Europe, it is mostly cultivated in Southern countries (Lazaridi and Bebeli, 2023) mainly for food, and mostly for grain (protein content = 22–30% dry weight; Dareus *et al*., 2021). Successful cultivation of cowpea in Southern Europe requires the development of new varieties with high yield and photo-thermal requirements for flowering and physiological maturity consistent with Southern Europe climates. Indeed, cowpea is generally a short-day plant, therefore adaptation should be tested for germplasm

imported from lower latitudes (Tropics and Equator). Nevertheless, neutral (photo-insensitive) and less demanding varieties and landraces exist.

Besides breeding and cultivating adapted legume varieties, further improvement of cropping systems, in terms of agronomic performance and provisioning of ecosystem services (including enhanced biodiversity), could be achieved by developing legume-based mixtures and testing their advantage over crop pure stands (Li *et al*., 2023). Advantages of intercropping compared to pure stands were associated with greater spatial and temporal efficiency of resource use, facilitation effects and limitation of weeds, pests and diseases (Picasso *et al*., 2011). However, wide adoption of multi-species mixtures is limited due to yield loss compared to cultivating the most productive species in sole-crop (Li *et al*., 2023), possible technical challenges (crop management), and difficulties in defining optimal legume/cereal mixtures (Annicchiarico *et al*., 2019). Information on useful plant traits that favor the compatibility of associated species is very limited for grain legumes, especially warm-season ones, while multi-species mixtures have been much less explored than binary mixtures. Cowpea has a long tradition of intercropping with warm-season cereals in subtropical areas (Singh, 2014). In Southern Europe, cowpea could be cultivated as a grain or a forage crop associated to summer cereals like sorghum (*Sorghum bicolor* [L.] Moench) or maize (*Zea mays* L.), with the aim of increasing the protein content of the forage or grain yield and the sustainability of the cropping system.

## Objectives of cowpea and soybean research (Task 1.6)

The first objective of the Task 1.6 was to evaluate a set of recent European soybean cultivars (Andrijanić et al., 2023) and a world collection of cowpea germplasm (Muñoz-Amatriaín *et al*., 2021) developed at the University of California (Riverside, CA) for yield ability in Northern Italy (as representative of Southern Europe) and other plant traits of interest for cultivation in organic farming systems and in intercropping. A restricted panel of genotypes with high yield and contrasting plant type were identified for cultivation in intercropping in Task 3.3. The second objective was to identify genomic areas and define genomic selection models for predicting traits of key interest for adaptation to organic environments of Northern Italy using molecular data for cowpea (Muñoz-Amatriaín *et al*., 2017).

## Material and methods of cowpea and soybean research (Task 1.6)

### Field experiments

Field experiments were established in Lodi (45°19’ N, 9°30’ E, 81 m a.s.l.) in summer 2021 for soybean (whose data were used for the present task) and in 2022 for cowpea, on a sandy loam soil with sub-acid reaction. Both experiments were designed as alpha-lattice with two replicates. Plots (1.5 × 1.0 m and 1.5 × 1.5 m for soybean and cowpea, respectively) were planted (four rows distant 37.5 cm) on the 6$^{th}$ of May for soybean (45 seeds m$^{-2}$) and the 28$^{th}$ of June for cowpea (18 seeds m$^{-2}$). A set of 50 recent European varieties was made up choosing the most yielding ones from a larger set of 250 varieties encompassing maturity groups (MG) 000–II (Andrijanić *et al*., 2023) evaluated at CREA in previous research projects for grain yield and biomass production under both optimal and drought-stress conditions (imposed at the onset of flowering up to physiological maturity). Two hundred twenty-two accessions of cowpea were selected from the UCR Minicore collection (Muñoz-Amatriaín *et al*., 2021), representing landraces and breeding material from Africa, Asia, Australia, Europe, and North and South America. Two additional accessions were from Central Italy, and two from Northern Italy.

## Plant traits

Plant traits (**Table 1.6.1**) were measured according to official guidelines for soybean (UPOV, 1998) and cowpea (IBPGR, 1983).

**Table 1.6.1 - List of plant traits measured for the two species.**

| Trait | Short name | Type[b] | Soybean | Cowpea |
|---|---|---|---|---|
| Onset of flowering (R1; days) | FLO | Quant. | Plot | |
| Pod maturity (R7; days) | SIL | Quant. | n.m.[c] | Plot |
| Physiological maturity (R8; days) | MAT | Quant. | Plot | |
| Growth habit | GRH | Qual. | n.a.[d] | 1=acute-erect; 2=erect; 3=semi-erect; 4=intermediate; 5=semi-prostrate 6=prostrate; 7=climbing; plot. |
| Growth pattern | GRP | Quant./ qual. | # nodes after R1; 2 plants | Determinate/ indeterminate; plot |
| Climbing plant | CLI | Qual. | n.a. | Yes/no; plot |
| Twining | TWI | Qual. | n.a. | Yes/no; plot |
| Leaf area ($cm^2$) | LEA | Quant. | Last expanded leaf; 2 plants | |
| Plant height (cm) | PLH | Quant. | 3 plants | |
| Shoot length (cm) | STL | Quant. | 3 plants | 2 plants |
| Aboveground biomass (t DM $ha^{-1}$)[a] | AGB | Quant. | At R8; plot | At R7; 2 plants |
| Grain yield (t DM $ha^{-1}$) | YNS | Quant. | Plot | |
| Grain yield under drought-stress (t DM $ha^{-1}$) [a] | YST | Quant. | Plot | n.m. |
| 1000 seeds weight (g DW) | TKW | Quant. | Subsample of harvested grain | |

[a]Measured in 2021 under managed environments in previous research project.
[b]Type of trait: qualitative or quantitative.
[c]n.m., not measured.
[d]n.a., not applicable.

Soybean varieties were grouped into three maturity classes according to their maturity group: 1) early-maturing (from MG 00 to MG 0); 2) intermediate-maturity (from MG 0 to MG I-); 3) late-maturing (from MG I to MG II). According to plant height, they were also classified as short (<81 cm), medium (81–97 cm; the mean ± 1 sd), and tall (>97 cm) varieties.

Cowpea accessions were classified according to the following classes of plant ideotype (**Figure 1.6.1**): 1) erect ideotype: non-climbing plant and large (>0.8) plant heigh to shoot length ratio (H/L); 2) bushy ideotype:

non-climbing plant and H/L≤0.8; 3) climbing ideotype: climbing plant and H/L≥0.3; 4) spreading ideotype: climbing plant and H/L<0.3. Thresholds for H/L were identified according to visual assessment of plant ideotype at maturity (pod and grain harvest) and cultivation techniques (all genotypes, including those climbing did not rely on a support for growing).

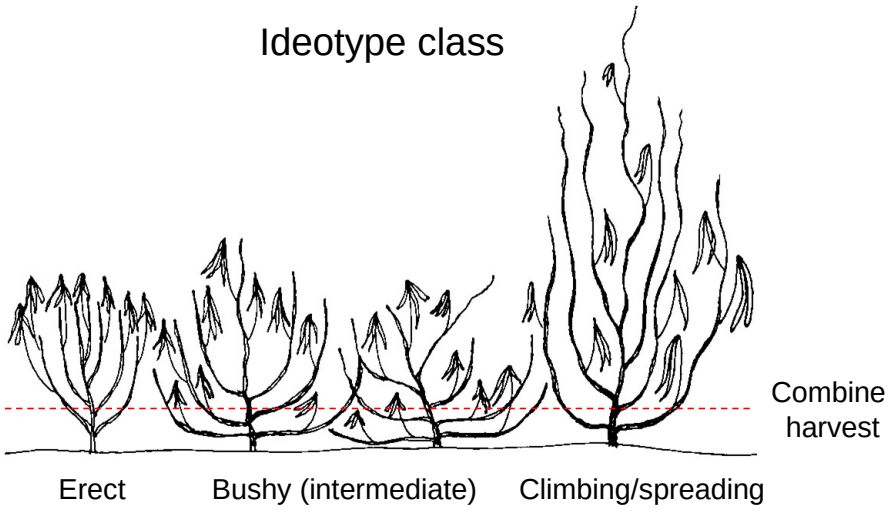



**Figure 1.6.1 - Habits of cowpea plants belonging to the four ideotype classes at physiological maturity.**

We restricted our selection of cowpea genotypes with potential interest for cultivation under organic farming and intercropping to the 77 best-yielding accessions that reached maturity before the 15$^{th}$ of October (excluding too late-maturing genotypes). Principal component analysis (PCA) was used to investigate patterns of morpho-physiological phenotypic variation among genotypes of soybean and cowpea genotypes.

For cowpea, a genome-wide association study (GWAS) was performed by Blink model for onset of flowering time, either measured as a continuous (number of days from the 1st of March) or discrete variable (classes), the ratio between plant height and stem length (representative of growth habit), individual seed weight, and grain yield. Moreover, a GWAS was performed for the climbing habit defined as a binary variable (climbing/non-climbing habit) by a mixed logistic regression model. Discriminant Analysis of Principal Components was employed to account for population structure. In addition, genomic selection (GS) models based on ridge-regression BLUP and ten-fold cross validation with ten replicates were developed for non-binary traits. Both GWAS and GS models relied on 220 genotypes and 41,188 SNP, obtained from genotyping by the Cowpea iSelect Consortium Array (Muñoz-Amatriaín et al., 2017).

## Results of cowpea and soybean research (Task 1.6)

### Phenotypic variability of soybean and cowpea

The set of 50 soybean varieties showed high variability for all the measured phenological, morphological and yield-related traits (Table 2). The onset of flowering spanned a period of 15 days, with early varieties flowering on average six days before late varieties. The range of physiological maturity was 37 days, with less clear differences between maturity classes. Plant height also exhibited a wide range of variability, with many varieties classified as tall (plant heigh > 87 cm) having different maturity date and that were potentially interesting for cultivation under organic farming and intercropping (higher competition with weeds and companion cereal crops). On average, biomass production and grain yield increased from early to intermediate and late varieties. High biomass-producing phenotypes are also desirable in intercropping, especially in the case of forage production.

**Table 1.6.2 - Descriptive statistics of the 50 soybean varieties. The mean and range (5$^{th}$–95$^{th}$ percentile; in parenthesis) are reported for the whole set and separately for each maturity class.**

| Plant trait[a] | All | Maturity class | | |
|---|---|---|---|---|
| | | Early | Intermediate | Late |
| FLO | 24 (15-30) | 20 (13-29) | 23 (15-30) | 26 (18-30) |
| HARV | 148 (130-167) | 146 (134-156) | 142 (129-160) | 151 (133-167) |
| PLH | 89 (74-102) | 96 (90-102) | 87 (77-96) | 89 (73-102) |
| AGB | 11.6 (7.7-15.4) | 10.1 (6.0-14.8) | 10.7 (7.8-14.5) | 12.5 (8.9-15.9) |
| YNS | 3.8 (3.2-4.9) | 3.4 (3.2-3.7) | 3.7 (3.2-4.0) | 4.0 (3.4-5.0) |
| YST | 1.0 (0.7-1.3) | 1.0 (0.5-1.3) | 1.0 (0.7-1.3) | 1.0 (0.7-1.3) |
| TKW | 169 (141-199) | 178 (150-203) | 169 (137-210) | 168 (145-187) |
| Number of accessions: | | 6 | 16 | 28 |

[a]Refer to Table 1.6.3 for trait name and description.

Most of the cowpea accessions (80%) reached maturity between September 19 and November 7 (the last harvest date), while the others did not flower at all. Late maturing varieties (later than October 15) were not considered suitable for cultivation in Northern Italy. Descriptive statistics of the remaining 77 accessions are reported in Table 1.6.3. Most of the accessions had a climbing/spreading (59%) or bushy (31%) ideotype, while erect ideotypes were less represented (10%). On average, flowering time followed the order erect < bushy < climbing < spreading ideotypes. Unlike soybean, which reaches a uniform maturity within plants and plots, most cowpea accessions had an indeterminate growth pattern (88%), presenting flowers, immature and mature pods at the same time (Figure 1.6.2); therefore, plots were harvested when most of the pods were at physiological maturity. Plant height ranged 39–79 cm; however, given

that all genotypes were cultivated without any support, more interesting is the variability of shoot length. Erect ideotypes had lower shoot length, followed by bushy, climbing and spreading ideotypes that presented shoots up to two meters long. Therefore, climbing/spreading ideotypes could potentially climb cereal stems of both sorghum and maize to grow vertically. There were no clear differences in average biomass production and grain yield among ideotype classes; however, larger biomass and grain were produced in climbing/spreading accessions compared to the erect ones. As observed for soybean, producing high biomass is a desirable feature for cultivation in organic-farming and in intercropping. Seed weight (100–253 g) was on average higher in erect ideotypes compared to others.

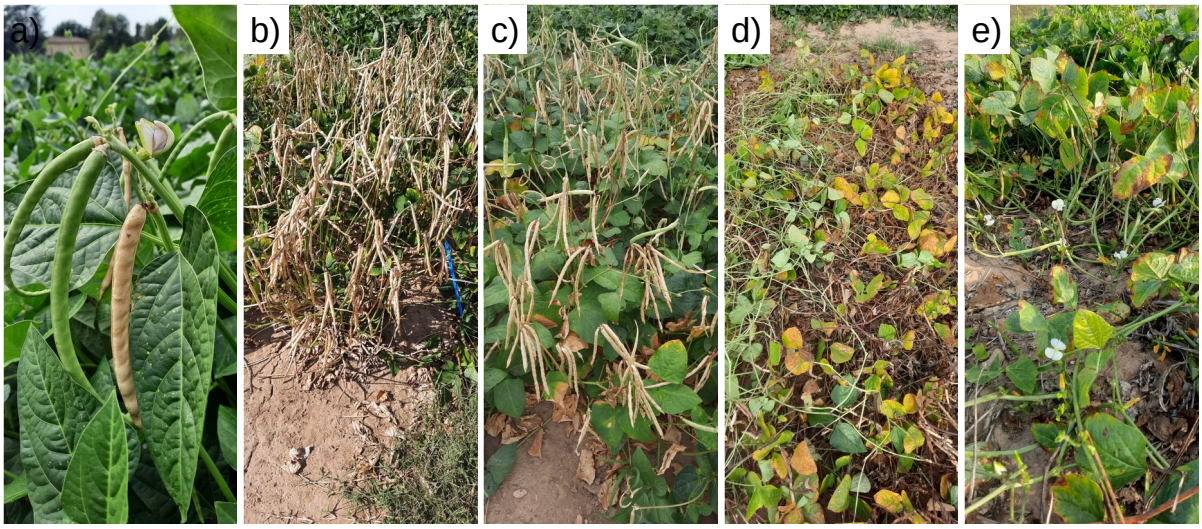



**Figure 1.6.2 – Variability in maturity-stage within plants: detail of a peduncle (a), erect accessions with uniform (b) and non-uniform (c) maturity-stage, climbing/spreading accessions with uniform (d) and non-uniform (e) maturity-stage.**

**Table 1.6.3 - Descriptive statistics of the selected 77 cowpea genotypes. The mean and range (5th–95th percentile; in parenthesis) are reported for the whole set and separately for each ideotype class.**

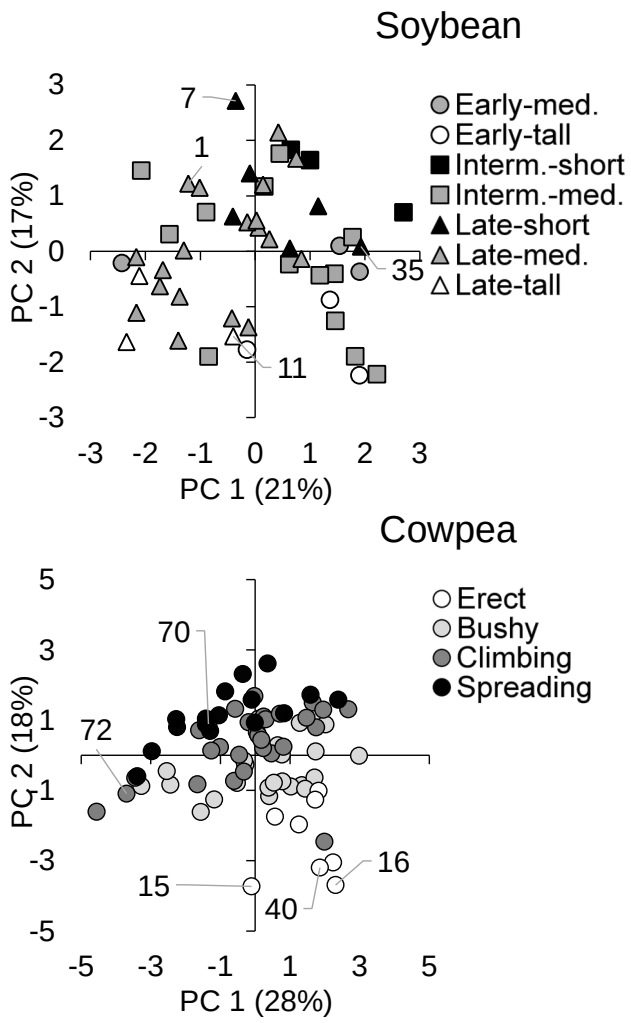
| Plant trait[a] | All | Ideotype class | | | |
|---|---|---|---|---|---|
| | | Erect | Bushy | Climbing | Spreading |
| FLO | 51 (37–68) | 46 (38–62) | 50 (38–64) | 51 (36–68) | 57 (42–67) |
| SIL | 82 (69–94) | 79 (70–96) | 80 (69–90) | 83 (72–95) | 87 (78–94) |
| MAT | 98 (86–110) | 103 (95–110) | 97 (86–109) | 97 (84–110) | 97 (86–107) |
| PLH | 58 (39–79) | 63 (48–82) | 57 (44–70) | 63 (46–83) | 47 (31–63) |
| STL | 151 (56–250) | 60 (45–92) | 144 (72–229) | 152 (78–222) | 205 (127–258) |
| PLT/STL | 0.5 (0.2–1.1) | 1.1 (0.8–1.4) | 0.4 (0.2–0.7) | 0.5 (0.3–0.9) | 0.2 (0.2–0.3) |
| AGB | 10.4 (6.7–16.0) | 9.8 (8.3–13.6) | 10.4 (6.3–13.1) | 10.3 (6.8–16.6) | 10.6 (7.3–16.2) |
| YNS | 3.1 (2.6–4.0) | 3.0 (2.7–3.3) | 3.2 (2.5–3.9) | 3.1 (2.6–4.0) | 3.2 (2.7–4.2) |
| TKW | 176 (100–253) | 200 (169–223) | 175 (103–273) | 171 (96–248) | 173 (121–248) |
| Number of accessions: | | 8 | 24 | 29 | 16 |

[a]Refer to Table 1.6.1 for trait name and description.

## Phenotypic selection of soybean and cowpea for cultivation in intercropping

The first two principal components (PC) used to summarize the data explained 38% and 46% of the variance in soybean and cowpea, respectively (Figure 1.6.3). In soybean, the first PC was negatively correlated with the onset of flowering and physiological maturity, plant growth pattern and biomass production, and positively correlated with seed weight. The second PC was negatively correlated with plant height, biomass production and seed weight, while it was positively correlated with the onset of flowering and grain yield. The two PCs separated well short from tall plants, and to a less extent early from late varieties.

In cowpea, the first PC was negatively correlated with the onset of flowering, leaf area, shoot length, biomass production and seed weight. The second PC was negatively correlated with days to physiological maturity, shoot length and seed weight, and positively correlated with plant growth pattern, climbing ability and shoot length. Principal component analysis separated well cowpea accessions with an erect ideotype from those having a climbing/spreading ideotype.




| Trait | PC1 | PC2 |
|---|---|---|
| FLO | **–0.48** | **0.42** |
| HARV | **–0.38** | –0.27 |
| GRP | **–0.46** | –0.21 |
| LEA | 0.16 | –0.04 |
| PLH | –0.28 | **–0.38** |
| AGB | **–0.38** | **–0.33** |
| YNS | –0.07 | **0.41** |
| YST | –0.11 | –0.10 |
| TKW | **0.38** | **–0.53** |

| Trait | PC1 | PC2 |
|---|---|---|
| FLO | **–0.50** | 0.03 |
| MAT | –0.12 | **–0.42** |
| GRP | –0.16 | **0.48** |
| CLI | –0.18 | **0.42** |
| LEA | **–0.32** | 0.01 |
| PLH | –0.26 | **–0.39** |
| STL | **–0.47** | **0.32** |
| AGB | **–0.40** | –0.13 |
| YNS | –0.19 | 0.00 |
| TKW | **–0.30** | **–0.38** |

**Figure 1.6.3 - Results of principal component analysis. For each component, the percentage of explained variance is reported in parenthesis. Numbers in the charts identify selected contrasting genotypes (Table 1.6.2). Refer to Table 1.6.1 for trait name and description.**

According to PCA, four contrasting soybean varieties (Figure 1.6.3) were identified for possible cultivation in Task 3.3. All varieties belonged to the late maturing group in order to reach maturity consistently with cowpea, and had grain yield higher or close to the average of the field (Table 1.6.2). Buenos and Adonai had greater plant height and biomass production compared to SY Victorius and Avril; in addition, Buenos was the most productive variety (for both biomass and grain) and therefore was selected for study in Task 3.3.

Five accessions of cowpea were identified following PCA, two with erect and two with climbing ideotype to explore, in Task 3.3, the effect of these two contrasting ideotypes on the performance of intercropping (Table 1.6.3). Within the erect accessions, IT4S-2246 was selected because it was

the highest, had longer shoots and provided satisfactory biomass and grain yield. The climbing variety Cp_5556 was selected instead of the Italian landrace Vazzano brown because had longer shoots and higher biomass production.

**Table 1.6.4 - Descriptive statistics of some contrasting genotypes of soybean and cowpea identified according to PCA. Refer to Table 1.6.1 for trait name and description.**

| Genot. | Name | Group | FLO | MAT | GRP | PLH | STL | AGB | YNS | YST | TKW |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Soybean | | | | | | | | | | | |
| 1 | Buenos | Late-med. | 30 | 149 | 7 | n.m.[a] | 94 | 14.0 | 5.0 | 1.4 | 157 |
| 7 | SY Victorius | Late-short | 30 | 154 | 8 | n.m. | 73 | 9.9 | 4.4 | 0.9 | 138 |
| 11 | Adonai | Late-tall | 20 | 167 | 8 | n.m. | 99 | 13.6 | 4.1 | 0.9 | 185 |
| 35 | Avril | Late-short | 20 | 154 | 8 | n.m. | 71 | 7.8 | 3.5 | 1.1 | 188 |
| Cowpea | | | | | | | | | | | |
| 15 | IT84S-2246 | Erect | 70 | 99 | Det. | 91 | 108 | 8.7 | 2.9 | n.m. | 200 |
| 16 | IT95K-1479 | Erect | 37 | 103 | Det. | 67 | 44 | 8.1 | 2.9 | n.m. | 217 |
| 40 | TVu-15610 | Erect | 44 | 94 | Det. | 66 | 51 | 9.9 | 3.4 | n.m. | 215 |
| 70 | Vazzano brown | Climb. | 64 | 94 | Ind. | 60 | 178 | 13.6 | 3.7 | n.m. | 149 |
| 72 | Cp_5556 | Climb. | 68 | 104 | Ind. | 81 | 215 | 17.7 | 2.8 | n.m. | 251 |

[a]n.m., not measured.

## Genome-wide association study and genomic selection for cowpea traits

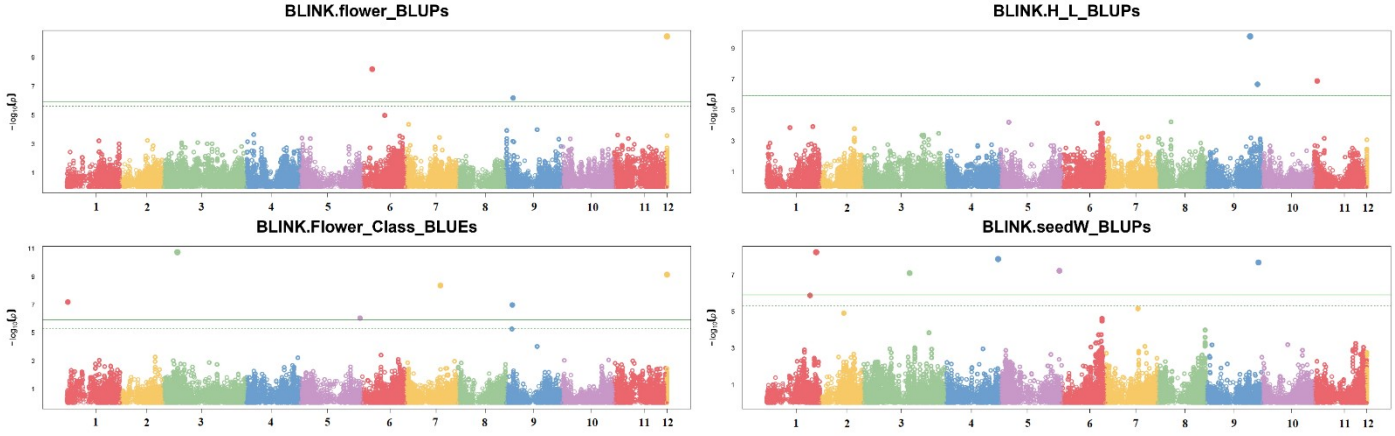
The GWAS revealed three significant SNP according to False Discovery Rate threshold for flowering time measured as a continuous variable and the ratio between plant height and stem length on three and two different chromosomes, respectively. Six significant SNP were detected both for flowering time expressed as a discrete variable and individual seed weight (mapping on six and five different chromosomes, respectively; Figure 1..6.4). No significant SNP was found for grain yield and climbing habit. The higher number of significant markers detected for flowering time expressed as a discrete variable relative to the same trait measured as a continuous variable could be attributed to the inclusion in the former analysis of the genotypes that did not reach the flowering stage because of marked climate misadaptation. The fact that no significant SNP was found for climbing habit classified as a binary trait, while several significant SNP were identified for the ratio between plant height and stem length, suggested that growth habit should be considered as a trait featuring continuous variation. Finally, the absence of significant SNP

markers for grain yield could be expected in the presence of polygenic trait control due to many small-effect genes. This hypothesis was confirmed by genome-base predictions, with a GS model for grain yield featuring moderate predictive ability (as correlation between predicted and observed values), i.e., 0.44. Moderately high predictions emerged for all the other non-binary traits (0.64-0.75) (Table 1.6.5), with an indication for a polygenic control also for these traits. On the whole, our results encourage the use of GS and, in some cases, just marker-assited selection, for the identification of cowpea germplasm accessions with putatively good phenological adaptation and grain yield in Southern Europe and a desired growth habit.

**Table 1.6.5 – Predictive ability of genomic selection models based on ridge-regression BLUP, 41,188 SNP, and 220 genotypes from a cowpea worldwide germplasm collection for five quantitative traits (based on a ten-fold cross-validation with ten replicates for phenotypic data derived from a single environment).**

| Flowering Time | Flowering Time Class | Stem height/length | Seed Weight | Grain Yield |
|---|---|---|---|---|
| 0.64 | 0.64 | 0.72 | 0.75 | 0.44 |

**Figure 1.6.4 - Manhattan plots showing the association scores of 41,188 SNP with four quantitative traits (top left: flowering time as a continuous variable; bottom left: flowering time as a discrete variable; top right: ratio between plant height and stem length; bottom right: individual seed weight) along the 11 cowpea chromosomes (chromosome 12 represents scaffolds) for a GWAS based on a worldwide germplasm collection of 220 genotypes. The green dotted line (not always visible due to the plot scale) represents Bonferroni threshold at 5%, while the green continuous line Bonferroni threshold at 1%.**



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# Farmer-participatory development of open-pollinated varieties of maize for Southern Europe (Task 1.7)

NARDI Fundulea, Romania: Victor Petcu

## Introduction to maize research (Task 1.7)

OPVs (open pollinated varieties) of cross pollinated crops are genetically heterogeneous and therefore likely to evolve over generations, under natural and human selection, which gives them a strong potential for organic and low input farming.

In Romania, there are a number of local maize populations, which are distinguished by a great adaptability and physiological properties specific to certain areas, as well as a production capacity and properties own quality (Hallauer and Miranda, 1981).

The Romanian local populations are very different, as are the conditions.

Ecological plants from our country, under the influence of which they were formed and over which the effects of the empirical selection made by thousands of growers, each in his own way, were superimposed.

Although they are very heterogeneous, they group themselves into distinct races, which occupy each a certain area (Criste a, 1975).

In this project we studied five local maize populations.

## Material and methods to maize research (Task 1.7)

In 2022, Romanian maize populations were sown in 5 locations from 4 localities different: Fundulea (Călărași), Solacolu (Călărași), Petrăchioaia (Ilfov), Cocani (Dâmbovița), in which maintained the cultivation conditions required by the organic farming system and had in view of the isolation in space and time - the cultivation of populations at a distance of at least 300m from the face of other maize crops or their sowing two weeks after other adjacent crops were established.


In 2023 (local population from Suceava), the multiplication of local populations will be taken into account to ensure the seed requirement. Physiological characterization will also be done, such as the height of the plants, the insertion of the spikelets, the date of flowering and silking, the production, the characterization of the grains and spikelets

The populations obtained in 2022 were multiplied in 2023, pollination being carried out manually. Within each population, pairs of plants were chosen in which female inflorescences and panicles before silking and flowering were isolated with special bags used for this purpose. This operation was performed to ensure that the pollen from each panicle would be used to pollinate the female inflorescence of the paired plant. At maturity the cobs from each population were harvested together, the resulting seed mixture representing the seed of the respective population.

Table 1. Description of the genetic material used to obtain open-pollinated maize lines

| Nr. Crt. | Denumire | Cod | Locul colecţiei | Statusul cultivarului |
|---|---|---|---|---|
| P1 | Românesc de Studina | SVGB-16224 | Fundulea, Călărași, Romania | Improved cultivar |
| P2 | Portocaliu 1 | SVGB-19978 | Fundulea, Călărași, Romania | Population/ Traditional |
| P3 | Portocaliu | SVGB-19967 | Fundulea, Călărași, Romania | Population/ Traditional cultivar |
| P4 | Cincantin | SVGB- | Fundulea, | Population/ |

| | | | | | |
|---|---|---|---|---|---|
| | | | 19964 | Călărași, Romania | Traditional cultivar |
| | P5 | Population Scorumnic de Olimpești | SVGB-16179 | Fundulea, Călărași, Romania | Population/ Traditional cultivar |
| | P6 | Population of Peretu 283 | SVGB-16181 | Pechea, Galați, Romania | Population/ Traditional cultivar |
| | P7 | Galben Timpuriu | SVGB-16216 | Sohatu, Călărași | Improved cultivar |
| | P8 | Românesc de Brezoaiele | SVGB-16196 | Brezoaiele, Dâmbovița, Romania | Population/ Traditional cultivar |
| | P9 | Moldovenesc de Pechea | SVGB-16168 | Fundulea, Călărași | Population/ Traditional |
| 0 | P1 | Population of Răzvani | SVGB-16182 | Peretu, Teleorman | Population/ Traditional cultivar |
| 1 | P1 | Bătrânesc de Progresu | SVGB-16129 | Fundulea, Călărași, Romania | Population/ Traditional cultivar |
| 2 | P1 | Population Pucheni Moșneni MI-315 | SVGB-16178 | Răzvani, Călărași, Romania | Population/ Traditional cultivar |
| 3 | P1 | Dintele Oii de Sohatu 80 | SVGB-16156 | Fundulea, Călărași, Romania | Population/ Traditional cultivar |
| 4 | P1 | Românesc de Butimanu 2 | SVGB-16198 | Brezoaiele, Dâmbovița, Romania | Population/ Traditional cultivar |
| 5 | P1 | Bănățean | SVGB-19979 | Fundulea, Călărași, Romania | Population/ Traditional cultivar |
| 6 | P1 | Românesc de Orlea (Roșu) | SVGB-16205 | Fundulea, Călărași, Romania | Population/ Traditional cultivar |

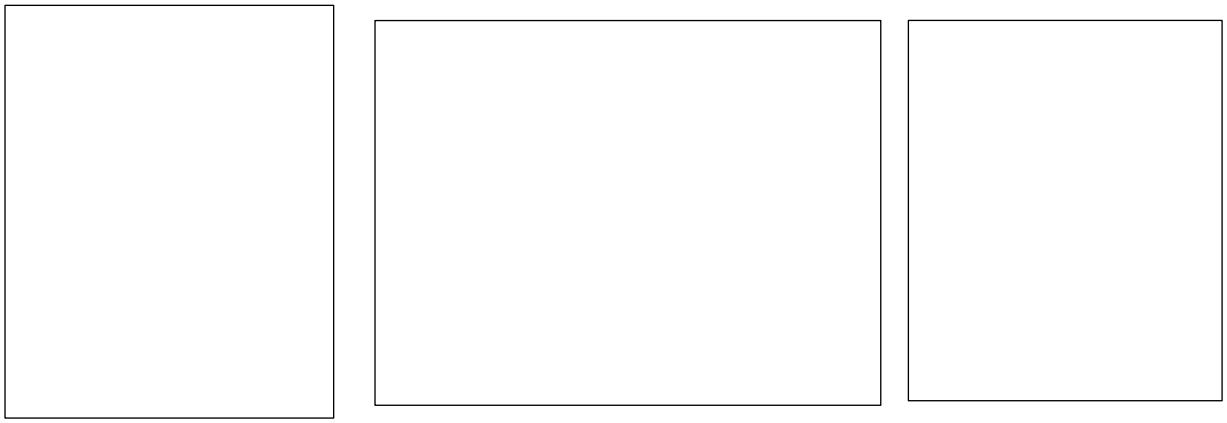


Foto 1. Aspect from the experimental field with isolated plants

## Results of the maize research (Task 1.7)

From a climatic point of view, the year 2023 in the Fundulea area was a very dry and hot year. The amount of precipitation that fell between January and August 2023 was 280.2 mm below the multi-year average for this period (407.8 mm), registering a deficit of 127.6 mm, and the average monthly air temperatures were above the multi-year average of the period (Figures 1 and 2).

These severe phenomena of drought and heat manifested this year throughout the corn vegetation period, had repercussions on the development and growth of corn plants from the experiences organized in the field at INCDA Fundulea.

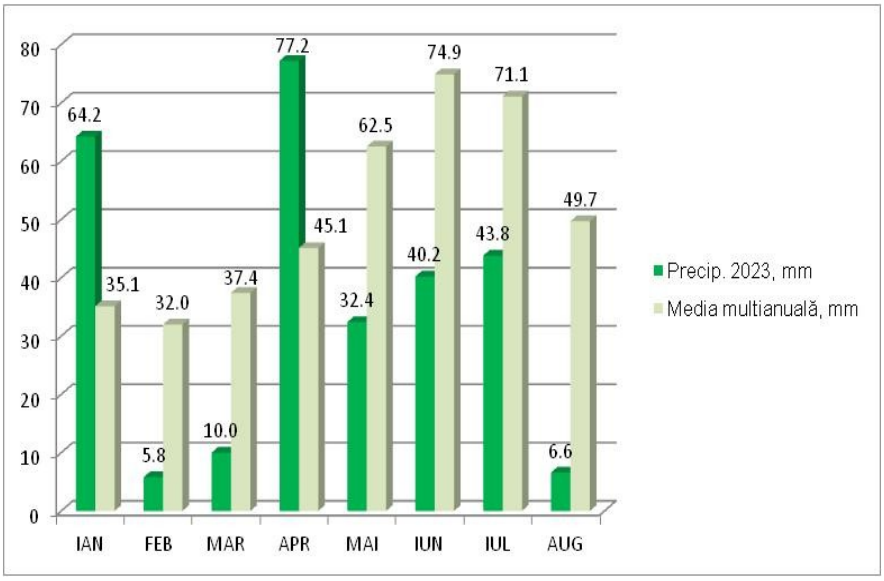



Fig. 1. Precipitation (mm) recorded during January-August, 2023 at INCDA Fundulea

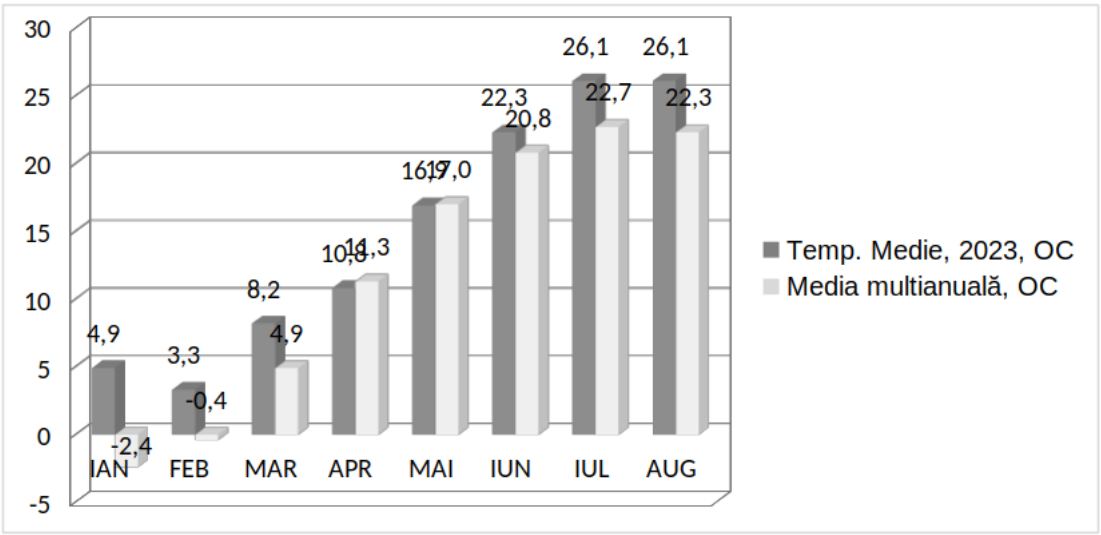


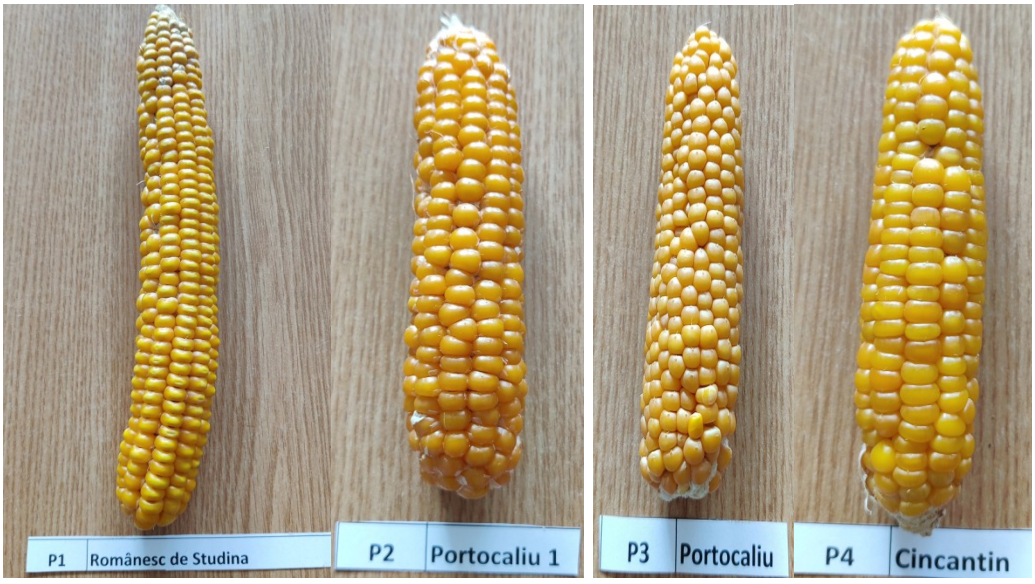

Fig. 2. Average monthly air temperatures (oC), recorded in the period January-August 2023, at INCDA Fundulea

Table 2 presents the characterization of the studied populations based on some morphological descriptors. Within this project, the following morphological descriptors were studied:
- for the plant: plant height, cob insertion height, flowering date, silking date;
- for cobs: length, diameter, number of rows of grains/cob, number of grains/row, rachis color;
-for grain: color, type of grain, MMB.

Table 2. The values of the morphological descriptors in 15 old local maize populations studied at INCDA Fundulea in 2023

| Nr. Crt. | Name of population | Plant heighti, cm | Cob insertion height , cm | Blooming date | Silky date | Cob length, cm | Cob diameter , cm | Number of rows of grains/ cobs | Number of grains/row | Color rachis | The color of the grain | The type of bean | TWG, g |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P1 | Românesc de Studina | 155 | 40 | 25.06 | 27.06 | 22 | 3,0 | 12 | 45 | alb | orange-yellow | indurat | 300 |
| P2 | Portocaliu 1 | 145 | 30 | 25.06 | 28.06 | 14 | 2,8 | 14 | 30 | alb | orange | indurat | 250 |
| P3 | Portocaliu | 140 | 30 | 24.06 | 26.06 | 11 | 2,5 | 18 | 34 | alb | orange | indurat | 240 |
| P4 | Cincantin | 140 | 35 | 24.06 | 25.06 | 15 | 2,6 | 14 | 28 | alb | yellow | indurat | 240 |
| P5 | Population Scorumnic de Olimpești | 148 | 36 | 29.06 | 2.07 | 14 | 2,8 | 14 | 29 | alb | yellow | indurat | 280 |
| P6 | Populație de Peretu 283 | 155 | 40 | 2.07 | 4.07 | 16 | 3,3 | 12 | 36 | alb | orange-yellow | indurat | 310 |
| P7 | Galben | 153 | 42 | 29.06 | 1.07 | 15 | 3,1 | 12 | 36 | alb | orange | indurat | 300 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Timpuriu | | | | | | | | | | | | |
| P8 | Românesc de Brezoaiele | 150 | 38 | 29.06 | 3.07 | 12 | 4,0 | 16 | 28 | roșu | yellow | indurat | 280 |
| P9 | Moldovenesc de Pechea | 175 | 52 | 29.06 | 2.07 | 16 | 4,0 | 12 | 30 | roșu | yellow | semiindurat | 300 |
| P10 | Populație de Răzvani | 180 | 55 | 29.06 | 1.07 | 12 | 3,9 | 18 | 30 | roșu | yellow | semidentat | 280 |
| P11 | Bătrânesc de Progresu | 182 | 50 | 1.07 | 3.07 | 11 | 3,5 | 12 | 24 | alb | yellow | semidentat | 280 |
| P12 | Populație Pucheni Moșneni MI-315 | 170 | 45 | 2.07 | 5.07 | 13 | 3,7 | 10 | 30 | alb | orange-yellow | semidentat | 300 |
| P13 | Dintele Oii de Sohatu 80 | 168 | 38 | 29.06 | 2.07 | 13 | 3,3 | 12 | 32 | alb | yellow | semidentat | 310 |
| P14 | Românesc de Butimanu 2 | 145 | 35 | 2.07 | 4.07 | 12.5 | 3,2 | 12 | 22 | alb | orange | indurat | 260 |
| P15 | Bănățean | 160 | 45 | 30.06 | 3.07 | 14 | 3,0 | 12 | 24 | alb | orange | indurat | 240 |
| Average (15 var.) | | 157.7 | 40.7 | x | x | 14 | 3.3 | 13.3 | 30.5 | x | x | x | 278 |
| Minim (15 var.) | | 140 | 30 | x | x | 11 | 2.5 | 10 | 22 | x | x | x | 240 |
| Maxim (15 var.) | | 182 | 55 | x | x | 22 | 4 | 18 | 45 | x | x | x | 310 |




P5


P6 Populație de Peretu 283


P7 Galben Timpuriu


P8 Românesc de Brezoaiele


P9 Moldovenesc de Pechea


P10 Populație de Răzvani


Photo 2. Images of the cobs of the populations studied at INCDA Fundulea in 2023

From the analysis of the results obtained this year, there are different levels of the descriptor values that characterize the architecture of the plants and the cobs.

The total height of the plants registers an average value of 157.7 cm, which indicates that the maize populations analyzed have, in general, a small to medium height. However, the populations with a tall waist, over 180 cm (Populatie de Răzvani, Bătrânesc de Progresu) are noteworthy.

The insertion height of the main cob is, on average, at 40.7 cm, with variations between populations, from 55 cm, in the tall population, Răzvani population, to 30 cm, in the short population, Orange and Orange 1.

Regarding the length of the cob, the Romanian population of Studina stands out with a cob length of 22 cm. .

The diameter of the cob registers a maximum value of 4 cm in the Romanian de Brezoaiele and Moldovan de Pechea populations and a minimum value of 2.5 cm in the Portocaliu population.

Three other descriptors are important for the assessment of productivity, namely: the number of rows of grains on the cob, the number of grains per row and the mass of 1000 grains (MMB).

The number of grain rows on the cob registers an average value of 13.3, the Orange and Răzvani populations stand out with 18 grain rows.

The number of grains per row registers an average value of 30.5, the Romanian population of Studina with 45 grains per row stands out.

The weight of 1000 grains also highlights local maize populations with values equal to and greater than 300 g: Romanian de Studina, Early Yellow, Moldavian de Pechea, Pucheni Moșneni population MI-315, Peretu population 283, Dintele Oii de Sohatu 80.

## ConclusIons of maize research (Task 1.7)

The morphological characterization descriptors of plant architecture, cobs and grains highlights local maize populations as sources of real interest for the improvement of the species.