**CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE**

**Faculty of Tropical AgriSciences**


# Development of an efficient slow-growth method for the *In vitro* storage of garlic *(Allium sativum)*

MASTER'S THESIS

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# Declaration

I hereby declare that I have done this thesis entitled “Development of an efficient slow-growth method for the *In vitro* storage of garlic *(Allium sativum)*” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 20.04.2022

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Yehor Nykyforov

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## Abstract

The present study aimed at developing an efficient slow-growth protocol for the medium-term storage of garlic *(Allium sativu*m) by testing the effects of various osmotic agents at various concentrations (sorbitol 20-60 g/l, mannitol 20-60 g/l, and sucrose 30-150 g/l) and plant growth regulators (chlormequat chloride (CCC) at 200-600 mg/l and abscisic acid (ABA) at 1-5 mg/l). Two cultivation temperatures, 5±1°C, and 18±1°C were also tested to observe their influence on the growth of the plants. Full-strength and 1/2 concentrated MS (Murashige and Skoog (1962) were used as basal culture media. Growth and development characteristics after 5-month slow-growth storage showed that garlic plantlets stored either on a concentration of 5 mg/l ABA in combination with full-strength MS medium, sucrose at 10% in half MS medium, or sorbitol at 4% in full MS, all at 5°C±1°C cultivation temperature prove to be most effective in reducing the growth of in-vitro plantlets. After the storage period, plantlets from these treatments were transferred to recovery media. Results showed that garlic plantlets stored on 1/2 MS medium supplemented by 10% sucrose had the strongest growth capacity. Relatively high regenerative abilities were also shown by plants stored in MS nutrient medium containing 4% sorbitol. As for the effect of ABA 5 mg/l in combination with full-strength MS medium on the regenerative capacity of garlic, results showed that the growth was weak, suggesting a need for long-term rehabilitation of garlic plants after the influence of this growth-regulating substance.

**Key words**: *Alium sativum,* slow-growth storage, medium-term conservation, osmotic agents, mannitol, sorbitol, sucrose, plant growth regulators, chlormequat chloride (CCC), abscisic acid (ABA).

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## List of the abbreviations used in the thesis

| | |
|---|---|
| **ABA** | Abscisic acid |
| **CCC** | Chlormequat chloride |
| **CRI** | Crop research institute |
| **CZU** | Czech University of Life Sciences Prague |
| **DNA** | Deoxyribonucleic acid |
| **FAO** | Food agriculture organization |
| **FTA** | Faculty of Tropical AgriSciences |
| **PCR** | Polymerase chain reaction |
| **PGR** | Plant growth regulator |
| **MS** | Murashige and Skoog (1962) medium |

# 1. Introduction

Garlic *(Allium sativum)* belongs to the *Allium* genus, which contains more than 600 species widely distributed throughout the world: Europe, North America, North Africa, and Asia (Ozturk et al. 2012). In many cultures of the world, garlic is used not only in cooking but also for medicinal purposes. Combining these factors has brought this plant to second place (Peter 2016) in cultivation and popularity from the *Allium* family, firmly consolidating its position immediately after the onion (Peter 2016). Garlic is unpretentious in cultivation, it does not require much care or extra fertile soil, preferring a temperate climate with a balanced nutrient and water regime ("Crop Profile for Garlic in Washington" 2002). But, like all crops, it has its own characteristics in its biology, which complicates its cultivation. Almost all types of cultivated garlic can only reproduce vegetatively due to their sterility (Kamenetsky 2007). Therefore, one of the main methods of maintaining varietal collections remains the field method with storage of bulbs (Olas-Sochacka & Kotlinska 2015). However, there is a big problem with field collections since it becomes necessary care for it every year, in addition, the genetic resources of garlic, being stored in field collections, are largely susceptible to losses due to the influence of weather conditions, fungal, bacterial or viral diseases (Olas-Sochacka & Kotlinska 2015).

Due to the difficulties and disadvantages arising from the classical conservation of plants there are studies demonstrating the possibility of applying innovative conservation methods under *in vitro* conditions (Ruta et al. 2020). Depending on the timing and methods of the proposed conservation of plants under *in vitro* conditions, three categories are distinguished: short-term (*in vitro* culture), medium-term (slow growth or minimal growth), and long-term (cryopreservation) (Cha-um 2007).

The maintenance of garlic plants material under standard *in vitro* growing conditions implies transfer to a new nutrient medium every 3-6 weeks as short-term storage (Ruta et al. 2020). If we want to store plants for many years, it would be advisable to use the cryopreservation method, but this will require specialized laboratory equipment: liquid nitrogen, dewar storage tanks, etc. (Ruta et al. 2020). In addition, if necessary, the preserved plant material cannot be used immediately since thawing and regrowth takes a rather long period, and the survival rate may be low.

These two methods are well studied and applied to garlic, which is not the case with the slow-growth technique. But despite this, the slow-growth technique has proven itself to be a reliable and efficient technique for the storage of plant germplasm. It has

begun to be widely used as a source of disease-free plants in international material exchange. since it can ensure high recovery rates and the immediate availability of high quality plant material. Furthermore, using this method allows the storage of cloned plant material from several months to years (depending on the type of plant) in sterile conditions with minimal costs (Chauhan et al. 2019). Based on the above, we can assume that *in vitro* culture under slow-growth conditions is the most effective alternative method to safeguard vegetatively propagated plant germplasm currently being preserved in field conditions (Chauhan et al. 2019).

Therefore, this masters thesis aimed at developing an efficient slow-growth method for the *in vitro* storage of garlic *(Allium sativum)* by testing the effects of various osmotic agents at various concentrations (sorbitol 20-60 g/l, mannitol 20-60 g/l, and sucrose 30-150 g/l) and plant growth regulators (chlormequat chloride (CCC) at 200-600 mg/l and abscisic acid (ABA) at 1-5 mg/l). Two cultivation temperatures, 5±1°C, and 18±1°C were also tested to observe their influence on the growth of the plants. Full-strength and 1/2 concentrated MS (Murashige and Skoog (1962) were used as basal culture media.

# 2. Literature Review

## 2.1. *Allium* genus and its representatives

### 2.1.1. Taxonomy of the *Allium* genus

The genus *Allium* contains many species, more than 500 in total (Fenwick et al. 2009). But humanity does not use all of this spectrum; only some species are used for food, including garlic, chive, notably, onion, rakkyo, leek (Fenwick et al. 2009), and different varieties of shallots (Perković et al. 2021). Probably, some of them have not yet been emulated and hide their potential as neglected crops. Nevertheless, it's no secret that many representatives of this family are actively used for culinary and medicinal purposes, especially the common onion (*Allium cepa*) and garlic (*Allium sativum*), which contributed to the formation of local characteristics of cultivation and active distribution throughout the world (Kamenetsky 2007; Hanelt 1990; Ozturk et al. 2012).

Karl Liney, the creator of a unified classification system for flora and fauna, also described the *Allium* genus in 1753 in his work "*Species plantarum: exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas ...* "(Linnaei 1753). This was one of the first attempts to classify plants, then many discoveries and changes followed, but in its modern form, the following hierarchy has been adopted and looks like this (Hanelt 1990):

1. Class Monocotyledones
2. Superorder *Liliiflorae*
3. Order *Asparagales*
4. Family *Alliaceae*
5. Tribe *Allieae*
6. Genus *Allium*

Besides, the genus is generally classified into two subgenera, *Rhizirideum* and *Allium*; the former includes the sections *Cepa, Schoenoprasum*, and *Rhizirideum* (species *cepa, fistulosum, schoenoprasum, tuberosum*) and the latter is comprised of the species *ampeloprasum, sativum* and *chinense* (Ozturk et al. 2012). However, the other classifications have their own followers, as seen in some literature. Hanelt (1990) states that *Allium* and its close relatives are recognized as the distinct family *Alliaceae*, close to the *Amaryllidaceae*, while Reveal & Chase (2011) already state that genus *Allium* is included in the family *Amaryllidaceae.*

### 2.1.2. Ecology, propagation, and cultivation of the main *Allium* species

The genus *Allium* is widespread, especially noticeable in the northern hemisphere over the warm-temperate and temperate climatic zones. Moreover, one or two species can also be found in the subarctic climatic belt. *Allium schoenoprasum L.* has attached itself even to such conditions (Hanelt 1990). But Representatives of the *Allium* genus adapted themselves to latitudinal zoning and to vertical, or rather mountainous. Such a representative of this genus as *Allium cassium* can grow near snow patches, rocky limestone slopes, mountain *pinus* and mixed forests, *Allium cupani* feels good on rocky places on limestone, serpentine and schist, alpine and gray steppe, *Allium frigidum* prefers crevices and rock faces, stony mountain ridges, grassy limestone ledges, while *Alliumkurtzianum* is pretentious and likes to grow on mountain slopes on marble (Ozturk et al. 2012)

However, the main center for the distribution of genus *Allium* is the territory from the Mediterranean to Turkestan. An interesting fact is that the distribution pattern in the world reveals that 211 Allium taxa are distributed in Central Asia, with 55% of these being endemic (Ozturk et al. 2012). It is worth noting that this genus is poorly distributed in the southern hemisphere, or rather in a single specimen. Only one species - *Allium dregeanum Kth.* has been described in South Africa (Ozturk et al. 2012), but this is not entirely reliable since there are doubts that this species was introduced to this territory from Europe by early colonialists.

In addition, a high concentration of species is observed in Turkey and the Irano-Turanian floristic region, for example, Middle-Asia Iran, north Iraq, Afghanistan, (including Kazakhstan), and West Pakistan. The further we move away from these regions the species composition will substantially decrease (Hanelt 1990).

Although most species are concentrated in one place, their biology is very different. Spring, summer, and autumn taxa are distinguished; there are also perennial species with a long and short life span, annual species with one or more life cycles of the formation of the green part. Some of them can show different manifestations of the dormant period. In some plants, it can manifest itself in the summer and others in the winter.

For most annual species, the growth period is limited to a short spring and early summer period. In 2-3 months, the plant manages to go from the beginning of growth to

the formation of seeds; in scientific terminology, such plants are called ephemeroids (Fritsch & Friesen 2002).

As for the conditions for seed germination, there is a very large variability depending on the species. The viability is a couple of years under unregulated conditions, but if certain temperatures and stable humidity are used, the life of such seed can be significantly extended. Evaluation of onion *(Allium cepa L.)* seeds after 10 years of storage at 5, −18, and −196°C based on germination rate, O2 uptake rates, root length and seed moisture content showed that seed deterioration was greater at 5°C, significantly less at -18°C and -196°C (Stanwood & Sowa 1995).

According to environmental requirements, plants of *Allium* genus are not whimsical. They can live in various ecological niches, mainly preferring open sunny places with fairly well-provided water conditions, but some species tolerate arid and waterlogged conditions. Even in aline and alkaline conditions, some taxa are tolerant (Fritsch & Friesen 2002).

### 2.1.3. World production of *Allium* species and their use

*Alliums* have been cultivated for millennia for their sustenance and flavor. But according to assumptions, firstly, these plants began to be cultivated not for nutritional but for medicinal purposes. Even today, plants of this group actively help in the treatment of dysentery bronchitis, abscesses, amoebic dysentery, skin diseases, cholera, tuberculosis (TB), cardiovascular disease (intake has been associated with significant reductions in blood pressure), cholesterol, and platelet aggregation, for asthma, blood contamination, cold restraints, colitis, cough, ear infections, fever, flu, liver and lung disorders, parasites, poisoning, prostate, respiratory problems, allergies, diarrhea, insomnia, diabetes, biliary problems, childhood diseases, ulcers, sinus problems, calluses, and the helminthic (Ozturk et al. 2012).

*Allium cepa* is one of the longest known and most cultivated vegetables. The first memories were already reported 4000 years ago and go deep into Egypt during the Old Kingdom (Fritsch & Friesen 2002). But this plant has not lost its significance even today, occupying a leading position among this genus. It is actively used in the food industry and for technical processing. Moreover, this vegetable is only increasing its production volumes.

According to the Food Agriculture Organization for 2019, the territory occupied by onions (Figure 1) was 5,192,651 hectares. The volume of cultivated products was 99,968,016 tons. In 2008 these figures were 4,066,189 hectares, and 75,013,844 tons are similar, while the increase in the territory was 21.7%, and in the harvest by 25%, that is, by almost a quarter ("FAO: Production/Yield quantities of Onions, dry in World + (Total)" 2021).

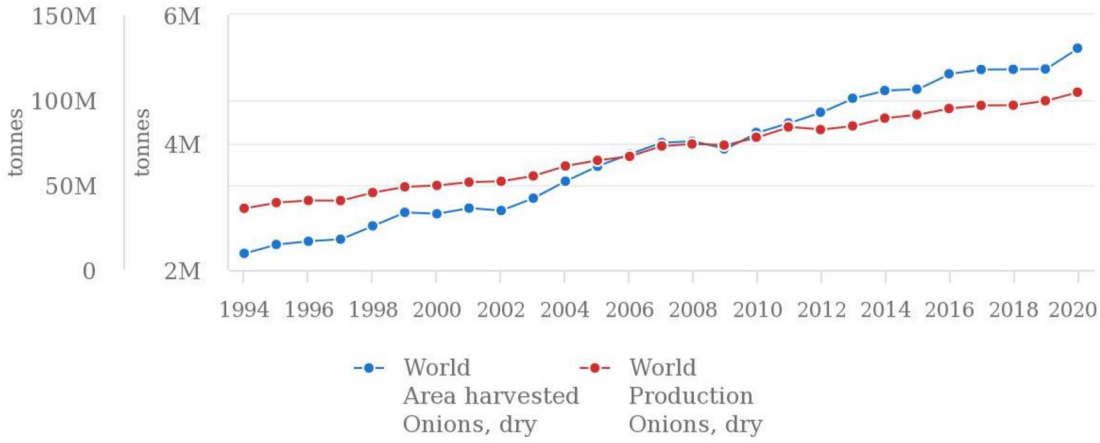


**Figure 1.** Production/Yield quantities of Onions, dry in World + (Total) 1994 – 2020.

*(Source: FAO: Production/Yield quantities of Onions, dry in World + (Total). 2021. FAO, FAOSTAT).*

The honorable second place is occupied by *Allium sativum* or simply garlic. Its production has also increased in recent years. For example, in 2019, the area under garlic (Figure 2) was 1,634,634 hectares. The gross harvest was 30,708,243 tons, which is significantly higher than in 2008: area – 1,403,732 hectares and harvest – 22,780,572 tons, while the increase in area was 14.1%, and in gross collections 25.8% ("FAO: Production/Yield quantities of Garlic in World + (Total)" 2020).

In many cuisines of the world, many species from the *Allium* genus are used in the ripe form and during the growing season - the green (leaf) part is eaten. The production quantities of onions and shallots on greens (Figure 3) in 2019 was at the level of 4,491,246 tons and occupied an area of 220,246 hectares. In 2008 the situation looked like this: the area was 233,645 hectares, and the harvest was 4,455,468 tons. In this case, there was a slight decrease in the amount of occupied space by 5.7%, while the collection of products increased by 0.8% ("FAO: Production/Yield quantities of Onions, shallots, green in World + (Total)" 2021). Here is a clear effect of the intensification of production.

As for leeks and other alliaceous vegetables (Figure 4), as of 2019, an area of 136,103 hectares was planted, and a harvest of 2,192,476 tons was obtained. Compared to 2008, the area occupied by the crop increased by 7.6%, and the yields increased by 5.1%; compared to 1994, it can be considered that there has been an enormous leap in the promotion of this type of vegetables. Over 15 years, the occupied area has increased by 32.4%, and the yields have increased by 23.8%. At the same time, it is worth noting that in the period from 1995 to 2004, there were jumps in production, and the gross harvest reached rather low levels (“FAO: Production/Yield quantities of Leeks, other alliaceous vegetables in World + (Total)” 2021).It should be noted that the production of *Allium* plants, or rather the occupied area under them, is variable in nature since the construction of farming strategies largely depends on the prices for these products in the previous season. Such a pattern occurs that at a low price in the previous season, a decrease in areas in next. It was reported that cobweb theory is valid for onion production (Hanci 2018). The cobweb theorem is an economic model used to explain how small economic shocks can become amplified by the behaviour of producers (“Cobweb theorem” 2020). In agriculture, this theory explains why price fluctuations occur in markets where there is a time interval between planting and marketing (Pashigian 1970).

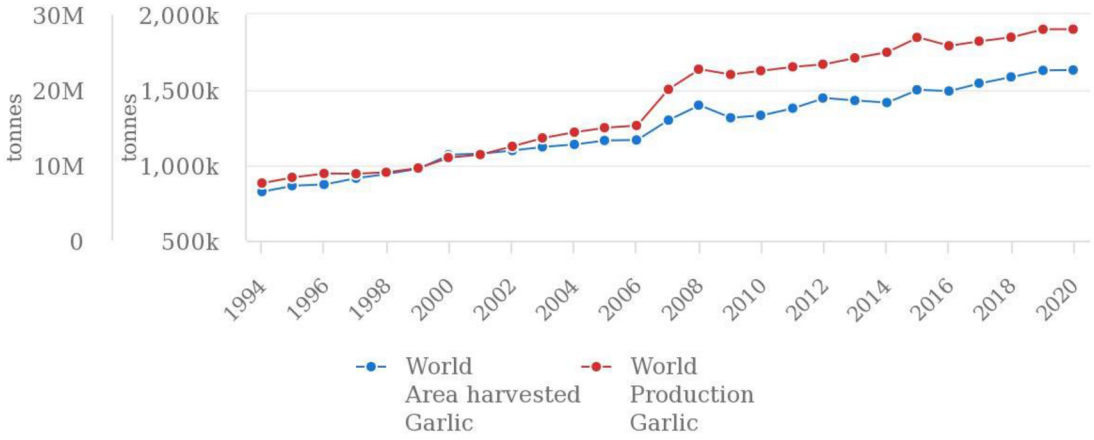


**Figure 2.** Production/Yield quantities of Garlic in World + (Total) 1994 – 2020.

*(Source: FAO: Production/Yield quantities of Garlic in World + (Total). 2021. FAO, FAOSTAT).*

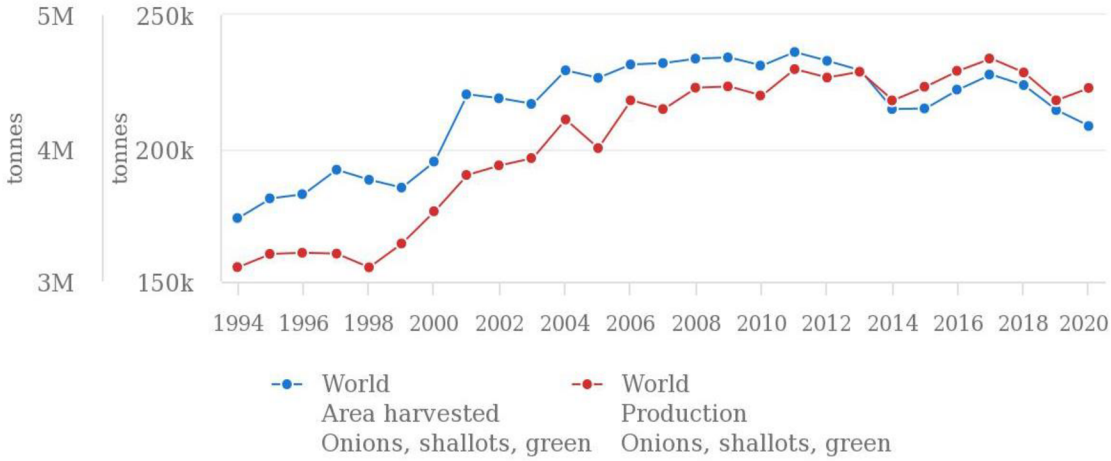


**Figure 3.** Production/Yield quantities of Onions, shallots, green in World + (Total) 1994 – 2020.

*(Source: FAO: Production/Yield quantities of Onions, dry in World + (Total). 2021. FAO, FAOSTAT).*

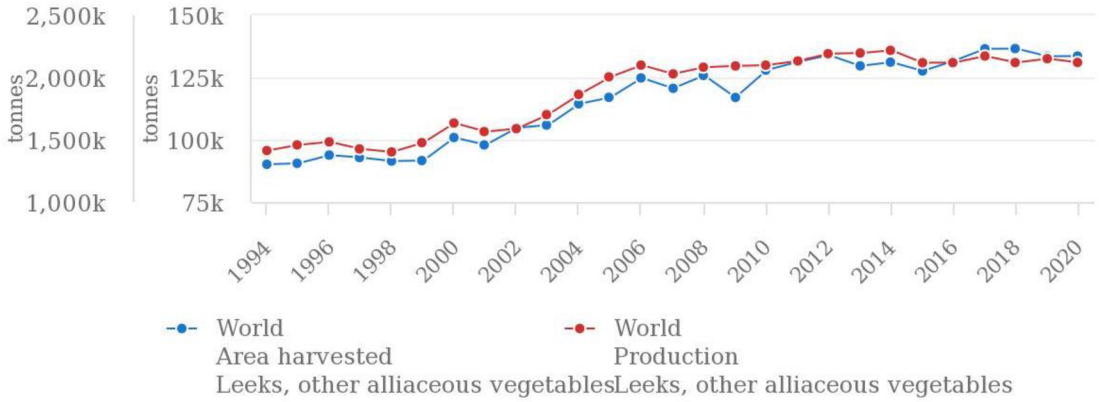


**Figure 4.** Production/Yield quantities of Leeks, other alliaceous vegetables in World + (Total) 1994 – 2020.

*(Source: 18. FAO: Production/Yield quantities of Leeks, other alliaceous vegetables in World + (Total). 2021. FAO, FAOSTAT).*

### 2.1.4. Conservation of *Allium* plants

Human health is directly related to diet, or rather to its diversity. Thanks to a balanced diet, the human body receives all the necessary micro and macro elements, amino acids and other organic compounds. The primary resource of these nutrients is obtained from vegetables and meat. In this case, vegetable biodiversity is the main and irreplaceable genetic resource in the food supply (FAO 2017) .

But due to vigorous economic activity, humanity has endangered many plant species. It is estimated that 21% of known species (L Pimm & H Raven 2017) are endangered and may disappear forever, among which there are many plants for food purposes.

Negative changes are taking place both in the wild and in artificial landscapes. First of all, such changes are provoked by climate change on the planet. Only since the beginning of the 20th century, the average temperature on Earth has risen by 1°C. Which made the last 16 years the hottest in the history of weather observations, and the period from 1893 to 2017 was the hottest in the northern hemisphere in the last 1400 years ("Climate Box" 2022). These changes affect many factors of plant life, including water availability, soil conditions, environmental aspects, which by 2100 year may lead to an 11% reduction in the number of days favorable for plant growth and development, which will complicate their survival (Worland 2015). But even now we can observe the first results of climate change, according to the International Union for Conservation of Nature (IUCN) climate change currently affects at least 10,967 species ("Species and Climate Change" 2021).

Along with this, there is a direct human impact on biodiversity. And first of all, this is active agricultural activity, or rather, changes in land use, the transition to large-scale food production, which led to a 30% of biodiversity decline globally ("How do humans affect biodiversity?" 2020). For example, the intensification of agriculture is actively displacing local varieties, replacing them with more productive and disease-resistant ones. But at the same time, we lose the possibility of future progress since local varieties can be actively used in breeding work, which can improve the quality and quantity of already existing varieties (Ruta et al. 2020).

But if we take climate change and the intensification of agriculture, a new threat appears - the active development of diseases and pests. Moreover, pests are becoming more destructive and posing that increasing threat to food security and the environment, about 40% percent of global crops are being destroyed by pests every year ("Climate change fans spread of pests and threatens plants and crops, new FAO study" 2021).

The processes of urbanization, pollution, spread of invasive species also have a great impact on the environment ("How do humans affect biodiversity?" 2020).

Therefore, back in 1992, the United Nations adopted the Convention on Biological Diversity. It has been signed by 196 nations and has become a critical regulatory

instrument for: "the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources" ("Convention on Biological Diversity"). Since then, the International Day for Biological Diversity has been celebrated annually on May 22.

Concerning biodiversity conservation, in the modern world, plant germplasm conservation can be performed in two ways: *in situ* and *ex situ* (Ruta et al. 2020). Let's consider them in more detail.

#### 2.1.4.1. *In situ* and on-farm conservation

This type of conservation implies the maintenance of species in their natural habitat through conservation practices and ecosystem management, or conservation is pursued by promoting farming landraces and ancient crop varieties (Ruta et al. 2020).

In other words, we can say that the conservation of germplasm can occur both under artificial conditions - on farms and in natural conditions - these can be national parks, game reserves, and special reserves and sanctuaries (Ajayi 2019).

This type of conservation, like everything in this world, has its advantages and disadvantages. The main advantage is that the protected object is located in natural conditions, where they have developed their distinctive properties. Therefore, this method can be used to conserve species that are difficult to conserve outside of their cultivation area. It is especially applicable for crop wild relatives (Amrapali & Babu 2016). In addition, it contributes to the conservation of the species natural ecosystem in which its natural genetic development occurs, including the cross-pollination of the protected plant with its closest wild relatives, which is good in terms of biological progress.

But as for the disadvantages, the main thing of all will be the inaccessibility of germplasm for research at a particular moment. Sometimes it takes about a year to get the desired specimen. In addition, there is a limited quantity, storage problems arise, etc. Also the problem of climate change is not one of the last places here. Every year it becomes more difficult to grow plants in areas where they have been cultivated for centuries, which exposes *in situ* and on-farm conservation practices to a high degree of danger. For example, in just a couple of days in 2012, the cherry industry of the Michigan State in the USA lost 90% of its tart cherry crop after a late freeze ("Climate Box" 2022). At the same time, this type of germplasm conservation is not immune from the invasion of pests and diseases. A good example is the locust invasion in Kenya that began in December

2019. These insects are able to multiply 20-fold in three months and reach densities of 80 million per square kilometer. At the same time, their appetite is also not small, each individual can eat up to 2 g of plants, which, in terms of a population of 80 million, will be equivalent to the daily diet of 35,000 people (BBC 2020).

In this case*, ex situ* conservation comes to the rescue.

### 2.1.4.2. *Ex-situ* conservation

*Ex-situ* conservation refers to the movement of endangered or rare species from their natural habitats to protected areas equipped for their protection and preservation (Ajayi 2019). That is, the growth and development of the required species occur under partially or completely controlled conditions.

This method involves many sequential steps such as sampling, transportation, storage of the investigated species from a specific location.

In addition, when using this method, a kind of genetic stability appears, which is suitable for research but not within the framework of nature. Also, the use of this type of conservation allows preserving biological material for a short (1-11 months), medium (1-2 years), and long term (unlimited case).

This biodiversity conservation strategy includes zoological gardens, botanical gardens, DNA banks, conservation stands, genes, pollen, seeds, seedling and *in vitro* tissue culture collections (IyyappanJaisankar et al. 2018).

#### 2.1.4.2.1. *Ex vitro* conservation

One of the most straightforward strategies in *ex vitro* conservation is to create seed germplasm banks for storing both wild and cultivated plants at low temperatures conditions. This method is simple but short-lived since the seeds of each species have their own lifespan. For example, onion seeds lifespan - 2 years; simultaneously, it requires its own storage conditions, which does not exempt seeds from replication and the need for their repeated reproduction. What is good about this method is that it does not require large investments, and it is possible to implement it for farmers and researchers (Tankley & Mccouch 1997).

Another direction in the conservation of biodiversity in this method is zoological and botanical gardens. In total, there are more than 1500 botanic gardens containing more than 80,000 species and more than 800 professionally managed zoos around the world

with about 3000 species (IyyappanJaisankar et al. 2018). Moreover, many of them are threatened and endangered species.

### 2.1.4.2.2. *In vitro* conservation

The *in vitro* conservation methods are one of the best ways to increase and conserve the available germplasm resources. This technique allows obtaining material free of pathogens and viruses and preserving this biological material for the desired period with the possibility of its subsequent use. In addition, such collections do not take up much space, are compact enough, and work regardless of the season.

In terms of duration, the *in vitro* technique acts as short-term (*in vitro* culture), medium-term (slow-growth or minimal growth method), and long-term (cryopreservation) (Cha-um 2007).

The *in vitro* propagation method, also known as "micropropagation", starts from primary explants, with an exponential increase in the number of individuals (shoots). The maintenance of such plant material under standard growing conditions implies transfer to a new nutrient medium every 3–6 weeks. The storage periods depend on the mortality of the biological material over time. Some species require more frequent transplanting since they quickly deplete the nutrient medium and die, which is unacceptable (Ruta et al. 2020). But most often the plant goes into senescence, a state when all growth processes in the cell slow down and its division stops. This phenomenon may be caused by salt stress, by decreases of soluble protein contents and by membrane permeability (Vieira Santos et al. 2001).

To avoid this phenomenon and reduce the time and money spent on transplantation, it is possible to use the slow-growth storage technique. The main task of this method is to reduce the growth of plantlets and prolong the subculture interval whilst minimizing or eliminating the risk of genetic instability generated through tissue culture manipulations (Agrawal et al. 2019).

Over time, this technique has led to the creation of *in vitro* gene banks, where clonally propagated plants are stored.

At the moment, medium conservation techniques have been developed for such plants as artichokes (*Cynara cardunculus*), cassava (*Manihot esculenta*), chicory (*Cichcorium intibus*), mint (*Mentha spp*.), potato (*Solanum tuberosum*), sweet potato

(*Ipomoea batatas*), thyme (*Thymus vulgaris*), and onions (*Allium cepa*) and many other plants (Ruta et al. 2020).

At the same time, it should be noted that for this storage method, depending on the species, different parts of the plants are used. In some cases, shoots, microtubers, synseeds, microcuttings, microbulbs, bulb portions. As for onions and garlic, in most cases, microbulbs are used, which is not very convenient for quick regrowth.

For example, to store onion microbulbs for 12 months, a standard nutrient medium (MS) is used, the osmotic substance was sucrose at a concentration of 100 g/L), the storage temperature was 1 °C, and the survival rate was 100% (Kästner et al. 2001).

But what about long-term storage of germplasm?

This option has been made by the use of the cryopreservation method. This method is considered one of the safest and most cost-effective long-term strategies for conservation (Agrawal et al. 2019). This technique is based on the reduction and subsequent arrest of metabolic functions of biological material stored at the ultra-low temperature of liquid nitrogen (-196 ° C) (Zamecnik et al. 2007).

The cryopreservation technique is actively used for many plants, especially for vegetatively propagated species. Such an example is garlic, whose plants do not produce fertile seeds, maintenance of field collections is laborious and expensive and at the same time classical *in vitro* conditions do not look promising in the long term storage (accumulation of endophytes) (Olas-Sochacka & Kotlinska 2015). Another good example of vegetatively propagated plants involved in cryopreservation is the *Musa* tree. (Panis, et al. 2004).

The cryopreservation method allows the preservation of organs and tissues from *in vitro* culture and the field by very rapid cooling. If done correctly, intact cells with stopped metabolic processes are obtained (Ruta et al. 2020).

This technique is quite difficult to perform since there is a great danger of destrdestroying plant cells by ice crystals; there is the so-called “ice nucleation“factor. But on the other hand, this technique allows storing plant material for an unlimited number of years (Popov et al. 2006).

Thanks to this technology, it is possible to create another type of germplasm bank, called “cryobanks”, where samples of living material are constantly stored in liquid nitrogen (“Cryo bank” 2021).

According to some reports, there are 22 conservation centers in the world, located in 16 countries (Europe, Asia, Africa, Oceania, North and South America), where more than 45,000 accessions are stored (in total: in *in vitro* banks + cryobanks) (Ruta et al. 2020). At the same time, of the total number of conserved samples, only about 8300 accessions are stored in cryobanks, while the remaining four-fifths are stored *in vitro*, under slow growth conditions (Ruta et al. 2020).

Many species have already been stored in banks using cryopreservation, including various varieties of apple, strawberry, and sweet potato, but the undoubted leader in this process is *Solanum spp*. Potato takes over 60% of the contents of cryobanks, the second place is occupied by cassava, representing 25.33% of all accessions, and the honourable third place, as strange as it may sound, is occupied by garlic with its 11.15% (Ruta et al. 2020).

## 2.2. *Allium sativum*

### 2.2.1. Taxonomic classification

Garlic (*Allium sativum*) belongs to the *Allium* genus, which contains more than 600 species widely distributed throughout the world: Europe, North America, North Africa, and Asia are the primary growth centres (Ozturk et al. 2012).

The position of Genus Allium is as follows: 1. Class – *Liliopsida*. 2. Subclass – *Liliidae*. 3. Superorder – *Liliianae*. 4. Order – *Amaryllidales*. 5. Family – *Alliaceae*. 6. Subfamily – *Allioideae*. 7. Tribe – *Allieae*. 8. Genus – *Allium* (Stavělíková 2008).

According to modern taxonomy, *Allium sativum* and its closest wild relative *Allium longicuspis* form a species complex (Kamenetsky et al. 2004). More specifically, this species complex consists of three main groups, the first being the common garlic group *(Allium sativum var. sativum and A. sativum var. typicum Regel)*, the second being the *Longicuspis* group, and the third one is the *Ophioscorodon* group, and two additional subgroups: subtropical and *Peking* (Fritsch & Friesen 2002).

From a morphological point of view, there are two basic morphotypes of garlic in horticultural practice: the morphotype of hard neck garlic and soft-necked garlic (Figure 5 and Figure 6). The botanical classification of these plants also distinguishes these two morphotypes as: *Allium sativum var. ophioscorodon* (hard-necked garlic) and *Allium sativum var pekinense (Prokh.*), another variety is distinguished as *Allium sativum var sativum* (soft-necked garlic) (Ovesná & Velát 2020).

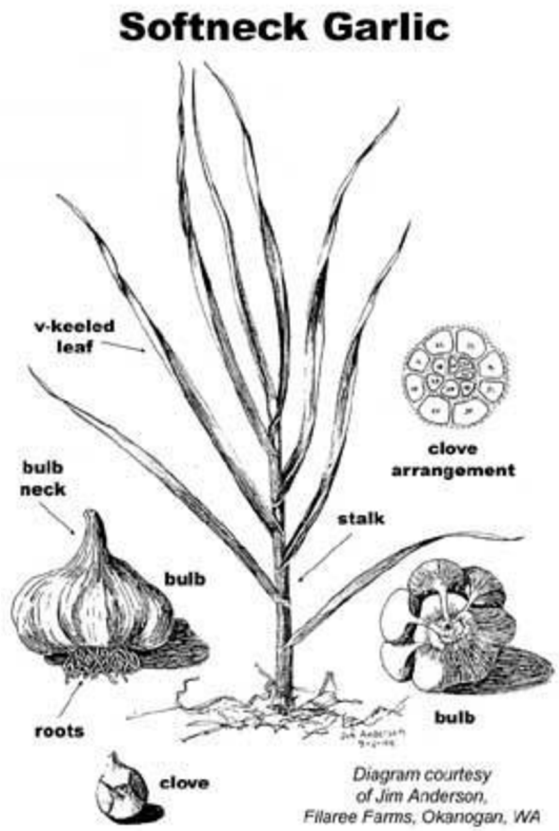


**Figure 5.** Softneck garlic.

*(Source: "Crop Profile for Garlic in Washington" (2002)).*

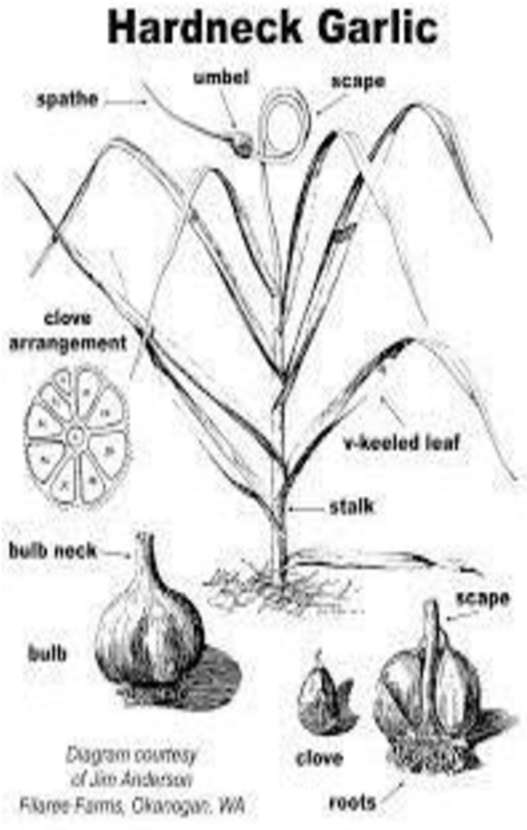


**Figure 6.** Hardneck garlic.

*(Source: "Crop Profile for Garlic in Washington" (2002)).*

## 2.2.2. Botanical description and cultivation

Garlic is a perennial monocotyledonous plant that forms onions composed of few to many densely packed elongated side bulbs - cloves. Garlic plants grow up to about 60 cm in height ("Garlic" 2021).

Depending on the varietal characteristics, new leaves usually emerge from a short, stiff stem above the bulb or from a softer pseudostem consisting of overlapping leaf sheaths. The bulb itself is covered with a membrane skin. The globular cluster of flowers is initially enclosed in a pair of paper-like conical bracts; when the green-white or pinkish

**Figure 7.** *Allium sativum* (garlic) plant. *(Source: "Allium sativum (garlic) plant" (1793))*

flowers open, the bracts open. Occasionally, flower stalks develop tiny bulbs (tiny secondary bulbs that form instead of flowers) and sterile inflorescences ("Garlic" 2021).

As previously mentioned, there are two basic morphotypes of garlic from a morphological point of view in horticultural practice: the morphotype of hard neck garlic and soft-necked garlic, which have their own differences in structure.

Hardneck cultivars differ in their growth and development; they produce a flower stalk (or scape) (Figure 6) and are often termed as "topsetting" or "bolting" cultivars (Kamenetsky 2007). In comparison to soft neck cultivars, they do not have a flower stem as such and the bulb generally contains a number of whorls with 10 to 50 cloves (Figure 5), while hardneck garlic cultivars produce bulbs comprising 1 or 2 whorls with 4 to 12 to 15 cloves surrounding the flower stalk (Kamenetsky 2007).

As for flowers, they usually abort during development, and small bulbs are formed in the inflorescence.

In general, the hardneck garlic morphotype has a more reliable yield, adapts better to the environment, and has a higher yield on average. For growers, this is the most basic division, represented by narrow-leaved and broad-leaved garlic, as well as winter and spring forms (Ovesná & Velát 2020).

But there are other classifications. For example, in the United States, garlic is classified by type, color, and number of cloves. "Rocambole morphotype" is one of the most common types of garlic in North America (Ovesná & Velát 2020). It is very popular and known for its robust, well-balanced garlic flavor. It usually has 6 to 10 cloves that peel well and belong to hardneck garlic morphotype (Ovesná & Velát 2020).

Regarding the preferred growth and storage temperature of bulbs, soft neck cultivars are commonly cultivated in different warm regions, and only some are suitable

for cold climates. Softneck garlic generally has a much longer shelf life than hardneck garlic and can be stored under standard conditions for 6 to 8 months (Kamenetsky 2007).

### 2.2.3. Reproductive biology

Almost all types of cultivated garlic can only reproduce vegetatively due to their sterility (Kamenetsky 2007). Therefore, one of the main methods of maintaining collections remains the field method, the second is the storage of bulbs, and the third is the storage of plant material under *in vitro* conditions. In the first case, the collection should be planted every year in spring or autumn, the garlic bulbs do not withstand the second year of storage (Olas-Sochacka & Kotlinska 2015). However, there is a big problem with field collections since it becomes necessary care for it every year, and, in addition, the genetic resources of garlic, being in the field, are largely susceptible to losses due to the influence of weather conditions, fungal, bacterial and viral diseases (Olas-Sochacka & Kotlinska 2015). This problem can be solved by collecting and placing varieties under *in vitro* slow growth storage conditions (Cha-um 2007).

Garlic is a so-called apomictic species; it uses asexual reproduction, in which sexual organs (such as a flower, pistils, etc.) are developed, but in fact, there is no fertilization at all. Apomixis leads to numerous somatic mutations. As a species that reproduces vegetatively, Garlic shows a wide range of diversity in its morphology, onion properties, and adaptability (Ovesná & Velát 2020).

For a long time, scientists have wanted to overcome the obstacle of sterility. It has been suggested that the presence of vegetative topsets are one of the major causes of sterility (Kamenetsky et al. 2004). Over time, they managed to do this with the help of chemicals. Fertile garlic plants were found in the Tien-Shan Mountains (between Kazakhstan and China) (Kamenetsky et al. 2004). In the latter work, 27 clones were classified as highly fertile, producing over 400 seeds per umbel, and seed germination ranged from 67% to 93% (Kamenetsky et al. 2004). Although plants capable of pollination were found, the problem of sterility remained. New varieties of garlic continue to be bred by clonal selection and by introducing varieties into different environments.

### 2.2.4. Genetic background and variability within the species

Among populations of cultivated garlic, genetic variation is very important for the economic utilization of both genes and genomes (Figliuolo et al. 2001). Genetic

assessment and collection of germplasm of cultivated garlic will allow identifying samples that can be useful for obtaining new varieties using clonal selection and for identifying samples with fertile inflorescences, which will be used in breeding work in the future (Kamenetsky et al. 2005).

By its nature, garlic is a diploid species and has 16 chromosomes (2n = 2X = 16) (Figliuolo et al. 2001). The main bases that distinguish garlic clones and allow selection are variable for morphophysiological traits, canopy structure and yield-related traits, which are especially important for commercial species (Figliuolo et al. 2001). But besides this, to identify genetic differences, a complex of analyzes is carried out, such as infraspecific differentiation by isozyme and analysis of Random Amplified Polymorphic DNA (RAPD) markers (Maaß & Klaas 1995). In addition, there is mention that in *Allium sativum*, the degree of heterozygosis for isozymes is high (Maaß & Klaas 1995).

Knowledge of the genetic traits and vegetative yield-related traits of garlic species in combination with the estimation of the degree of heritability will improve and steer breeding work in the right direction.

## 2.2.5. Origin and geographic distribution

According to some assumptions, wild garlic began its distribution in early times, in central Asia, as early as 10,000 years ago, when the sami-nomadic tribes began to cultivate it (Kamenetsky 2007). They most likely were the first to spread garlic to new lands, namely to the Mediterranean region, India and China. Evidence has also been that garlic was used in Egypt as early as 2000 years BC, and in China and India even earlier than 5000 years BC. (Kamenetsky 2007).

Over time, in Europe, garlic gained great popularity, overgrown with myths and legends. Then, along with the European colonists, it came to sub-Saharan Africa, North and South America (Kamenetsky 2007).

Garlic is known to be able to adapt to different climatic conditions and as a cultivated plant is grown all over the world (Ozturk et al. 2012).

Garlic can be found in almost every country, so the geography of its cultivation is vast and covers almost all climatic zones, from the equator to 50° parallel (Kamenetsky 2007). This plant is very unpretentious, but like all living organisms, it has its favorite conditions and can be grown in regions where there is nutritious soil, not too cold winters,

warm summers with moderate rainfall, which contributes to the filling and ripening of the bulbs(“Crop Profile for Garlic in Washington” 2002).

### 2.2.6. Uses and nutritional value

Our world is very gastronomically diverse; each nation has its dishes and recipes. Although all dishes look different, the ingredients may be the same. A good example is garlic, which is used in almost all cuisines of the world and is a universal enhancer of taste and aroma, is suitable for preparing side dishes and main dishes and is widely used in canning and pickling and can also be consumed fresh and dried. Moreover, sometimes it is even used as an ornamental plant (Kamenetsky 2007).

For the convenient use of this spice, the modern market offers various forms of garlic products, such as garlic extract, garlic powder, garlic oil and of course, raw garlic.

In many cultures of the world, garlic is used not only in cooking but also for medicinal purposes. Combining these factors has brought this plant to second place (Peter 2016) in cultivation and popularity from the *Allium* family, firmly consolidating its position immediately after the onion (Peter 2016).

Due to these features and the actively growing population, the amount of garlic planted every year is growing. According to the Food Agriculture Organization (FAO), in 2019, about 30,708,243 million tons were harvested in the world. The total occupied area under the crop was 1,634,634 ha, which is 7,927,671 tons more than in 2008 and more by 230,892 hectares over the same period (“FAO: Production/Yield quantities of Garlic in World + (Total)” 2020). This underlines the growing interest in this crop among both farmers and researchers.

Interestingly, a simple survey showed that consumers in the Czech Republic prefer garlic of the hard neck morphotype, preferably purple (Ovesná & Velát 2020), despite the complexity of the agricultural technique, compared to soft neck garlic, it does not form a peduncle. The latter is grown more intensively outside the Czech Republic.

Also, many of us know from childhood about the therapeutic properties of garlic, that it helps with colds and strengthens the immune system. And indeed it is, it exhibits antioxidant, anticarcinogenic, antimicrobial, antifungal, hepatoprotective, neuroprotective, antimutagenic, immunomodulatory, hypolipidemic, anti-hypertensive, antiviral, antidiabetic, cardioprotective, antiasthmatic, anti-inflammatory, anti-pro-amnesic properties (Bisen & Emerald 2016).

In terms of the nutritional value of garlic, standard bulbs contain fiber, protein, ash, oil, raw energy, dimethyl sulfite, essential oils, and minerals including K, P, Mg, Na, Ca and Fe (Haciseferogullari et al. 2005).

A historical fact, during the war, soldiers were given garlic, it was mandatory in the diet of snipers since in conditions of poor nutrition, it helped to avoid night blindness. "Russian penicillin" - this name was given to garlic during World War II ("Body Watch: The Scent of Garlic Is in the Air" 1995).

The average water content of garlic is 58.6 g, the energy value of garlic is 149 kcal per 100 g or 623 kJ, while the sugars content is 1g, and vitamin C ascorbic acid in total) is present in amount - 31.2 mg. ("Garlic, raw (SR LEGACY, 169230)" 2019).

As for the quantitative composition, it contains crude protein - 17.2 %, oil content - 0.14 %, dimethyl sulfite is present in the amount of 1779 mu cg/kg, and the trace element content is as follows: phosphorus - 6009.37 mg/kg, potassium - 21,378.84 mg/kg, magnesium - 1056.15 mg/kg, sodium - 532.78 ppm and calcium - 363.61 ppm (Haciseferogullari et al. 2005).

### 2.2.7. Legislative nuances of production

Since ancient times, garlic has had a dual purpose; first of all, it was used as a medicine, and secondly, as food. Since then, not a lot has changed, now it is consumed in much larger quantities. According to nutritional and medicinal properties, garlic products are marketed in different ways; for example, in the European Union (EU), it's marketed as foodstuffs and as herbal medicinal products (Kroes 2005).

Imports of garlic into the EU are subject to a system of import licenses and certificates of origin. Implementing Regulation (EU) No 341/2007 and Implementing Regulation 2016/2243 (Ovesná & Velát 2020) lay down the conditions for the administration of tariff quotas and the introduction of a system of import licenses and certificates of origin for garlic and certain other agricultural products imported in EU.

Also quite interesting is the legislation regarding garlic in the Czech Republic; according to Vyhlášky o podrobnostech uvádění osiva a sadby pěstovaných rostlin do oběhu "(2012), there are some nuances in the placement of canned varieties on the market, which directly concerns field collections. It says the following.

The number of seeds of each canned variety of agricultural species placed on the market may not exceed the following percentages of the number of seeds of the same

species used in the Czech Republic during one growing season, or may not exceed the amount required to sow on an area of 100 hectares, depending on which of these two quantities is greater. The percentages are set as follows: 0.5% for other species, including garlic. Also, the total amount of seeds of all canned varieties of one type of agricultural species that can be sold in the Czech Republic cannot exceed 10% of the number of seeds of the same type that is used in the Czech Republic for one year or cannot exceed the amount required for sowing. an area of 100 hectares, whichever is greater. The number of seeds of each canned variety of vegetables placed on the market per year cannot exceed the number of seeds required for growing vegetables in a certain area: 10 hectares for onions, garlic, chervil, celery, celery, asparagus, vines, escarole, parsley, scarlet beans, radishes, radishes, rhubarb, black root, gooseberries, corn, and popcorn. Also, the Institute shall conduct a subsequent verification of the varietal authenticity and varietal purity of the stored variety placed on the market by means of vegetation tests within at least 5% of the lot of seeds placed on the market.

## 2.3. Plant material and its main quality indicators for *in vitro* conditions

In recent years, *in vitro* culture has become a powerful tool for both science and commercial use. Indeed, it is difficult to disagree with the words of Kumar & Reddy (2011) that micropropagation is an alternative means of propagation that can be employed in the mass multiplication of plants in a relatively shorter time.

Plant micropropagation is a sequence of steps in which tissues, organs, and cells are amenable to specific manipulations such as isolation, sterilization, and placement in a controlled environment for subsequent cloning. In general, this process can be divided into five stages (Kumar & Reddy 2011).

The first step is the cultivation of the mother plant in the field or greenhouse conditions. In the case of garlic, getting healthy and ripe bulbs (Olas-Sochacka & Kotlinska 2015).

The second step is culture establishment, which includes the selection of the best samples, disinfestations, and sterilization for subsequent growth. Again, in the case of garlic, the growth meristem is released from the cloves (one in one clove) and sterilized (Olas-Sochacka & Kotlinska 2015; Kumar & Reddy 2011).

The third stage is a multiplication phase, which implies a rapid resumption of the development of culture. In this phase, the culture begins to sprout for subsequent reproduction (cloning). But not in the situation with garlic. Garlic sprouts are not formed on every variety, therefore, after the plant is strengthened, occurs an artificial division of the lower part of the stem into 2-6 parts, depending on the degree of development (Olas-Sochacka & Kotlinska 2015; Kumar & Reddy 2011).

The fourth stage is the elongation and induction of roots, the penultimate phase before moving to *ex vitro* conditions, the main goal of which is to obtain healthy and well-developed plants. In this study, will also be used, garlic will be placed on regrowth after slow growth conditions (Olas-Sochacka & Kotlinska 2015; Kumar & Reddy 2011).

And, finally, the fifth stage - the transfer of plants from *in vitro* conditions to *ex vitro* (Cha-um 2007).

The term "meristem culture" itself means a meristem without leaves primordial or at most 1-2 leaf primordial, which are excised and cultured (Kumar & Reddy 2011). As for garlic, a monocotyledonous plant, its introduction into *in vitro* can be carried out with microbulbs, the culture of extracted central meristems, or culture of shoots (Ruta et al. 2020).

It is important to observe the quality of plants at each stage during micropropagation: presence or absence of infection, color, the strength of plant growth, presence of hypohydration (Kumar & Reddy 2011). Especially it is worth paying attention to the formation of callus, cause shoot bud formation without any callus phase from appropriate explants is of great success for large-scale clonal multiplication of desired plants (Kumar & Reddy 2011). If it is formed, this can lead to genetic mutations and the main rule *in vitro* is that we must get the same plant at the end of the process, which will be violated at the beginning.

## 2.4. *In-vitro* establishment of *Allium sativum*

To start shoot culture under *in vitro* conditions, it is necessary to perform surface sterilization. This procedure is necessary to disinfect the source material from harmful fungi and bacteria. This procedure can be carried out in different ways. One of them is dipping the sample in ethanol and followed by flaming. In the case of garlic, this operation is carried out with peeled cloves, then the protective leaf is removed from each clove and the central meristem is planted on a nutrient medium (Bhojwani et al. 1982).

In this procedure, various approaches can be used, at the beginning, before disinfection with ethanol, garlic cloves can be placed under running water for a certain period of time and then subjected to various types of detergents and antiseptics (Dixit et al. 2013).

It is also possible to use sodium hypochlorite (NaClo) for surface disinfection. For example, for sterilization of banana plants (*Musa spp.)*, it is possible to use NaClo in such concentration as 0.01% at pH 5.4, but even when using a concentration of 0.002%, an inhibitory effect on bacteria and their subsequent death is achieved (Matsumoto et al. 2009).

It is also possible to use the above-mentioned chemicals in a complex way. In the study by Metwally et al. (2014) "*In vitro* propagation of garlic (*Allium sativum* L.) through adventitious shoot organogenesis", surface sterilization of garlic cloves was carried out using ethanol and sodium hypochlorite in combination. The cloves were initially immersed in ethanol for 30 seconds and then immersed in a 3.5% sodium hypochlorite solution for 20 minutes.

Instead of ethanol and NaClo and their combinations, it is also possible to use other substances. One of these is PPM™.

Plant Preservative Mixture ™ (PPM ™) has established itself as a phyto-safe and highly effective microbicide, effective against bacteria and fungi by killing multiple enzymes, which in turn does not lead to the development of resistance (Agrawal et al. 2017). The solution contains two isothiazolones, namely 2-methyl-3 (2H) isothiazolinone (MIT) and 5-chloro 2-methyl 3 (2H) isothiazolinone (CMIT) (Agrawal et al. 2017).

But for each culture, it is necessary to select their own concentrations. In an experiment with establishing *in vitro* cultures from papaya field plants different concentrations of PPM ™ were used: 5% for 4 hours, 50% for 10 minutes, or 100% for 10 minutes also. As a result, all concentrations failed. As result, all concentrations were failures, in the first case the contamination was 80%, in the second 90%, and in the third case, it was 95% (Agrawal et al. 2017).

It is also possible to use excipients to improve the sterilization effect, it can be carbendazim cetrimide ($HgCl_2$ or polysorbate 80 (Metwally et al. 2014; Agrawal et al. 2017).

## 2.5. Application of slow-growth method on *Allium sativum*

### 2.5.1. Biodiversity conservation practices for *Allium sativum*

The number of garlic varieties is very large, each of which has its own characteristics: color, shape, shelf life, taste, etc. But it is the 21st century, all we know is that an increasing population, modern agriculture, and industry worldwide damage plant habitats while forgetting that it also, as stated by Heywood & Iriondo (2003), directly reduce plant genetic diversity. In other words, some local wild and cultivated varieties can be supplanted by more productive ones or destroyed by human negligence.

To prevent such consequences, special protective territories are created, plant collections in botanical gardens and research institutions, etc. The so-called "plant conservation" takes place, where varieties are not only preserved but multiplied. Cha-um (2007) has a very good opinion on this: "Plant conservation is one of the most attractive ways to concern and intensively manage plant genetic resources for sustainable usages". Garlic is unpretentious in cultivation, it does not require much care or extra fertile soil, preferring a temperate climate with a balanced nutrient and water regime. But, like all crops, it has its own characteristics. Firstly, this plant can be grown both as an annual or biennial crop, and secondly, garlic requires vegetative propagation, by dividing the cloves, since it is natural sterile (Sendl 1995).

Due to the difficulties and disadvantages arising from the classical conservation of plants: annual planting of collections is required, which is accompanied by losses as a result of attacks by pathogens or changes in climate, as well as large losses during storage, new approaches have emerged in creating such collections. There are numerous studies demonstrating the possibility of applying innovative conservation methods, and one of them is in-vitro slow-growth storage (Ruta et al. 2020).

Very interesting is the concept mentioned by Prabhakaran Nair (2013), which claims that *in vitro* culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells.

*In vitro* culture provides many opportunities, the first of which is the ability to obtain "clean" plant material, free from pathogens and viruses. According to scientists, *in-vitro* meristem culture is a progressive channel to eliminate bacteria, fungi, and viruses from host plants (Cha-um 2007). Depending on the timing and methods of the proposed conservation of plants under *in vitro* conditions, three categories are distinguished: short-

term (*in vitro* culture), medium-term (slow growth or minimal growth), and long-term (cryopreservation) (Cha-um 2007).

Using the slow-growth and cryopreservation methods helps in storing germplasm, but this is only the visible part of the iceberg. Using these methods not only aids storage, but also serves several other equally important functions, such as germplasm collecting, future multiplication, and exchange (Withers 1991).

Each germplasm storage method has its own advantages and disadvantages, the choice of which depends on the goals pursued, the availability of the necessary equipment and space, the time and labor required for transplantation, viability tests and, of course, where without it, the money to cover the costs. But despite this, the slow-growth technique has proven itself well and has begun to be widely used as a source of disease-free plants in international material exchange, since it has high recovery growth and a high level of genetic stability compared to cryopreservation (Cha-um 2007). Using this method allows the storage of cloned plant material from several months to years, depending on the type of plant, in sterile conditions with minimal costs (Chauhan et al. 2019).

Of course, if we want to preserve plants for many years, it would be advisable to use the cryopreservation method, but this will require specialized laboratory equipment: liquid nitrogen, dewar storage tanks, etc. In addition, if necessary, the preserved plant material cannot be used immediately since thawing and regrowth takes a rather long period, and the survival rate may be low. Based on the above, we can assume that *in vitro* culture under slow-growth conditions is the most effective alternative method for plant germplasm conservation. It is cost-effective, requires less time for recovery and multiplication and ensures the immediate availability of the plant material under conservation (Chauhan et al. 2019). But how is the slow-growth effect achieved? The effect of slowing down the metabolic processes of the cultured shoots is achieved both: by modifying the chemical composition of the nutritive medium and by manipulating the physical conditions of the environment (temperature, light, etc.). The modification of the nutrient medium includes changes in the concentration of micro and macro elements, the modification of the carbon source content, and the use of different combinations and concentrations of plant growth regulators (Ruta et al. 2020).

## 2.5.2. Temperature conditions and their influence on growth processes

Humanity has learned to use the temperature storage factor for a long time. Even in ancient times, people stored food in special earthen rooms with a lower temperature, but with the advent of technical progress, all this led to the use of refrigerators. Therefore, decreasing the temperature, in conjunction with decreasing the light availability or even storing in the dark, is one of the most common techniques (Engelmann 2011).

The temperature factor limits and affects all physiological processes occurring in the plant; this effect is especially strong on respiration and photosynthesis. The most commonly used temperature range for plant tissue culture is between 20 °C and 27 °C, depending on the plant material (Kumar & Reddy 2011).

Also, depending on the temperature, *in vitro* plants undergo changes in metabolic rate, membrane composition and function, protein content. Sometimes these changes can be irreversible and destructive, if the effect of low temperatures is long-term, as in slow-grow conditions. This is especially true for tropical plants, which are very sensitive to low temperatures, so the recommended range for them is 15-22 °C, depending on the sensitivity of the crop (Ruta et al. 2020). Empirically, it was found that for medium-term conservation, the most suitable temperature for storing plant material of temperate species ranges from 2 °C to 12 °C (Lambardi & Ozudogru 2013), but the most commonly used temperature is in the range of 4-5 °C (Lambardi & Ozudogru 2013).

## 2.5.3. Culture medium and its variations

Active work on the research of root cultures or cell and callus cultures led to the creation of various culture mediums, the active use of which continues to this day with some modifications. The culture medium named MS medium, the full name of which is Murashige and Skoog 1962, is the most commonly used. It was initially adapted for tobacco callus culture, but due to its versatility, MS medium has been adapted for organogenesis and micropropagation of a wide range of plant species (Aitken-Christie, et al. 1995).

Depending on the objectives of the study and the selected plant, the modification of the nutrient medium is possible in different directions, for example: changes in the concentration of mineral salts (micro and macroelements), in carbon source content, , and the use of different combinations and concentrations of plant growth regulators and growth retardants (Ruta et al. 2020).

At the same time, changes in the components of the nutrient medium can occur not only in the direction of decreasing the concentrations of certain components but also in the direction of mutilation, leading to intoxication or various types of stress. This feature can also be used in the slow-growth (minimal growth method) (Ruta et al. 2020).

Sucrose is present in almost every nutrient medium; since its initial role as the main carbon resource (Kumar & Reddy 2011), it acts as a building material for plant growth and development. But it has been found that high sugar concentrations of 6-9% lead to the so-called osmotic stress similar to sugar alcohols (Bonnier & Tuyl 1997). There is a decrease in water potential, or in other words, the level of water availability in the nutrient medium for plants decreases. This phenomenon is based on the basis of plant physiology, namely the laws of osmosis (Cha-um 2007).

To achieve the desired effect, reducing and slowing down the growth activity, a half or quarter composition of MS medium can also be used. In this case, there is a decrease in nutrient absorption, translocation, and utilization, as a result, growth reduction (Bonnier & Tuyl 1997).

Strength reduction of the culture medium is the first aspect to result in fruitful minimal growth preservation: a half and one-fourth strengths of media are generally modified in many species, also for banana (*Musa spp.*) (Cha-Um et al. 2007), a monocotyledonous plant like garlic (Sendl 1995).

A good example of increasing sucrose concentration and decreasing nutrient saturation is the study by Bonnier and van Tuyl (1997), which successfully stored the *in-vitro* bulblet of *Lilium spp.* for a period of 28 months at 25 °C on ¼-strength MS medium supplemented with 9% (w/v) sucrose.

### 2.5.4. Osmotically active substances and their use

Equally interesting is the use of active chemicals that contribute to the limitation of the available water, thus providing the desired effect of reducing growth. The mechanism of action of such osmotically active substances is quite simple, osmotic shrinkage occurs through the semi-permeable membranes (Ruta et al. 2020). It is worth noting that the higher the concentration of such substances, the more quickly the plant cell loses its turgor and cannot be restored, as a result, the death of the sample. Therefore, the goal of slow-growth is to find the optimum at which the plant cell can still consume water and mantian

its vitality, being in a semi-dehydrated state, which will slow down all metabolic processes and the desired effect will be achieved (Ruta et al. 2020).

Osmotic agents such as mannitol, sorbitol, or sucrose, in combination with low temperature has also been effective in limiting the growth of cultures in potato (6°C and 4% mannitol), garlic (4°C and 10% sucrose), and sweet potato (5% sorbitol) (Pandey et al. 2015).

Considering the issue of sucrose in the previous section, since it plays a double role, depending on the concentration, first of all, it provides the plant with carbon, but when the concentration increases, it exhibits osmotic properties and can thus be used for slow-growth (Ruta et al. 2020).

Sorbitol is a sugar alcohol and is naturally found in many fruits. But it can be also produced by the bacterium *Zymomonas mobilis* from glucose and fructose (Silveira & Jonas 2002). This compound is actively used in the food industry as a texturizer, softener, humectant, and sweetener. (Silveira & Jonas 2002). But recently it has found its application in *in vitro* culture as a provocateur of osmotic stress, which makes it possible to use it in research related to plant drought resistance (Cha-um 2007).

The use of sorbitol in *in vitro* culture of *Musa* species has shown that this substance is not an energy resource and can be used as a neutral osmotic inducer (Rukundo 2009). Different stress levels have already been observed by Rukundo (2009) at sorbitol concentrations from 0.1 to 0.5 M, but research has shown that 0.2 M is the most optimal concentration for obtaining different growth rates (Rukundo 2009).

Mannitol, like sorbitol, is a polyol or sugar alcohol. It also acts as a non-processed sweetener, exhibits antioxidant properties, and also acts as an osmoregulating substance.

Mannitol is also use for the technology of in vitro slow growth on monocotyledonous plants, where it has proven itself to be quite efficient. This chemical was used in the experiment with: "*In vitro* conservation of enset (*Ensete ventricosum*) under slow-growth conditions" and it was found that it can be used as a growth inhibitor at a concentration of 0.1 or 2 %, which made it possible to maintain *in vitro* culture for 6 months at a temperature of 15 °C, and then successfully revived and transferred to *ex vitro* conditions (Krens et al. 2001).

## 2.5.5. Plant growth regulators (phytohormones or their synthetic analogs)

But we should not forget about the existence of other chemicals that could have a positive effect on the application the slow-growth conservation method. These are the so-called plant growth regulators (PGRs) which are actively used both in field and laboratory conditions (Cha-um 2007). For example, in the field, the use of phytohormones on cereals avoids the active growth of the green mass, which redirects the flow of nutrients to the ear, while preventing the plants from sticking out (Mc Millan et al. 2019). As for the method of production, plant growth regulators are of natural and synthetic origin.

Some argue that plant growth regulators are usually the key to control plant growth and development under *in vitro* conditions (Aitken-Christie et al. 1995).

Phytohormones can have both a stimulating effect, activating growth processes, and an inhibitory effect, slowing them down (Niazian & Shariatpanahi 2020).

Introducing growth retardants in the culture medium changes the hormonal balance, which in turn changes vital processes in an *in-vitro* plant (Espindula et al. 2009).But the desired effect is not always achieved; it all depends on the type of hormone and the type of plant on which it is applied. For example, the use of growth retardants like chlormequat chloride (CCC) has proved useful in limited cases (Cha-um 2007).

Chlormequat chloride (CCC) inhibits gibberellin metabolism by blocking cyclases copalyl-diphosphate synthase and ent-kaurene synthase and has a proven effect on stem growth, stem elongation, flowering, and somatic embryogenesis (Niazian & Shariatpanahi 2020).

Other growth retardants or inhibitors can also be added to the nutrient medium. It can be abscisic acid (ABA), a compound belonging to the anti-giberelin group, ancymidol, acetylsalicylic acid as an alternative to mannitol (Cha-um 2007).

ABA hormone plays an important role in the processes of germination and maturation of seeds, in adaptation to environmental conditions, since it contributes to the mechanism of stomatal closure, and also affects gene expression (Xiong & Zhu 2003).

Researchers have found that CCC, like ABA, when applied to garlic *in vitro* culture, can provoke the formation of bulbs, accelerating the entry into a dormant state, which is an important factor in the application of the slow growth technique. The study showed that at all CCC concentrations: 0.5, 1, 10, and 100 mg/l, bulbs were formed, but most of

all, at a concentration of 100 mg/l against the background of a consequence of leaf and shoot growth inhibition (Hahn et al. 2003).

Whereas when using ABA at concentrations of 0.01, 0.1, 0.5, and 1 mg/l, the concentration of 0.1 mg/l was found to be most effective for bulb formation ("Abscisic Acid Signal Transduction" 1998). Therefore, the most effective way for medium-term preservation has been to modify both physical and chemical factors to slow the rate of *in-vitro* growth and development (Ruta et al. 2020).

## 2.6. Possible deviations in plant growth and development

First of all, under *in vitro* conditions a problem of late contamination may arise in the plant material, or rather, the manifestation of latent, endogenous infections with a change in chemical and physical factors such as temperature, pH of the media, illumination, different concentrations of chemical pathogen inhibitors. In this case, the pathogen finds its favorable ranges for its development The causative agents of contamination can be both bacteria and fungi (Agrawal et al. 2017).

The next problem may be the manifestation of viral diseases since it is sometimes not possible to test each plant separately. For example, garlic can be susceptible to viruses such as *Carlavirus, Allexivirus* and *Potyvirus*, exposure to which leads to a 78% bulb weight reduction (Conci et al. 2005). Symptoms can be different, from a change in the color characteristics of plants to a deviation of all sorts of physical parameters, a change in the shape of leaves can be observed, internodes decrease (the plant becomes dwarf), the straightness of the plant can be disturbed, uncharacteristic twists appear, chlorotic streaking, mosaics, etc. (Pramesh & Baranwal 2015).

Analysis of samples for the presence of viruses is carried out using polymerase chain reactions (PCR), including reverse transcription-polymerase chain reactions (RT-PCR), and enzyme linked immunosorbent assay (ELISA) (Pramesh & Baranwal 2015).

Also, in the course of working with a culture *in vitro*, a problem with hyperhydricity (Figure 8) may arise, which leads to overfilling of the plant with water, a change in the density of tissues and their structure, deformation, discoloration, etc.

This phenomenon is not uncommon with garlic, such plants can be easily identified by color, the stem changes its color from light green to dark green and acquires certain transparency. The size of the stem changes, it significantly thickens and deforms, takes on an irregular shape, or begins to grow too actively.

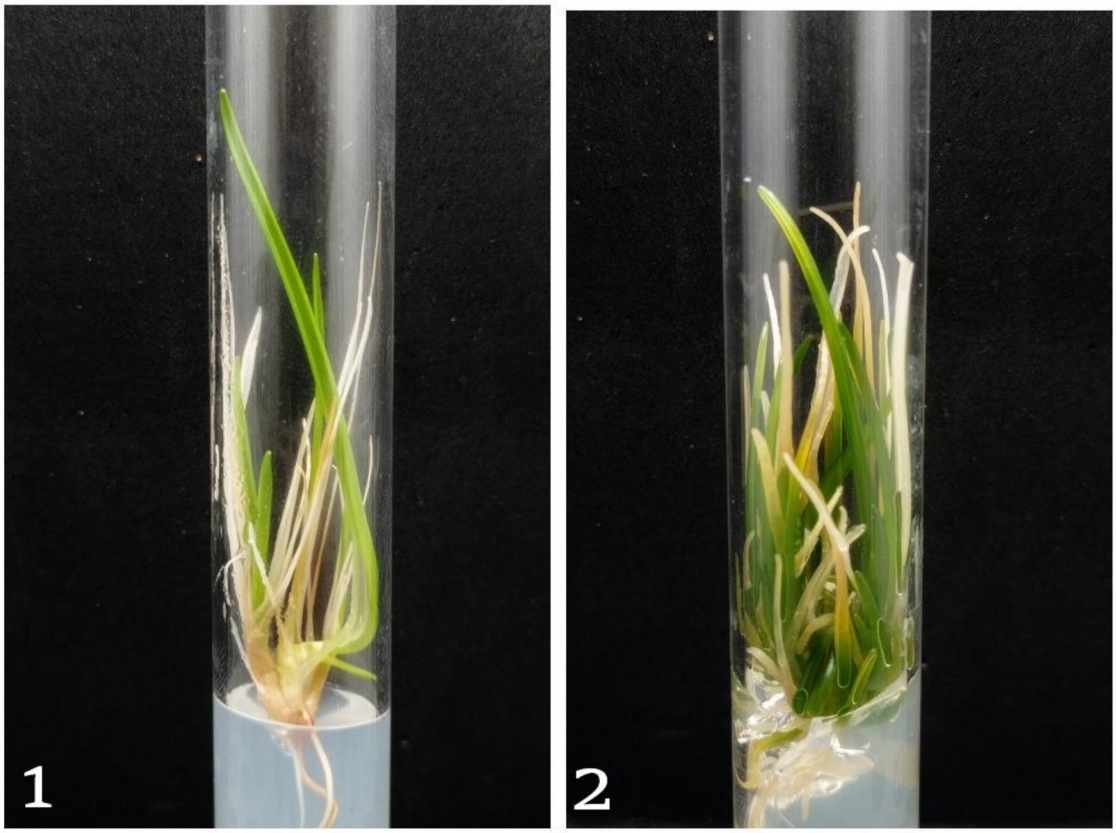


**Figure 8.** *Allium sativum* (garlic) plants: 1 – healthy plant, 2 – hyperhydrated plant. *(Source: Author)*

The roots of this problem, may relate to culture ventilation, or rather the created microclimate in the middle of the test tube. Poor ventilation can lead to the accumulation of harmful ethylene and disturb the humidity of the microclimate in vitro, which can lead to such a phenomenon as hyperhydricity (E. Benson 2000).But according to Grigoriadou & Leventakis (2000), the problem of hyperhydricity can also be triggered by tissue age, they compared *in vitro* shoot proliferation in adult and seedling material of *Myrtus communis* and found that the old plant material showed symptoms of hyperhydricity, while seedling-derived explants did not show signs of this morphological abnormality.The development and regrowth of cells *in vitro* is an asexual process, which implies only mitotic cell division. Based on this, the possibility of uncontrolled and spontaneous variation is quite large (Leva et al. 2012).

*In vitro* conditions can be mutagenic and regenerated plants derived from such *in vitro* cultures: organ cultures, protoplasts, calli, and somatic embryos sometimes can show genotypic and phenotype variation. These variations are called "somaclonal variation". Such changes usually occur spontaneously and can be permanent (genetic

changes in cells) or temporary (reversible). Temporal changes are non-hereditary and occur as a physiological response to some factor (Leva et al. 2012).

This problem also occurs when using the slow-growth technique. *In vitro* slow-growth storage of *Ananas comosus* germplasm including 66 accessions performed wide variability in the response of the plants to storage parameters, showing somaclonal variation (Chauhan et al. 2019).

Therefore, the most important thing is not to lose genetic stability during the entire process of slow growth. According to Pandey et al. (2015), there is a mandatory requirement to verify the genetic stability of *in vitro* slow grown and cryopreserved-regrown plant germplasm.

# 3. Aims of the Thesis

The main aim of the research: optimization of a protocol for the medium-term *in vitro* conservation of *Allium sativum* using two basal culture media (either full-strength MS or half-strength MS medium) supplemented by osmotic agents (sucrose, mannitol and sorbitol), plant growth inhibitors (chlorcholinchlorid (CCC) and abscisic acid (ABA)) as culture medium supplements, and cultivation temperatures of either 5°C±1°C or 18°C±1°C.

The specific aims of the research:

1) determine which basal culture medium and media supplement is optimal for developing an efficient medium-term conservation protocol for *Allium sativum*.
2) determine the optimal cultivation temperature for medium-term conservation of *Allium sativum*.

# 4. Methods

## 4.1. Plant material

The plant material of *Allium sativum* (garlic) was obtained from the Crop Research Institute (CRI), where the world collection of garlic species maintained at the Olomouc Genebank are being transferred to *in vitro* conditions for subsequent multiplication and cryopreservation. This activity is conducted within the framework of the National Program for the Conservation and Use of Plant Genetic Resources and Agrobiodiversity with a separate chapter Cryopreservation of gene pools of vegetatively propagated crops. Currently, the CRI Cryobank of vegetatively propagated plants holds one of the largest collections of garlic varieties according to the number of entries in the European Allium Database.

This research was carried out at the laboratory of Research Group 11: Plant Physiology and Cryolbiology of the Crop Research Institute in Prague.

## 4.2. *In vitro* establishment of *Allium sativum*

The selected genotype of *Allium sativum* was established *in vitro* according to the sterilization protocol of the Crop Research Institute for *in vitro* establishment of excised plant meristems from garlic cloves maintained in field conditions.

The sterilization protocol for *in vitro* establishment is based on the level of sterility, various concentrations of NaClo (sodium hypochlorite) and PPM (plant preservation mixture), on the duration of action of these agents, as well as on the survival rate of plants/meristems. PPM and NaClo – sterilization agents used in the Laboratory of the Crop Research Institute in Prague, which established itself as a phyto-safe and highly effective bactericed, effective against microbes and fungi. The proposed method for sterilization for *in vitro* establishment of the selected *Allium sativum* accession involves three steps: 1) The cloves of the selected genotype were pealed and placed in a pre-treatment overnight consisting of 0.5% NaClo these were placed on a shaker at low speed, followed by the excision of shoots which were then placed in 5% NaClo for a 5 minute treatments time duration 2) the shoots were then submerged in 3% PPM mixture for a 5 minute treatment time, 3) thereafter the shoots were washed 3 times in sterile distilled water at room temperature for 1 minute. All stages were carried out in a laminar flow

cabinet. To establish the *in vitro* cultures, a minimum of 30 isolated garlic shoots were used (Figure 9).

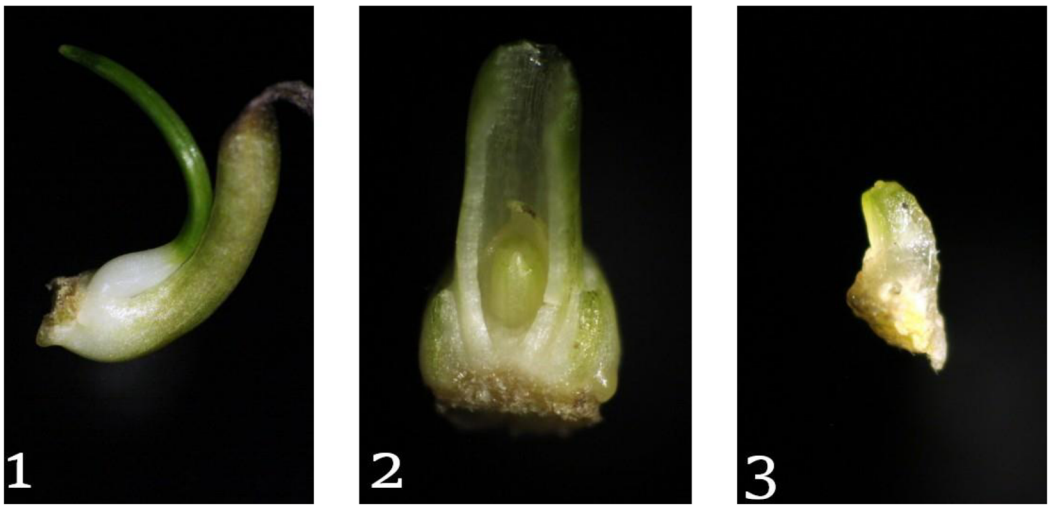


**Figure 9.** *In vitro* establishment of excised plant meristems from garlic cloves maintained in field conditions. 1 - shoot contained in the middle of a clove, 2 -apical meristem in the middle of the shoot, 3 - isolated apical meristem with basal part.

*(Source: Author)*

After this procedure, excised shoots from cloves were placed in tubes, containing 5 ml of MS (Murashige and Skoog, 1962) medium cantaining 30 g/l sucrose, 100 mg/l Myo-inositol, 8 g/l agar, and pH of 5.7. The culture medium was sterilized by autoclave at 121 °C and a pressure of 100 kPa for 20 min before being used. All plants were kept in cultivation conditions of approximately 85% humidity, a temperature of 18±1°C, the cultures were maintained under a 16/8 h light/dark regime, at a photosynthetic photon flux density of approximately 35 µmol $m^{-2}$ $s^{-1}$ provided by cool-white fluorescent tubes. The *in vitro* culture were visually evaluated every week after establishment for a duration of four weeks.

## 4.3. *In vitro* propagation of *Allium sativum*

Once the aseptic plant cultures have been established *in vitro,* the shoot segments of *in vitro* plants were regularly sub-cultured every 3-4 weeks in Erlenmeyer flasks (100 ml), containing 25 ml of sterile MS (Murashige and Skoog, 1962) media, in other to obtain sufficient plant material to carry out the slow-growth experiment. This procedure

was carried out by the artificial division of the lower part of the stem into 2-4 parts, depending on the degree of development (Figure 10).


**Figure 10.** Artificial division of garlic under *in vitro* conditions. 1 – shoot (bulb) before cutting, 2 – shoot after artificial division, 3 - regrown shoots.

*(Source: Author)*

## 4.4. Slow-growth treatments for garlic medium-term conservation

To optimize the slow-growth protocol for medium-term *in vitro* conservation of *Allium sativum,* various osmotic agents in different concentrations were used such as sorbitol in 20 g/l, 40 g/l, and 60 g/l, mannitol – 20 g/l, 40 g/l, and 60 g/l, and sucrose – 30 g/l, 50 g/l, 100 g/l, and 150 g/l. Plant growth inhibitors such as chlormequat chloride (CCC) at concentration 200 mg/l, 400 mg/l, and 600 mg/l and abscisic acid (ABA) at 1 mg/l, 3 mg/l, and 5 mg/l were also used. Full-strength and ½ concentrated MS (Murashige and Skoog, 1962) were used as basal culture media for treatments. Samples placed on full-strength and ½ concentrated MS medium without the addition of osmotics

and plant growth inhibitors were used as control. To carryout the experiment the growth regulators and osmotic agents were added to the nutrient medium during its preparation. The pH was adjusted to 5.7. Thereafter the media was dispensed into test tubes (15 × 160 mm). All treatments were sterilized by autoclave at 121 °C and a pressure of 100 kPa for 20 min before being used.

To set up the experiment for medium-term conservation of garlic (*Allium sativum*), individual *in vitro* shoots of 0.5 cm in length were used (Figure 11). The shoots were matched according to the principles of equal development and standardized to the above mentioned size by top cutting with updating the bottom cut and removal of the root system. The age of the original garlic plant material after last multiplication was 14 days. The experiment was carried out in two repetitions.

Thereafter the standardized shoots were placed and the treatments containing

**Figure 11.** Standardized in vitro shoot of 0.5 cm for experimental setup.

*(Source: Author)*

different concentrations of growth regulators and osmotics, all samples were distributed and placed in equal numbers in different temperature conditions. A total of 40 plants were

planted in each tested treatment (20 on MS and 20 on 1/2 MS for each tested growth inhibitor and osmotic agent), 20 of which were placed in a cultivation room at 5±1°C and 20 at 18±1°C (10 from MS and 10 from 1/2 MS treatments).

The experiment was carried out in two repetitions (40 plants per tested treatment on MS and 1/2 MS basal media divided in two cultivation temperatures).

Plant height (cm), shoots, bulbs, number and length of roots were evaluated every two weeks for six consecutive months, after which the data obtained were used to determine what basal media, treatment and cultivation condition is most appropriate for the medium-term conservation of *Allium sativum*.

Plant height measurements were taken with a ruler and recorded in centimeters. Shoots and bulbs were counted up to 15 pieces, since it was not possible to accurately count a larger number, the length of the root system was measured up to a maximum of 5 cm.

Any observed morphological abnormalities during the experiment were also evaluated. Abnormalities such as coolling of plants that, during growth, lost contact with the nutrient medium or were with obvious signs of hypohydration (HH) were recorded. In further measurements, the symbol "0" was used to designate such plants.

Throughout the entire period of measurements, the quality of the studied plant material was monitored. Changes such as yellowing of the top leaf - "y top" and its complete drying - "d top" was recorded.

The general color characteristics of the plants were also observed and recorded to further assess the quality of the conserved plant material. When a completely whitened sample was encountered, marked as "w", such a plant was considered dead and was designated as "0" in further measurements, yellow plant was designated as "y", and normal green plant as "g". Combinations of different colors were also seen and were designated as "g/y", "y/g", and "y/w".

In the case of the formation of a microbulbs and the death of the vegetative part of the plant, the plants went dormant, in which case the sample was recorded with the letter "d", and in the case of resumption of vegetation or regrowth, it was marked with the letter "r". The active formation of bulbs in conjunction with the death of the vegetative part and subsequent dormancy is an undesirable factor. Such samples were not counted as successful and were not regarded as a deviation from the desired goal of the slow-growth technique.

At the end of the six-month conservation period, a comparative analysis of the growth parameters and quality of plant material was carried out.

## 4.5. Regrowth of garlic shoots after slow-growth storage

After the 6 months conservation period of the experiment on the *in vitro* slow-growth storage of garlic *(Allium sativum)*, the best treatments for growth and quality characteristics were identified, the samples of which were tested for regrowth potential. The selection of the best treatments for post-storage regrowth was based on the level of plant survival and the basis of quality characteristics such as no morphological abnormalities, best plant color and least number of shoots and bulbs. The overall best controls and treatments with mannitol, sorbitol, sucrose, ABA, and CCC were selected from 18±1°C and 5±1°C cultivation temperatures.

Ten plants were from each selected treatment were then place on regrowth media consisting of sterile MS (Murashige and Skoog, 1962) media dispensed into test tubes (15 × 120 mm). The culture medium was sterilized by autoclave at 121 °C and a pressure of 100 kPa for 20 min before being used. Then all samples were placed in a cultivation room with a temperature of 22±1°C The cultures were maintained in an incubator at 22±1°C under a 16/8 h light/dark regime for 28 days. .

For the regrowth experiment, single shoots were standardized to a length of 0.5 cm and the same thickness by artificial division of the lower part of the stem into 2 parts.

Subsequently, growth and development parameters such as survival rate, plant height, number of shoots, presence of roots and color of plants were measured every week during the 28 days regrowth period. Based on these data, *ex vitro* transfer to field conditions and DNA analysis will be carried out in the future.

## 4.6. Statistical analysis

For statistical processing of this research, the software "Statistica" was used.

The one-way and two-way analysis of variance (ANOVA) were used to determine whether there are any statistically significant differences between the means of the parameters obtained as a result of the influence of various chemical agents, temperature factors, and factors of the nutrient medium as independent (unrelated) groups and two independent groups, in combination.Then, if a significant difference was found, Post Hoc Tests with ANOVA were used to find the statistical difference within the groups.

# 5. Results

Effect of different concentrations of growth regulators on slow-growth storage of garlic (*Allium sativum*) under different temperature and nutrient media conditions.

## 5.1. Control

### 5.1.1. Effect of cultivation temperature

During the five-month slow-growth conservation period, the survival rate in both temperature ranges, 18°C±1°C and 5°C±1°C, was at a high level and amounted to 99%. However, control samples placed in the tested temperature regimes differed significantly in their growth and development both at the half and at the full composition of the MS (Murashige and Skoog 1962) as a basal culture medium.

With the full composition of the nutrient medium MS, the control under temperature conditions of 18°C±1°C reached a maximum average height of 4.65 cm, while under conditions of 5°C±1°C, this indicator was 1.59 cm (Figure 12, Figure 13).

The qualitative characteristics also differed significantly; there was a more active growth and development of shoots at a higher temperature. At the end of the measurements, there were 6 shoots per plant, while at a low storage temperature, this indicator averaged 1.4 shoots. In the first case average standard deviation was 2,67, and in the second case, 0,51. It is worth noting that at a higher temperature, the development of the root system was more active, but it was not expressed in all samples, while at a low temperature, the processes of root formation almost did not occur. Another important fact is that at 18°C±1°C, there was a fluctuation in the height of the samples due to the death of the upper leaf, which occurred in the fourth month of the experiment, while at a temperature of 5°C±1°C, this process did not occur (Figure 13).

Regarding the quality of the plants, some irreversible changes were seen more often at 18°C±1°C. The most common process was hyperhydration. The percentage of which in the control samples ranged from 5 to 10%.

### 5.1.2. Effect of full and half composition of the culture medium

The composition of the culture medium had a significant effect on the development of garlic shoots (Figure 12), which shows a comparison of the controls of the full and half

composition media under the same temperature conditions. Different concentrations of micro and macro elements, sucrose, and vitamins led to interesting results. After 6 months of slow-growth storage, it was found that at a temperature of 18°C±1°C, the nutrient medium of the half composition provoked a more active growth of garlic shoots compared to the full MS. The maximum height of plants in the first case was about 6.09 cm, while in the second, the maximum average height was only 4.65 cm (Figure 12). At the same time, the number of formed shoots did not differ much. According to this indicator, the half composition of the MS medium induced a higher growth rate. The latest measurements showed that there were 6.75 new plants per sample on average, while with full MS medium 6.15 plants. According to external quality indicators, in both cases, the plants looked healthy without any deviations. The root system was formed only by single specimens.

As for the temperature of 5°C±1°C, the growth and development was reversed in the beginning after the first month; higher growth rates were observed on samples cultivated on a complete nutrient medium, the difference was 0.2 cm, but with the last measurements, everything changed on the contrary. With regard to the formation of shoots, no difference was found (standard deviation was 0.49 on 1/2 MS and 0.53 on MS media). Although the plants looked healthy in appearance, the root system did not develop.

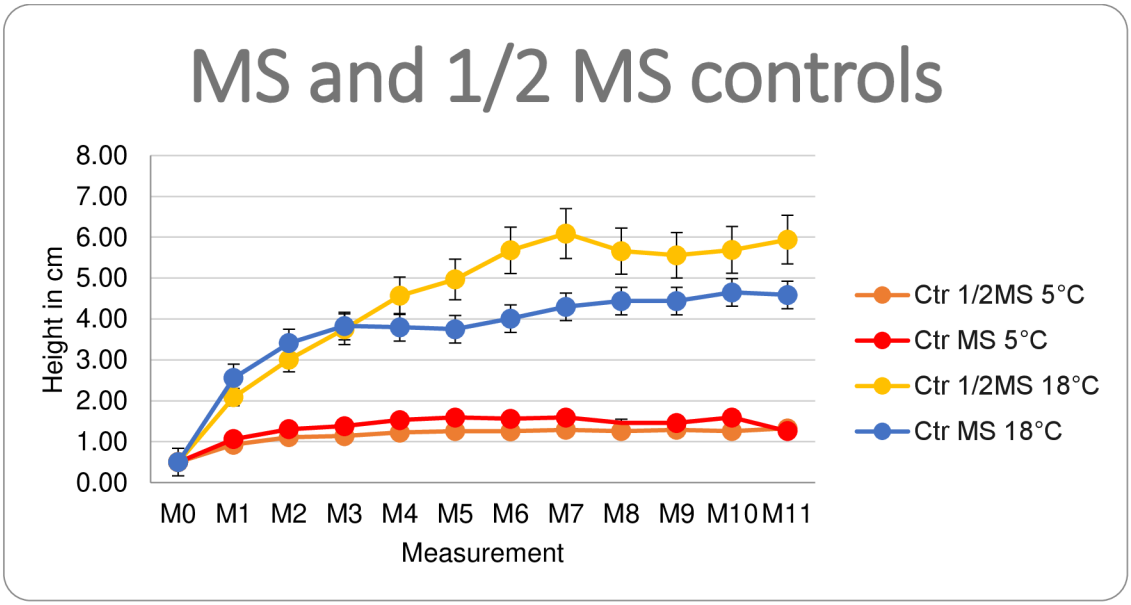


**Figure 12.** Effect of culture medium and culture temperature on plant height during the 5-month conservation period.

*(Source: Author)*

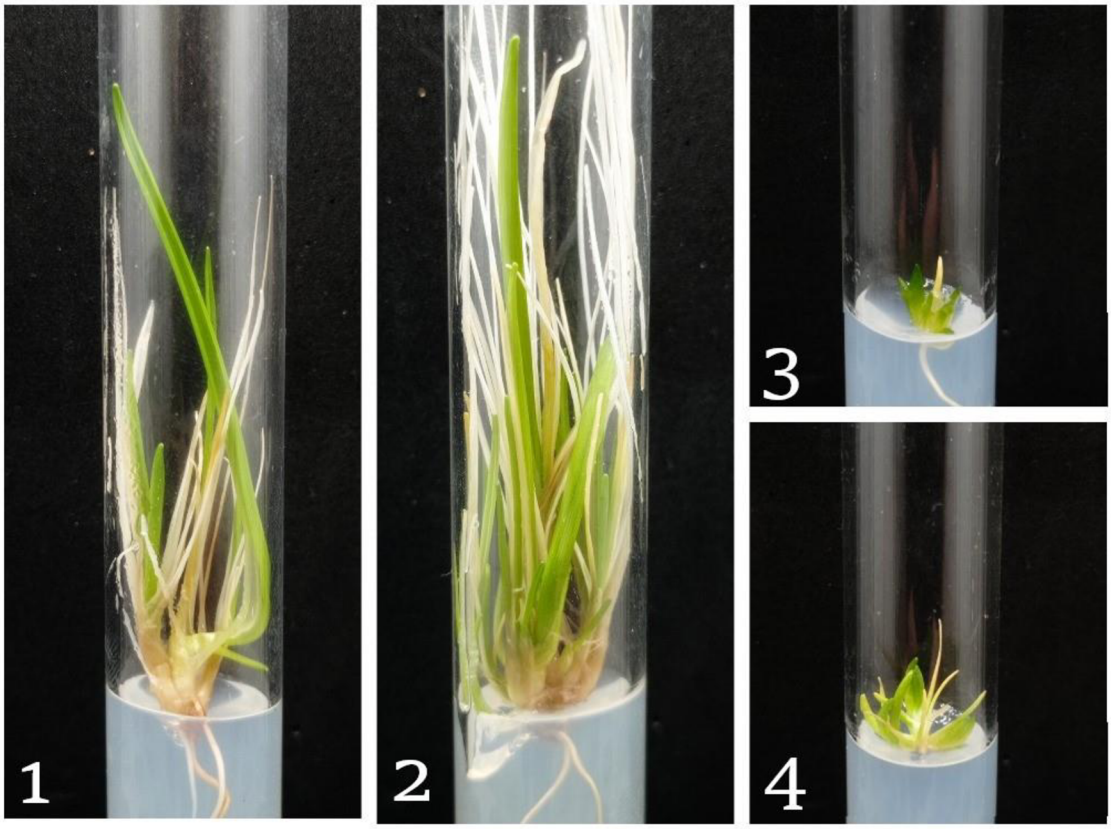


**Figure 13.** Control plants on different nutrient media at different temperature conditions after 5 months of storage: 1 - garlic plant on 1/2 MS medium at 18°C±1°C, 2 - garlic plant on MS medium at 18°C±1°C, 3 - garlic plant on 1/2 MS medium at 5°C±1°C, 4 – garlic plant on MS medium at 5°C±1°C.

*(Source: Author)*

## 5.2. Mannitol

### 5.2.1. Effect of cultivation temperature

Culture media supplied with different concentrations of mannitol placed in two temperature regimes had a significant difference in the qualitative and quantitative characteristics of garlic stored for a 5-month slow-growth conservation period. The temperature factor significantly affected plant height (Figure 14); for example, at a concentration of mannitol 2% in 1/2 MS medium at 18°C±1°C, the average maximum plant height was 3.99 cm, while at 5°C±1°C 1.10 cm, at concentrations of 4% - 3.05 cm, and 1.13 cm, and at a concentration of 6% - 2.68 cm and 1.09 cm, respectively (Figure 15 & Figure 16). During the first three months on a full culture medium with 4% mannitol and a temperature of 5°C±1°C, the average gain was +0.83 cm, while at a temperature of 18°C±1°C, the gain was much stronger and amounted to +2.67 cm. But in subsequent

observations, it was noticed that there was a progressive growth with small deviations in quality characteristics at a lower temperature, while at a high temperature, the quality indicators deteriorated; many samples, about 90%, were hyperhydrated after 5-6 months.

The higher temperature also had a strong influence on the rate of shoot formation; within the first month of storage on 1/2 MS medium with 2% mannitol at a temperature of 18°C±1°C, stored samples induced 1.5 shoots per sample, while at temperature 5°C±1°C were formed 0.3 shoots per sample (Figure 14). At the end of the sixth month storage period, the formation of the shoots was at the level of 7.8 and 2.10, respectively (standard deviation of 3.05 and 0.84, respectively).

The formation of the root system also occurred more actively at high temperatures.

In combination with mannitol as a media supplement, the high temperature also induces morphological abnormalities, mainly in the form of hyperhydration of plants. At a high temperature at the end of the measurements, an active manifestation of this phenomenon was observed, while in control and at a lower temperature, this was not very noticeable.

### 5.2.2. Effect of full and half composition of the culture medium supplemented with mannitol

Full and half MS nutrient medium supplemented with different concentrations of mannitol at a temperature of 18°C±1°C affected the growth and development of garlic at the initial stages of the experiment differently (Figure 15). Still, in subsequent measurements, the trend of this factor could not be traced correctly due to the high amount of hypohydrated samples (Figure 14). For example, at a concentration of mannitol at 6% in 1/2 MS, the increase at the end of the first month was +1.70 cm, while MS showed a result of +1.44 cm, at a concentration of 4%, 1/2 MS showed an increase of + 1.71 and MS +1.99 cm, and at a concentration of 2%, 1/2MS showed +1.70 cm, MS: +2.50 cm (Figure 15, Figure 16).

The concentration of the nutrient medium at 18°C±1°C also did not particularly affect the quality characteristics.

As for 5°C±1°C, the full and half culture medium also did not significantly affect the growth of garlic in the initial stages, but by the end of the conservation period, differences appeared. The final mean plant height at 2% mannitol was 1.03 cm on 1/2 MS and 1.44 cm on MS, at 4% of mannitol, 1.08 cm on 1/2 MS and 1.38 cm on MS, and at

6% 1.02 cm on 1/2 MS and 1.39 cm on MS. This trend shows that the full composition of the culture medium supplemented with mannitol has a stronger effect on garlic growth. However, the formation of shoots looked the other way around; at 1/2 MS media, they formed slightly more than a full MS (2.1 and 1.45 shoots per sample, respectively, standard deviation 0.6 and 0,84).

### 5.2.3. Effect of mannitol at various concentrations

During the study, it was found that with an increase in the concentration of mannitol in culture media at a temperature of 18°C±1°C, a decrease in the growth activity of the samples was observed. But at the same time, a detrimental effect of this component was also found, which provoked 90-100% hyperhydration of the samples at various concentrations. For example, on full strength MS medium at a concentration of mannitol 2% after the first month, the growth of plants was +2.51 cm, on the second +0.76 cm, and on the third +0.69 cm, at a concentration of 4%, the growth was +1.99 cm, + 0.31 cm and +0.37 cm, and at a concentration of 6%, the increase was +1.44 cm, +0.46 cm and +0.33 cm.

While at a temperature of 5°C±1°C, the effect of different concentrations of mannitol was not so strong. The difference in growth at different concentrations of mannitol in 1/2 MS medium was only 0.01-0.16 cm. Interestingly, growth increased at MS with the increasing concentration of mannitol. On full MS media, no difference in growth was found.

Regarding the effect of various concentrations of mannitol on the formation of shoots, no effect was found either at full and half media at 18°C±1°C or at 5°C±1°C.

If we compare different concentrations of mannitol on different nutrient media at different temperatures with controls, then the effect of mannitol is also noticeable; most garlic samples were lower than control values or almost at its level, as was the case with 4% mannitol in MS at 5°C±1°C (Figure 15, Figure 16). For example, at a temperature of 5°C ± 1°C on 1/2MS medium with the addition of 6%, the maximum average plant height was reached at the 4th month of measurements and amounted to 1.09 cm, while the control plants, on the same nutrient medium, reached the maximum height on the third month of the experiment and it was 1.29 cm. However, using mannitol at 18°C ± 1°C in nutrient media for growing garlic is not recommended due to low-quality characteristics resulting from high hiperhydration.

The best mannitol slow growth effect in terms of growth and quality was achieved at 5°C ± 1°C per 1/2 MS and 6% mannitol concentration (Figure 14).

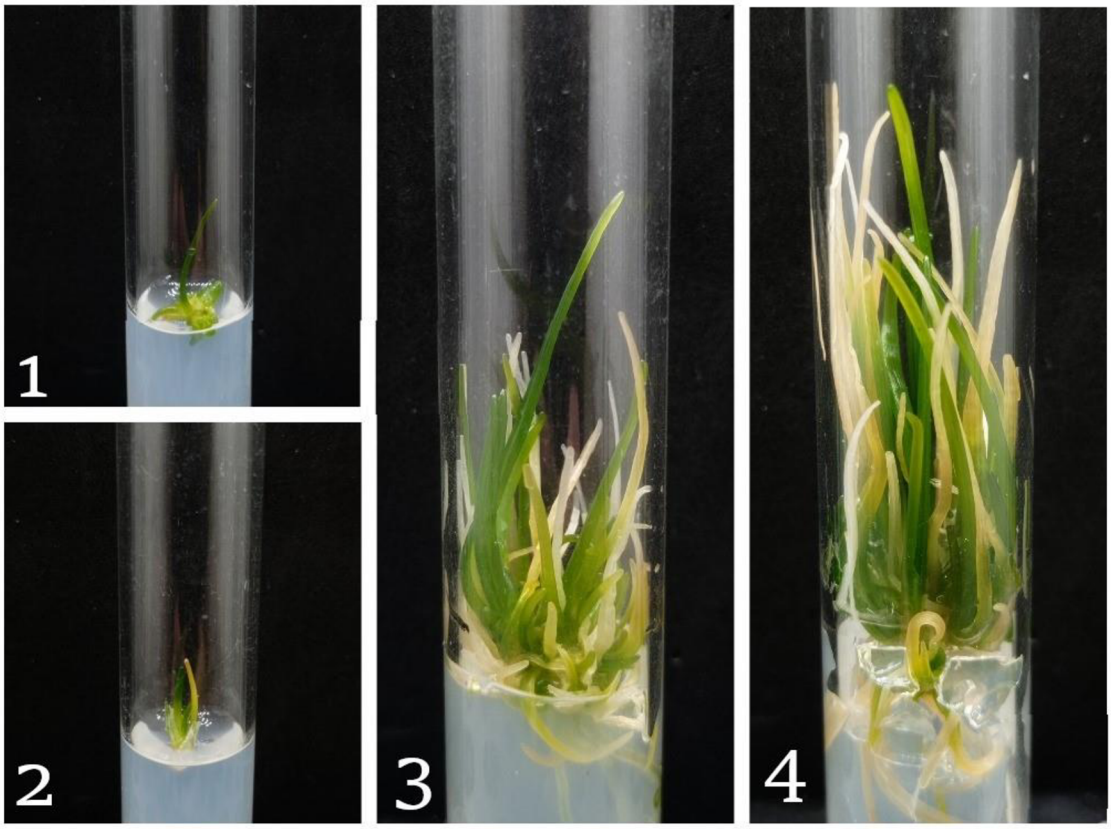


**Figure 14.** Effect of mannitol combined with full and half composed MS media at different temperature conditions on garlic after 5th months conservation period: 1 – effect of 6% mannitol in combination with 1/2 MS medium at 5°C±1°C, 2 – effect of 6% mannitol in combination with MS medium at 5°C±1°C, 3 – effect of 6% mannitol in combination with 1/2 MS medium at 18°C±1°C (hyperhydrated plant), 4 – effect of 6% mannitol in combination with MS medium at 18°C±1°C (hyperhydrated plant).

*(Source: Author)*

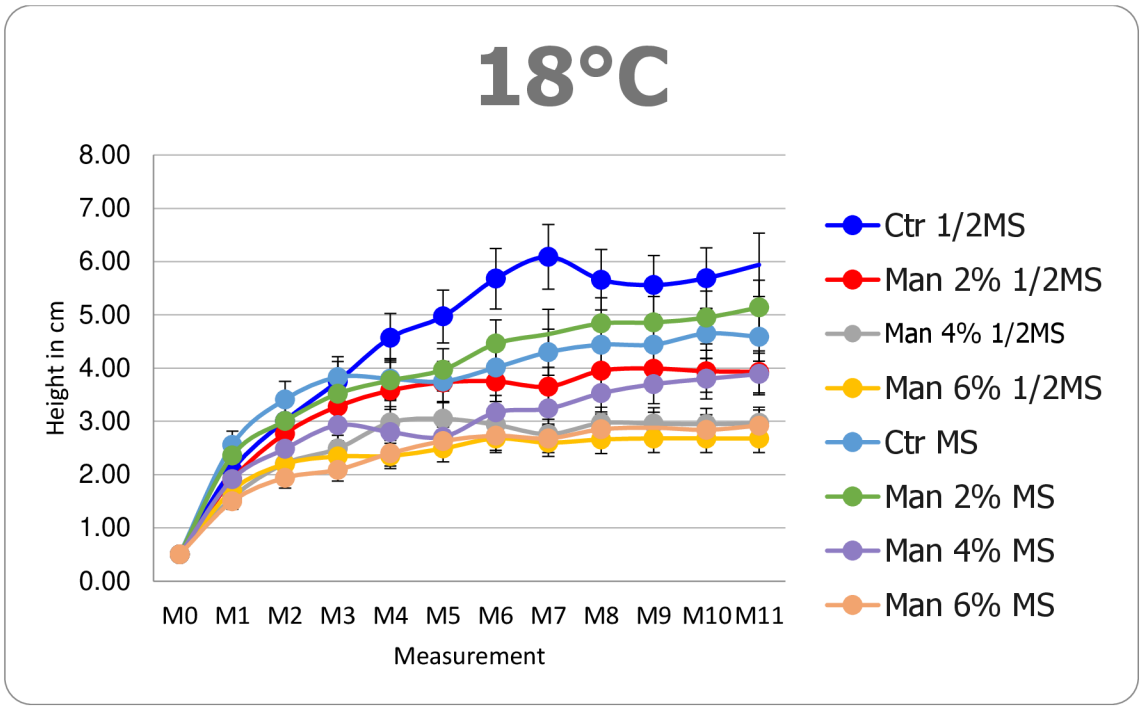


**Figure 15.** Effect of mannitol combined with full and half composed MS media at 18°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

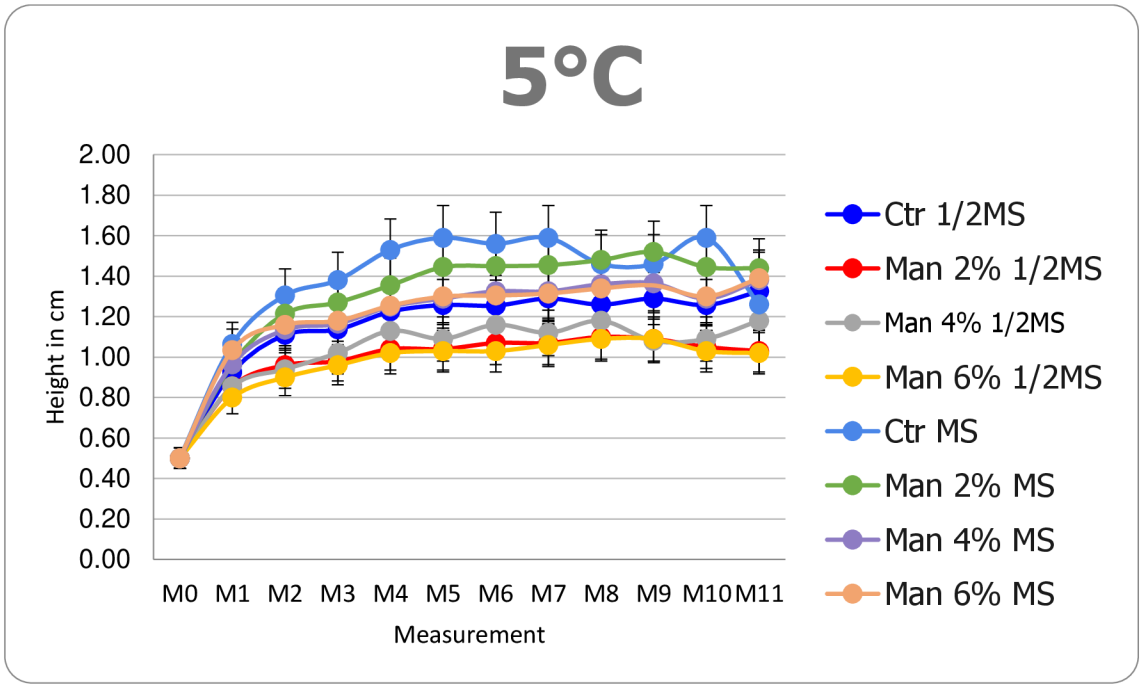


**Figure 16.** Effect of mannitol combined with full and half composed MS media at 5°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

## 5.3. Sorbitol

### 5.3.1. Effect of cultivation temperature

The temperature factor seriously influenced the height of garlic samples supplied with sorbitol on full and half MS medium (Figure 19). The final height difference of the samples on MS at 18°C±1°C and 5°C±1°C and concentrations of 2%, 4% and 6% were +2.40 cm, +2.98 cm and +2.14 cm in favor of MS (Figure 17, Figure 18). But as for the development of shoots, their number was 3-4 times higher at all concentrations of sorbitol at a higher temperature.

Compared to the control, all samples showed a similar trend in their growth.

As for the cultivation temperature of 18°C±1°C, it also provoked an increased level of hyperhydration, the indicator of this effect was higher than the control level, especially with half composition of the MS medium. In addition, this temperature range, as in the control, affected the death of the upper leaf at 3-4 months of the experiment. While at a temperature of 5°C±1°C, the processes of hyperhydration occurred singly and were also at the control level, the top leaf did not die.

### 5.3.2. Effect of full and half composition of the culture medium supplemented with sorbitol

Half and complete MS culture medium with various concentrations of sorbitol had a significant effect on the growth and development of garlic at a temperature of 18°C ±1°C. The difference between MS and 1/2 MS at 18°C±1°C with 2% sorbitol at the end of measurements was +1.12 cm, with 4% sorbitol +0,25 cm, and with 6% concentration +0.35 cm (Figure 17). But in the beginning, after the first month, the situation was as follows, the increase between MS and 1/2 MS with 2% sorbitol was +0.57 cm, at 4% -0.41 cm, and at 6% -0.36 cm (Figure 17). We can see that at the initial stages, the complete nutrient medium with sorbitol had a more inhibitory effect on plants. At the same time, the situation changed on the contrary at the end of the measurements. It follows from this that, as in the case of the control, MS has a more inhibitory effect. But if we compare the last plant height measurements with control, they will be different. In the control, plants on 1/2 MS medium were ahead of plant height per MS, while in the presence of sorbitol, nutrient media showed opposite results. This can be explained by the fact that sorbitol and more active plant nutrition and the lower concentration of nutrients on 1/2 MS, sorbitol penetrated faster into shoots, and osmotic stress occurred a little earlier than on

MS medium. But at a temperature of 5°C ± 1°C, no significant effect of the nutrient medium on plant height was observed (Figure 18).

Culture media also had an ambiguous effect on *Allium sativum* at 5°C±1°C. In the first month of measurements, the difference in growth between MS and 1/2 MS at 2% sorbitol was +0.05 cm, at 4% -0.34 cm, and at 6% +0.01 cm. At the end of the measurements, these indicators looked like this: at 2%, the gain between MS and 1/2 MS was +0.04 cm, at 4% -0.66 cm and at 6% -0.03 cm. From this, it can be concluded that the nutrient medium's effect against the background of sorbitol at a lower temperature did not manifest itself.

Considering the situation with the formation of shoots in different temperature conditions, at 18°C±1°C, no nutrient medium was influenced. At the end of observations, MS and 1/2 MS with 2% sorbitol had 7.30 and 5.50 shoots per sample (standard deviation 2.70 and 2.12), with 4% 7.30 and 7.10 (standard deviation 3.09 and 2.89), and with 6% 8.60 and 5.80 shoots per sample (standard deviation 3.63 and 2.36). The MS environment provoked a greater formation of shoots with an average difference of 1 shoot per sample.

In terms of root formation, more roots at 18°C±1°C were formed on MS medium compared to 1/2MS. In recent measurements at all concentrations of sorbitol, the average difference was 1-1.5 roots between samples. At 5°C±1°C, no difference was observed.

### 5.3.3. Effect of sorbitol at various concentrations

During the study, it was found that sorbitol had a significant effect on the growth and development of plants under different temperature conditions compared to the control; the only exception was the concentration of sorbitol 4% in 1/2 MS medium at 5°C±1°C, where plant height at the end of storage exceeded the control by 0.3 cm (Figure 19). Furthermore, after 5 months of storage, measurements showed that the mean plant height at 18°C±1°C at full MS averaged 3.67 cm, while the MS control showed a height of 4.59 cm at the same period (Figure 17).

But it's important to note that the effect of sorbitol at 18°C±1°C in both full and half nutrient medium had an undulating character on plant height with increasing concentration. With increasing concentration, the height of the plants increased and then fell again. For example, at a sorbitol concentration of 4%, the maximum plant height was achieved. At 1/2 MS, it was 3.70 cm, and at MS, 3.95 cm, while at a concentration of 2%,

plant height was 2.53 cm and 3.65 cm, and at 6%, 3.06 cm and 3.41 cm, respectively (Figure 17). At 5°C± 1°C, a similar effect was observed.

At different concentrations of sorbitol, the formation of shoots also occurred unevenly. At 18°C±1°C and MS nutrient medium, their number also increased with increasing concentration; at 2% sorbitol, on average, there were 7.30 shoots per sample, and at 6%, there were already 8.6 of them (standard deviation 2.70 and 3.63 respectively). But at 1/2 MC, as in the case of plant height, shoots developed in waves, reaching a maximum number of 7.10 shoots per sample at a sorbitol concentration of 4%. In all cases, sorbitol had an increased effect on the formation of shoots since the control values were 6.75 pieces per sample at 1/2 MS and 6.15 at MS. At 5°C±1°C, on the opposite, with increasing concentration, a decrease in the formation of shoots was observed, but the indicators still exceeded the control values by 1.5–2 times.

One thing worth noting is that sorbitol had a stimulating effect on root formation processes at a temperature of 18°C±1°C for both nutrient media; at the end of the measurements, there were from 0.85 to 2.5 roots per plant, while the control did not exceed 1.2 roots per plant. At low temperatures, this phenomenon was not observed.

As in the case of mannitol, sorbitol was one of the factors that provoked an increased level of hyperhydration of the samples at high temperature compared to the control, but this happened only at a concentration of 4% sorbitol and 6% sorbitol in 1/2 MS medium.

As can be seen from the above, the effect of slow growth was more pronounced at a lower temperature of 5°C±1°C. Based on both qualitative and quantitative indicators, the most effective concentration of sorbitol was 4% against the background of a complete nutrient medium, the average plant height at the end of the storage period was 1.27 cm, number of shoots 2.00 per plant, hyperhydration level below control level (Figure 19).

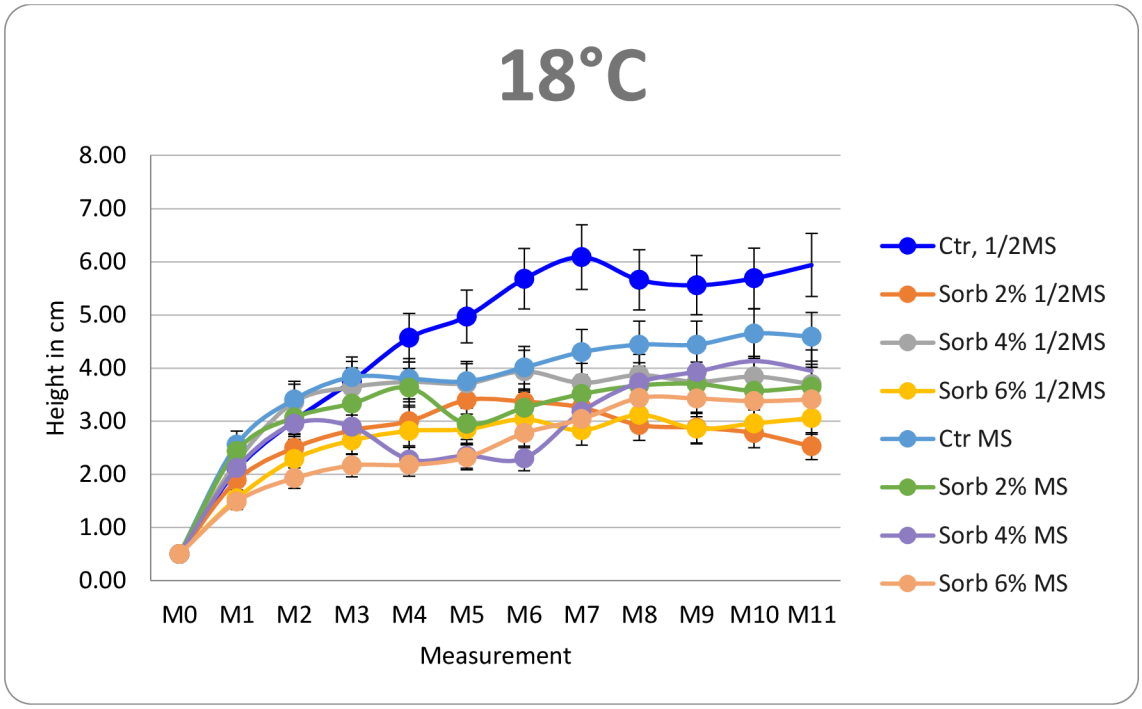


**Figure 17.** Effect of sorbitol combined with full and half composed MS media at 18°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

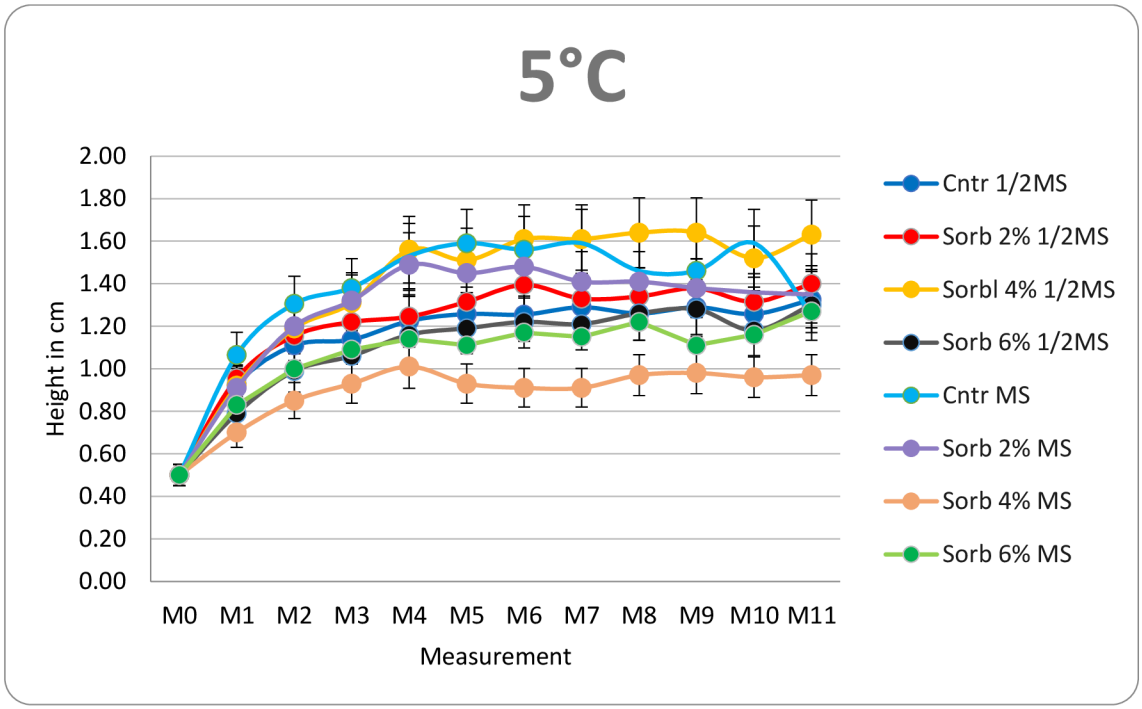


**Figure 18.** Effect of mannitol combined with full and half composed MS media at 5°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

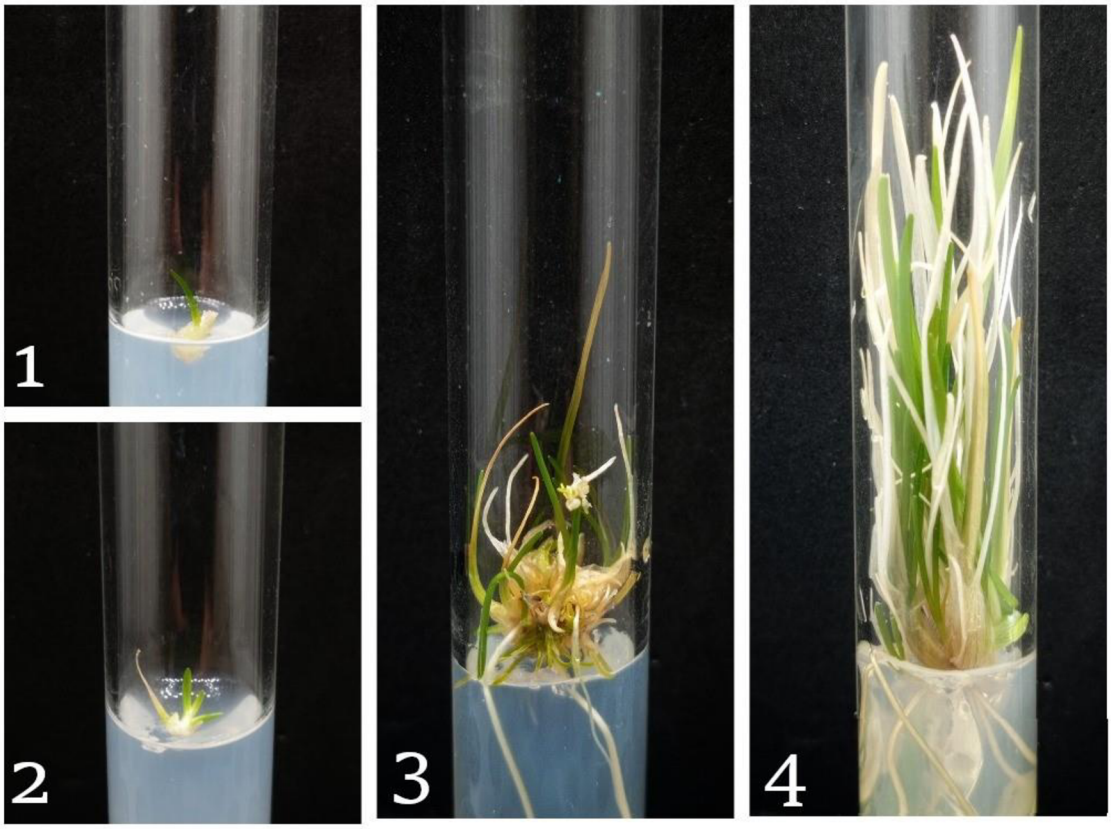


**Figure 19.** Effect of sorbitol combined with full and half composed MS media at different temperature conditions on garlic after 5th months conservation period: 1 – effect of 6% sorbitol in combination with 1/2 MS medium at 5°C±1°C, 2 – effect of 4% sorbitol in combination with MS medium at 5°C±1°C, 3 – effect of 6% sorbitol in combination with 1/2 MS medium at 18°C±1°C, 4 – effect of 6% sorbitol in combination with MS medium at 18°C±1°C.

*(Source: Author)*

## 5.4. Sucrose

### 5.4.1. Effect of cultivation temperature

Shoots of garlic planted on full and half MS medium with various concentrations of sucrose placed in temperature regimes of 18°C±1°C and 5°C±1°C showed different growth rates, different strength of shoots formation, different degree of root formation (Figure 22). Also differed and qualitative indicators, such as the color of plants and leaves' death rate. At the same time, the difference in growth between these temperature regimes directly depended on the concentration of sucrose.

At a sucrose concentration of 3% in MS medium at 18°C±1°C, the plant height at the end of measurements was 2.93 cm, at 5% 3.08 cm, at 10% 2.09 cm, and at 15% 1.21

cm, while at 5°C±1°C these figures were 2.85 cm, 1.85 cm, 1.05 cm and 1.0 cm respectively. As can be seen, at a sucrose concentration of 3%, there was no significant difference between the average heights of the last measurements. However, with an increase in concentration, a difference appeared, already at 5% sucrose, the increase at a high temperature was 1.24 cm higher than at lower, and at 10%, the difference was 1.4 cm. However, at a maximum concentration of 15%, the temperature effect was no longer strong; the growth difference was only 0.21 cm.

From this, we can conclude that the temperature effect on the growth and development of *in vitro* garlic plants directly depends on the concentration of sucrose in the nutrient medium; the effect of the temperature factor is especially prominent at a sucrose concentration of 5-10%, then the effect of temperature is levelled.

A similar situation occurred with 1/2 MS nutrient medium.

But now let's look at the growth rate of plants after the first month, at 3% sucrose concentration in MS medium and 18°C±1°C, plant growth were +2.37 cm, at 5% +1.90 cm, at 10% +1,36 cm, and at 15% +0.53 cm, while at 5°C ± 1°C and 3% of sucrose the increase was + 0.86 cm, at 5% +0.47 cm, at 10% +0, 32 cm, and at 15% +0.31 cm. But in the last month on the same medium, the growth looked a little different; at 18°C±1°C on MS media with 3% of sucrose, the growth was negative and amounted to -0.16 cm due to the death of the apical leaf, at 5% +0.24 cm, at 10% -0.06 cm, and at 15% -0.08 cm also due to the death of the apical growth leaf, and at 5°C ± 1°C the situation looked like this: with 3% of sucrose increase was +0.04 cm, at 5% +0.03 cm, at 10% +0.06 cm, and at 15% +0.04 cm. As can be seen in the first month of observations, the temperature factor seriously affected the growth of plants, but in the last month, its effect has decreased.

Especially the temperature conditions affected the formation of shoots (Figure 22). On the MS medium with 3% sucrose at 18°C±1°C, at the end of measurements, it was formed 10 pieces per sample, at 5% 8.3 pieces, at 10% 7.40 pieces, and at 15% 8.15 pieces, while at 5°C±1°C and 3% of sucrose were formed 2.85 pieces, at 5% 1.85 pieces, at 10% 1.05 pieces, and at 15 % 1 piece per sample. As a result, the difference in the formation of shoots is higher at 18°C±1°C by 4-8 times than at 5°C±1°C.

As for the root system, it was also more readily formed at a higher temperature, on average were formed 0.8 roots per plant at all sucrose concentrations, and only 0.15 roots at low temperature.

Also, the manifestation of hyperhydration was not a rare phenomenon, which at high concentrations of sucrose was higher than the control level. As regards the temperature factor in this situation, it manifested itself more strongly at a temperature of 18°C±1°C in combination with 1/2 MS medium, and on MS nutrient medium, on the contrary, at a temperature of 5°C±1°C.

In combination with sucrose, different nutrient media at a higher temperature provoked more active bulb formation. On MS medium at 18°C ± 1°C and a sucrose concentration of 3%, an average of 0.85 bulbs was formed per sample, at 5% 0.80 bulbs, at 10% 1.25 bulbs, and at 0.45 bulbs. At a temperature of 5°C±1°C, the formation of bulbs occurred singly.

### 5.4.2. Effect of full and half composition of the culture medium supplemented with extra sucrose

The nutrient medium had an ambiguous nature of influence.

Half nutrient medium at a temperature of 18°C±1°C with 3% sucrose in the first month provoked an increase of +2.14 cm, with 5% +1.52, with 10% +0.97 cm, and with 15% +0.74 cm. MS at the same temperature with 3% sucrose gave an increase of +2.37 cm, with 5% +1.90 cm, with 10% +1.36 cm, and with 15% +0.53 cm. At the second month, 1/2 MS with 3% sucrose gave +0.84 cm, with 5% +0.88 cm, with 10% +0.49 cm, and with 15% -0.26 cm. At that time, MS with 3% sucrose gave an increase of -0.44 cm, with 5% -0.30 cm, with 10% +0 cm, and with 15% -0.09 cm.

As we can see, the complete nutrient medium provoked a more active growth of garlic; however, already in the second month, the situation changed entirely with a negative increase in the MS medium. This was due to the active death of the apical leaf during this period on MS medium, while on 1/2 MS medium, this phenomenon actively manifested itself after two weeks, which proves the direct effect of the nutrient medium in combination with high concentrations of sucrose on the growth and development of garlic.

At a temperature of 5°C±1°C, no effect of the nutrient medium was found, both at the beginning of the measurements and at the end. For example, after the first month at a sucrose concentration of 5%, the increase on the MS medium was +0.47 cm, at 10% +0.32 cm, and on 1/2 MS +0.48 cm and +0.33 cm, respectively. After the second month, the

increase on MS was +0.13 cm at 3% sucrose and +0.7 cm at 10%, and for 1/2 MS at 3%, the increase was +0.09 cm, and at 10% +0.04 cm.

Also, the nutrient medium influenced the formation of shoots, but not in all cases. At 5°C±1°C, a significant difference in their formation was observed only at a sucrose concentration of 3%. Moreover, they formed more on the MS medium (standard deviation with 3 % sucrose on MS medium was 1.09, while on 1/2 MS with the same concentration 0.49). In other cases, their number did not differ much. At 18°C±1°C, similar results were obtained; MS medium with 3% sucrose provoked their more active formation; however, at this temperature, an increased amount of them was also observed on MS medium with 5% sucrose (standard deviation 3.23).

Regarding the development of the root system, at low temperatures on two nutrient media with different amounts of sucrose, their number was equal.

The formation of bulbs on both nutrient media occurred at the same level.

### 5.4.3. Effect of sucrose at various concentrations

With an increase in the sucrose concentration, a decrease in the intensity of growth of garlic plants was observed (Figure 20, Figure 21). Therefore, increased concentrations of sucrose in the nutrient medium directly affect the decrease in the intensity of garlic growth under *in vitro* conditions. But what is important, the quality indicators are also significantly deteriorated (Figure 22).

Cultivated garlic at 1/2 MS with 3% sucrose at 18°C±1°C at last measurements showed an average height of 3.35 cm, with 5% 2.78 cm, with 10% 1.91 cm, and with 15% 1.22 cm; however, the same concentrations, but in combination with MS medium, showed slightly different results, namely: with 3% sucrose plant height reached 2.93 cm, with 5% 3.08 cm, with 10% 2.09 cm, and with 15% 1.21 cm. Such small differences are explained by the uneven death of the leaves of the samples. But at 5°C±1°C, increasing the concentration of sucrose, namely 5%, 10% and 15%, on 1/2 MS and MS medium did not affect the difference in growth, and the plants have had the same height. The significant difference in growth was observed only between 3% and 5% sucrose on both nutrient media.

With an increase in sucrose concentration, monthly increases in growth also decreased. For example, after the first month of storage, the increase in plant height at 1/2 MS medium with 3% sucrose at 18°C±1°C was +2.14 cm, at 5% +1.52 cm, at 10% +0.97

cm, and at 15% + 0.74 cm. At 5°C±1°C, this trend is also clearly observed, at 3% sucrose the increase was +0.63 cm, at 5% +0.48 cm, at 10% +0.33 cm, and at 15% +0.31 cm (Figure 20).

If we compare the height of plants with controls, then the effect of increased concentration of sucrose is also noticeable at 18°C±1°C. Already at 1/2 MS with 3% sucrose, the maximum average height was reached after the third month of the experiment and amounted to 4.05 cm. At the same time, the control reached the maximum height also in the third month, but it was as much as 6.09 cm, at 5%, the maximum height was 3.24 cm after 4 months, and already at 10% the maximum height was 2.02 cm after 4 months also. But at 5°C±1°C, the situation looked different. The sucrose concentration of 3% in 1/2 MS and MS medium provoked a stronger increase than in control; the concentrations of 5%, 10% and 15% were less than controls, but not significantly. The difference was up to 0.02-0.3 cm.

Also, according to the research results, it was noted that different concentrations of sucrose against the background of different nutrient media did not affect the formation of shoots. At all concentrations, the same amount of them was formed. Their number was almost at the control level, about 6 per sample.

It may seem that with almost all concentrations of sucrose, it is possible to achieve the effect of slow-growth. Still, if we look at the qualitative indicators, then with an increase in sucrose concentration, the quality indicators are falling. At 18°C±1°C, with increasing concentration, more and more samples in both MS and 1/2 MS medium changed their color characteristics and looked unhealthy. Plant color varied from yellow-green to light yellow. The maximum number of such plants was observed at a sucrose concentration of 15% (about 70%) under both temperature conditions; many plants even looked white while remaining alive. Also, at this concentration, the plants twisted and bent into atypical shapes.

The development of the root system at 1/2 MC and 18°C±1°C at all sucrose concentrations were not too different and corresponded to the intensity of root development in control. On average, one plant had 1.05 roots. But on the MS medium, their number exceeded the control level by 0.25-4 times, but without dependence on sucrose concentration. At 5°C±1°C on different nutrient media with different concentrations of sucrose, the formation of the root system almost did not occur or was below the control level.

But most importantly, such a sucrose concentration provoked the plant to go dormant and actively form bulbs, which is an undesirable phenomenon in the slow-growth technique. At 18°C ±1°C, 1/2 MS medium per sample had the maximum number of bulbs, 4.25 pieces; this is the largest number of all treatments. With an increase in the concentration of sucrose in nutrient media and temperature regimes, an increased tendency to bulb formation was found. For example, at 18°C ± 1°C and 3% 1.3 bulbs were formed per sample, at 5% 2.25 bulbs, at 10% 2.45 bulbs, and at 15% 4.25 bulbs.

The phenomenon of hyperhydration was increased at almost all concentrations of sucrose at all temperature conditions compared with the control, but this phenomenon was especially manifested at 15% sucrose.

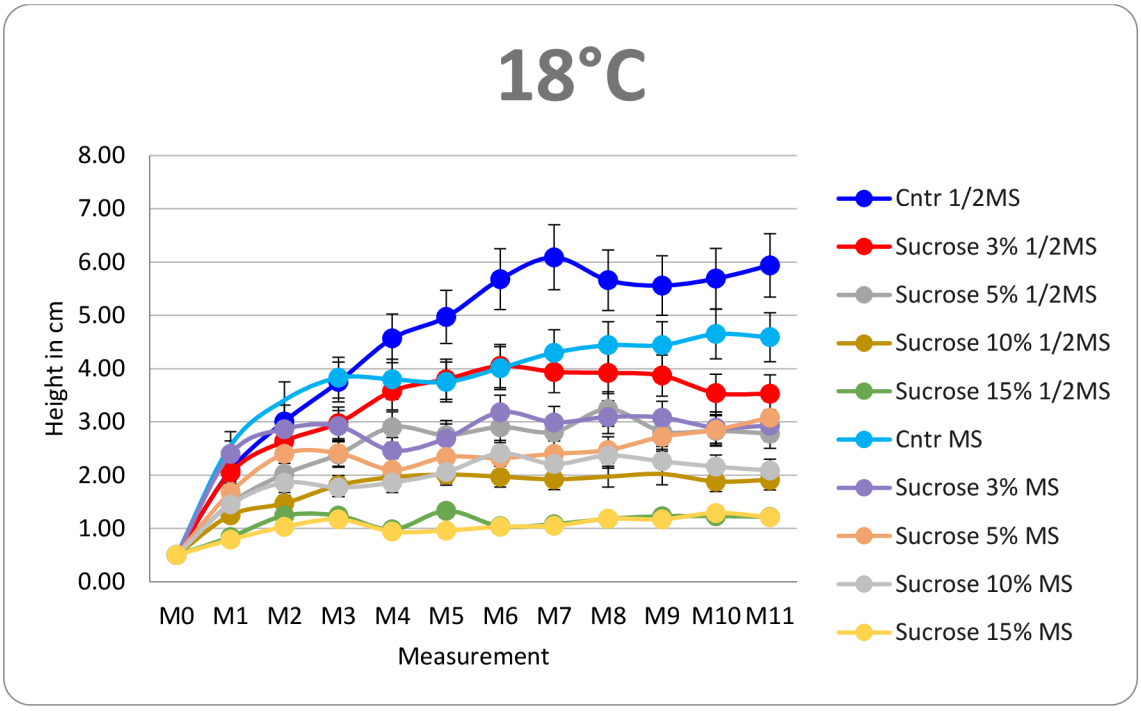


**Figure 20.** Effect of sucrose combined with full and half composed MS media at 18°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

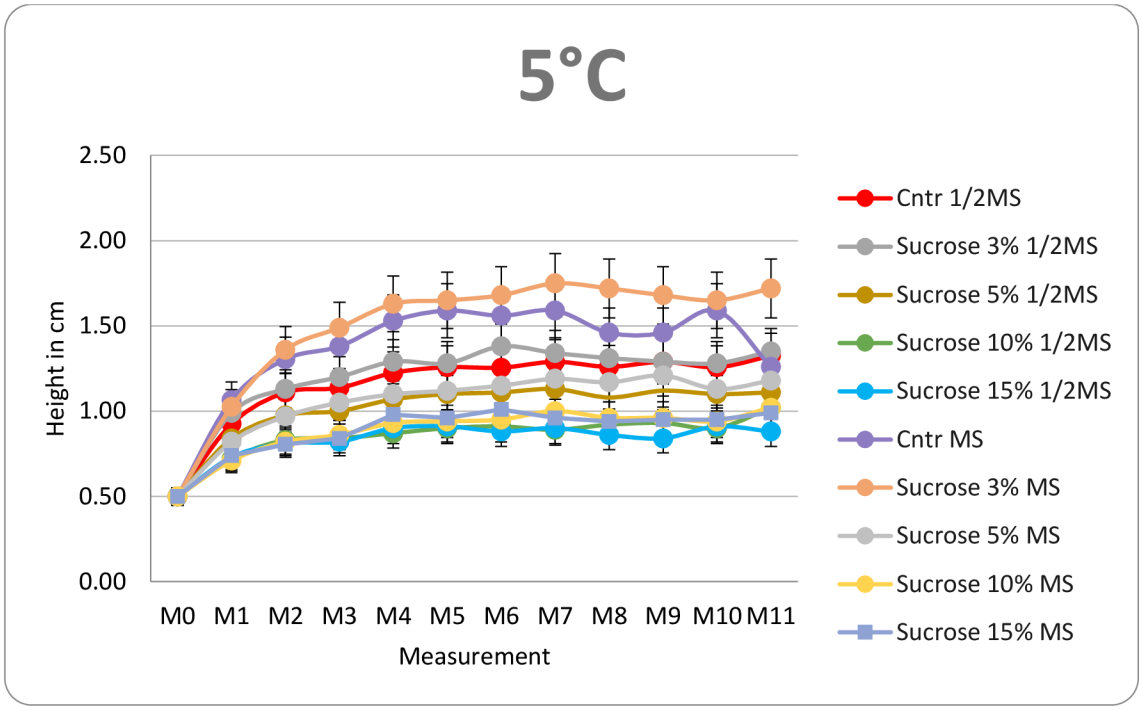


**Figure 21.** Effect of sucrose combined with full and half composed MS media at 5°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

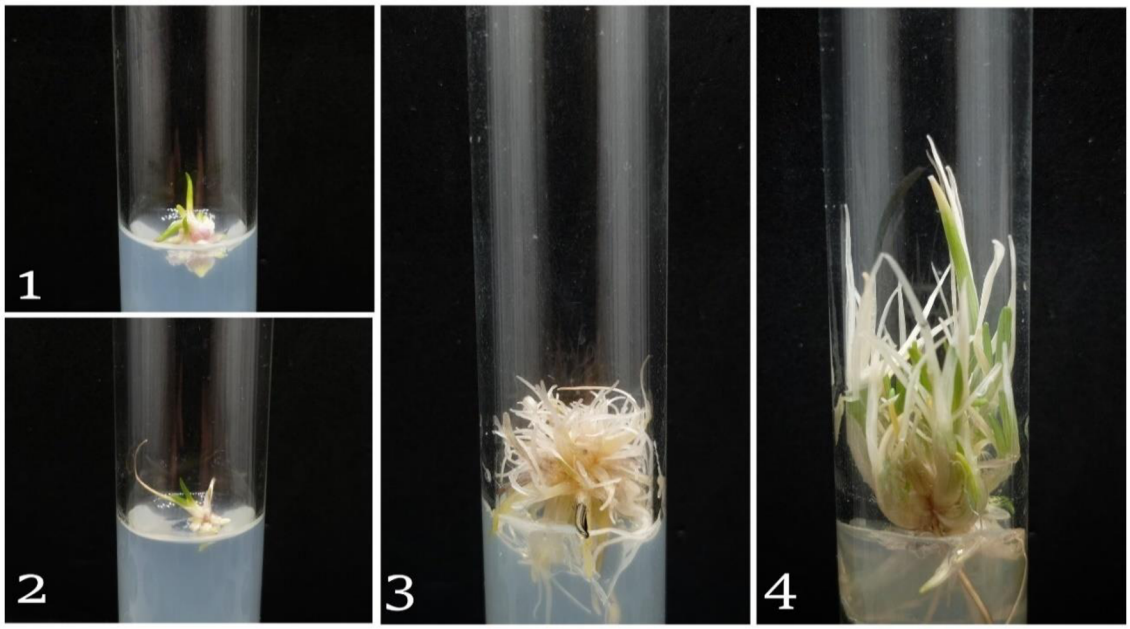


**Figure 22.** Effect of sucrose combined with full and half composed MS media at different temperature conditions on garlic after 5 months conservation period: 1 – effect of 10% sucrose in combination with 1/2 MS medium at 5°C±1°C, 2 – effect of 10% sucrose in combination with MS medium at 5°C±1°C, 3 – effect of 10% sucrose in combination with 1/2 MS medium at 18°C±1°C, 4 – effect of 10% sucrose in combination with MS medium at 18°C±1°C.

*(Source: Author)*

## 5.5. Chlormequat chloride (CCC)

### 5.5.1. Effect of cultivation temperature

The temperature factor directly impacted the development of garlic plants on different nutrient media with the presence of CCC in different concentrations. Especially the temperature factor affected the height of plants (Figure 23). On 1/2 nutrient medium at 18°C±1°C in the presence of CCC in the amount of 200 mg/l, the average plant height at the end of measurements was 4.83 cm, at 400 mg/l 4.23 cm, and at 6 mg/l 5.0 cm, but at 5°C±1°C the plants reached a much lower height, at 200 mg/l CCC it was 1.33 cm, at 400 mg/l 1.23 cm, and at 600 mg/l 1.33 cm. Samples cultured on full MS showed similar results (Figure 24, Figure 25).

The development of shoots in the two temperature ranges also differed significantly. More often, they were formed at a temperature of 18°C±1°C. On a complete nutrient medium, their average number was 9.2 shoots per sample, and at 5°C±1°C, their number was 1.2 shoots per sample (standard deviations were 3.5 and 0.7, respectively).

The formation and development of the root system were also formed depending on the ambient temperature. At a low temperature, the roots developed only in single plants, and the phenomenon was not of a mass nature. Still, the roots developed more willingly and intensively at a high cultivation temperature. At the end of the measurements on half the nutrient medium, their number per sample reached an average of 0.51 roots, and low temperature provoked the development of only 0.06 roots per sample (Figure 23).

The phenomenon of hyperhydration also took place; most of all, it occurred at 18°C±1°C. The formation of bulbs did not have a massive character and proceeded singly at both low and high temperatures.

### 5.5.2. Effect of full and half composition of the culture medium supplemented with different concentrations of chlormequat chloride (CCC)

Half and full nutrient media did not significantly affect the monthly growth of plants against the background of different concentrations of CCC at different temperature conditions. For example, at 18°C±1°C per 1/2 MS medium and a CCC concentration of 200 mg/l, the increase after the first month of the experiment was +3.66 cm, at 400 mg/l +2.67 cm, and at 600 mg/l +3.03 cm (Figure 8). MS medium with the same concentrations of CCC showed a similar increase in plant height, which amounted to +3.36 cm at 200

mg/l, +3.61 cm at 400 mg/l, and at 600 mg/l +2.76 cm. In the second month, the situation with plant growth looked like this: 1/2 MS with 200 mg/l CCC +0.56 cm, with 400 mg/l +0.54 cm, and with 600 mg/l +0.98 cm, and MS with 200 mg/l CCC +0.52 cm, with 400 mg/l -0.21 cm, and 600 mg/l +0.22 cm. The above heights showed that the increase did not have a specific vector depending on the nutrient medium; all measurements have an undirected character.

At 5°C±1°C 1/2 MS with 200 mg/l CCC, the increase after the first month was +1.03 cm, at 400 mg/l +0.66 cm, and at 600 mg/l + 0.60 cm, and MS with 200 mg/l CCC +1.44 cm, with 400 mg/l +0.87 cm, with 600 mg/l + 0.89. On the second month, the increase by 1/2 MS with 200 mg/l CCC was +0.18 cm, with 400 mg/l +0.17 cm, and with 600 mg/l +0.15 cm, and with MS with 200 mg/l CCC +0.09 cm, with 400 mg/l +0.23 cm, and with 600 mg/l +0.11 cm (Figure 25). There is also no clear relationship between the nutrient medium and plant growth rate at low temperatures.

The concentration of the nutrient medium in combination with CCC did not affect the growth of plants. However, such dependence can be traced during the formation of shoots. Moreover, such dependence is observed at a higher and lower temperature. At 18°C±1°C and 1/2 MS with 200 mg/l CCC, an average of 7.55 shoots were formed per sample, with 400 mg/l 7.70 shoots, with 600 mg/l 7.25 shoots, and on complete MS medium with 200 mg/l CCC, 9.35 shoots were formed, with 400 mg/l 9.50 shoots, and with 600 mg/l 8.75 shoots. At a temperature of 5°C±1°C, the difference in the formed shoots on MS and 1/2 MS was also 0.5-2 shoots, in favor of MS, standard deviations between MS and 1/2 MS were 0.81 and 0,92.

The intensity of root formation and the formation of bulbs on the full and half nutrient medium remained at the same level. The number of hyperhydrated plants also did not depend on the factor of the nutrient medium.

### 5.5.3. Effect of chlormequat chloride (CCC) at various concentrations

Against the background of full and half nutrient media at different temperatures, different concentrations of CCC did not show the desired effect to achieve the technique of slow-growth on garlic. At 18°C±1°C against the background of MS and 1/2 MS of the medium, all concentrations of CCC provoked an almost uniform growth of all samples (Figure 23). At the end of the measurements, it was found that on the MS medium with a concentration of CCC of 200 mg/l, the average plant height was 4.87 cm, at 400 mg/l 4.02

cm, and at 600 mg/l 3.96 cm, and on 1/2MS medium and CCC 200 mg/l 4.83 cm, at 400 mg/l 4.23 cm, and at 600 mg/l 5.0 cm (Figure 24).

At 5°C±1°C, different concentrations of CCC did not affect the growth of plants on 1/2 nutrient medium at all; in all cases, almost the same plant heights were obtained. At 200 mg/l CCC, the average height at the last measurement was 1.33 cm, at 400 mg/l 1.39 cm, at 600 mg/l 1.33 cm. But on the MS medium at the same temperature, the CCC proved to be not so unambiguous. At 200 mg/l CCC, the final height was 1.87 cm, at 400 mg/l 0.96 cm, and at 600 mg/l 1.41 cm. Although the values differ, CCC has no clear influence since with an increase in the concentration of CCC, the growth force increases again.

Also, if we compare the growth rates of garlic against the background of CCC with the control, then in many cases, the CCC samples exceeded the control or were at its level, which also confirms the absence of the influence of CCC in the slow-growing development of garlic.

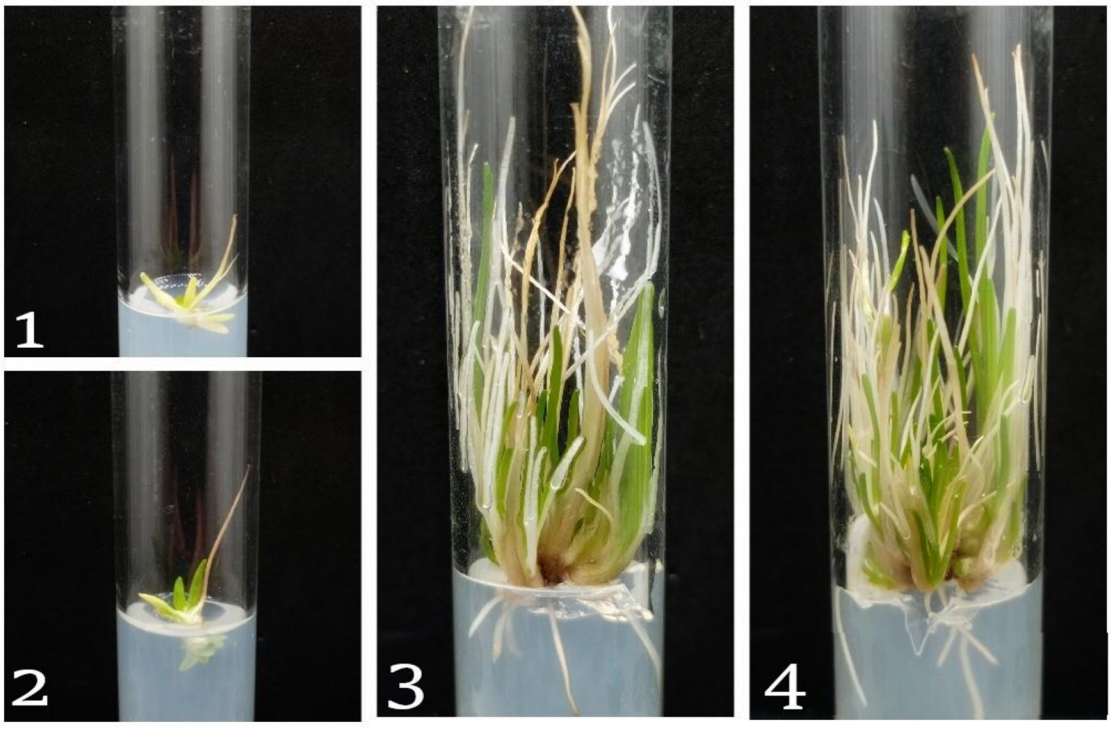


**Figure 23.** Effect of chlormequat chloride (CCC) combined with full and half composed MS media at different temperature conditions on garlic after 5th months conservation period: 1 – effect of 400 mg/l CCC in combination with 1/2 MS medium at 5°C±1°C, 2 – effect of 400 mg/l CCC in combination with MS medium at 5°C±1°C, 3 – effect of 400 mg/l CCC in combination with 1/2 MS medium at 18°C±1°C, 4 – effect of 600 mg/l CCC in combination with MS medium at 18°C±1°C.

*(Source: Author)*

Regarding the formation of shoots, the effect of CCC at a temperature of 5°C±1°C is traced only in the MS medium. With increasing concentration, their average number per sample decreased. On MS with 200 mg/l CCC, 3.05 shoots per sample were formed, at 400 mg/l 2.10 shoots, and at 600 mg/l 1.75 shoots (standard deviations were 1.19, 0.78, and 0,67). But at a temperature regime of 18°C±1°C on different nutrient media, the effect of CCC was completely leveled; at all its concentrations, the same number of shoots was formed.

The root system, against the background of different concentrations of CCC, was formed at the control level, which negates the influence of this chemical on this process in both temperature regimes on different nutrient media.

Also, CCC did not provoke the plants to form bulbs at all at different temperature conditions.

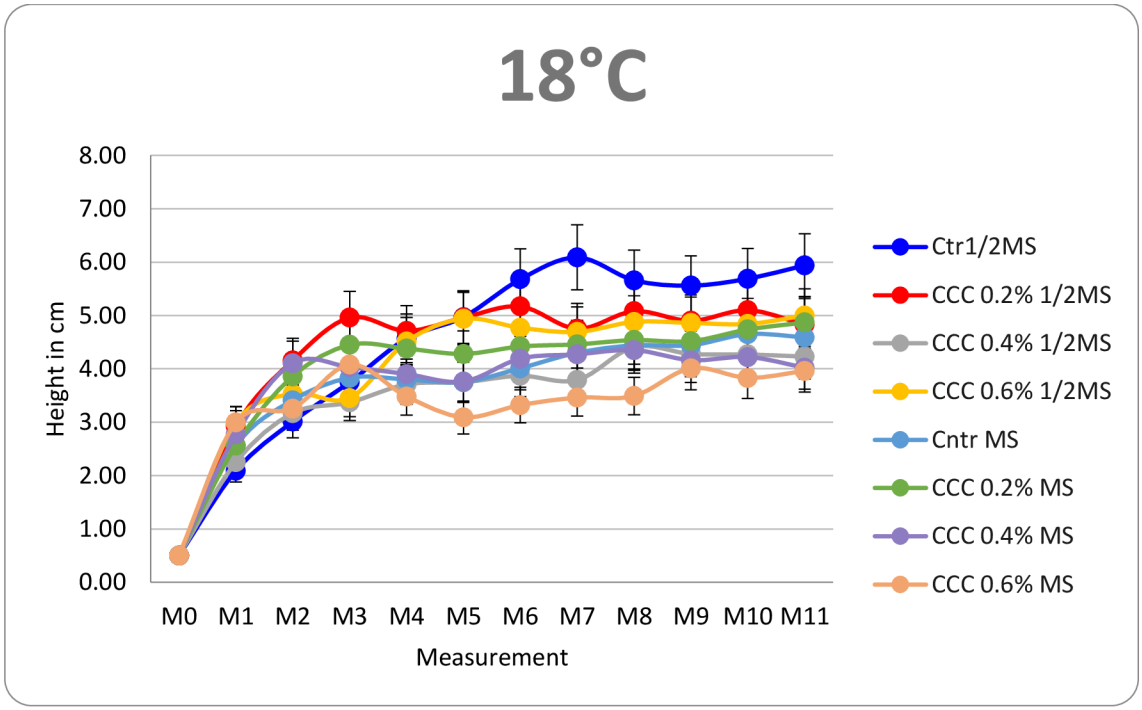


**Figure 24.** Effect of chlormequat chloride (CCC) combined with full and half composed MS media at 18°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

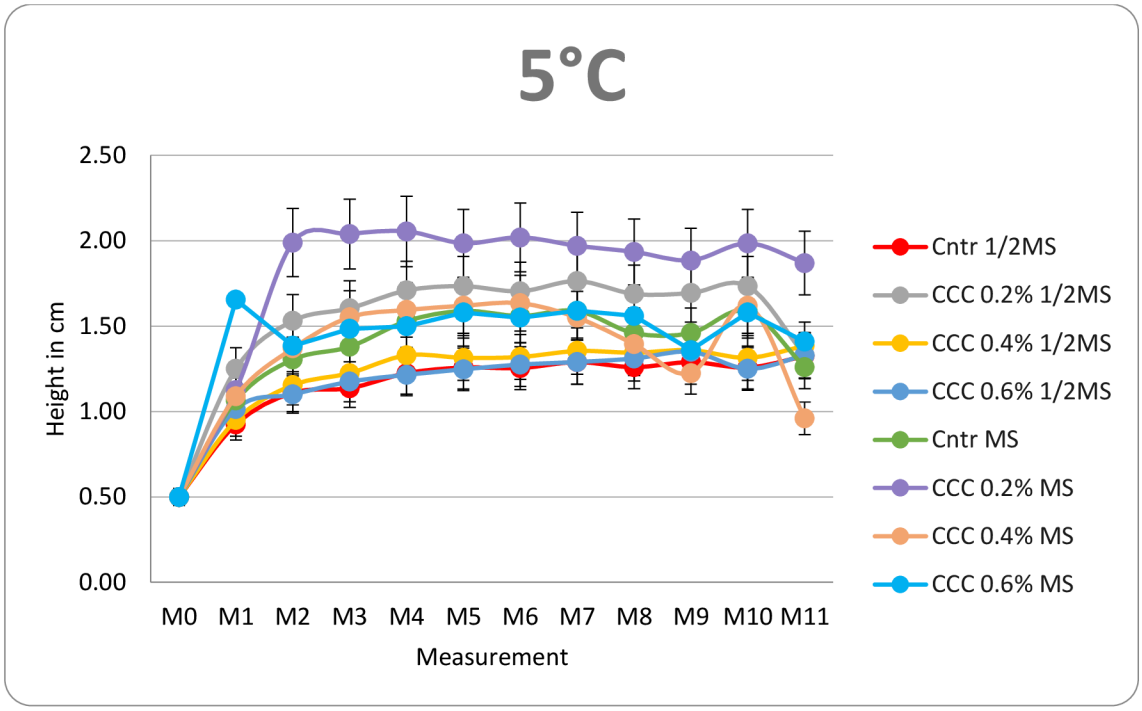


**Figure 25.** Effect of chlormequat chloride (CCC) combined with full and half composed MS media at 5°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

## 5.6. Abscisic acid (ABA)

### 5.6.1. Effect of cultivation temperature

Different cultivation temperatures affected the growth of *Allium sativum* plants on both full and half nutrient media supplied with different concentrations of ABA (Figure 26). At a temperature of 18°C±°C, a complete nutrient medium with 1 mg/l ABA at the end of measurements showed an average plant height of 2.10 cm, with 3 mg/l 2.66 cm, and with 5 mg/l 1,63 cm (Figure 27). The same nutrient medium with the same ABA concentrations, but at 5°C±1°C, already showed a lower height, which at 1 mg/l ABA was 1 cm, at 3 mg/l 1.19 cm at 5 mg/l 0.99 cm (Figure 28). A similar effect of temperature was also exerted on plants with 1/2 MS medium with the same concentrations of ABA.

Monthly gains also differed depending on the temperature regime. For example, after the first month of the experiment at 18°C±1°C on a complete nutrient medium with 1 mg/l ABA, the increase was +2.08 cm, with 3 mg/l +1.40 cm, and with 5 mg/l +1.02,

and at 5°C±1°C, respectively, with 1 mg/l +0.27 cm, with 3 mg/l +0.56 cm and with 5 mg/l +0.36 cm.

The ambient temperature also had a significant effect on the formation of shoots. At 18°C±1°C, 3-7 times more shoots were formed than at 5°C±1°C. For example, MS medium with 3 mg/l ABA at 18°C±1°C at the end of the measurement generated 7.5 shoots per sample, while at 5°C±1°C, 2.15 shoots, standard deviations were 3.27 and 0.85, respectively.

Root formation under different temperature conditions against the background of MS and 1/MS of nutrient media with different concentrations of ABA occurred at the same level or did not occur at all, which indicates the absence of a relationship between temperature and root growth.

Hyperhydration in both temperature ranges occurred at the control level; the color characteristics of plants also did not differ. But on the formation of bulbs, the higher temperature played a stronger influence.

### 5.6.2. Effect of full and half composition of the culture medium supplemented with abscisic acid (ABA)

Nutrient media of full and half composition did not affect plant growth in different temperature ranges. At 18°C±1°C, the average maximum plant height at 1/2 MS medium with 1 mg/l ABA was 1.99 cm, with 3 mg/l 2.13 cm, and with 5 mg/l 2.07 cm, but with MS medium and 1 mg/l ABA the plants reached 2.58 cm, with 3 mg/l 2.29 cm, and with 5 mg/l 1.63 cm (Figure 27). The increase in the first month of the experiment was also not unambiguous. 1/2 MS medium with 1 mg/l ABA allowed to obtain an increase of +2.47 cm, with 3 mg/l +1.27 cm, and with 5 mg/l +0.77 cm, and MS medium with the same concentrations ABA +2.08 cm, +1.40 cm, +1.02 cm. At 5°C±1°C, plant heights were similar, with minimal differences.

The intensity of shoots formation was the same on both nutrient media against the background of different concentrations of ABA. For example, at 18°C±1°C and 1/2 MS medium with 5 mg/l ABA formed 6.85 shoots, and MS medium with the same concentration of ABA 7.5 shoots (standard deviations were 0.24 and 0.16). Due to the temperature factor, a smaller number of shoots formed at 5°C±1°C, but there was no difference in their formation from nutrient media.

Root formation took place regardless of the type of nutrient medium in two temperature regimes and at the control level. Bulb formation also took place regardless of the factor of the nutrient medium.

### 5.6.3. Effect of abscisic acid (ABA) at various concentrations

Abscisic acid (ABA) positively reduced the growth rate of *in vitro* garlic plants (Figure 26), which is a key factor in achieving the slow growth technique. Almost all concentrations of ABA against the background of different nutrient media and temperature ranges provoked plant growth below the control values.

At a temperature of 18°C±1°C, plants grown on nutrient media with the addition of ABA had almost 3 times lower height compared to the control, but at 5°C±1°C, the difference between them was not so noticeable. The results obtained on both full and half nutrient medium with ABA concentrations of 1 mg/l and 3 mg/l were particularly similar to the control values obtained on 1/2 MS medium (Figure 27). For example, at the end of measurements, the height of plants on 1/2 MS medium with 1 mg/l ABA was 1.23 cm, with 3 mg/l 1.21 cm, and with 5 mg/l 0.98 cm, while the control plant height indicators on 1/2 MS medium were 1.33 cm.

Comparing different concentrations of ABA at 18°C±1°C, there is a tendency that with an increase in concentration from 1 mg/l to 3 mg/l, growth rates slightly increase, and then from 3 mg/l to 5 mg/l stabilize on 1/2 MS medium, but drops again on MS medium. Thus, on MS medium, plant growth with increasing ABA concentration has the character of an inverted parabola. The following plant height was achieved: 1 mg/l 2.10 cm, 3 mg/l 2.66 cm, and at 5 mg/l 1.63 cm. But at 5°C±1°C, the situation looked a bit otherwise; with an increase in ABA concentration from 1 mg/l to 3 mg/l, the growth parameters remained at the same level, while when the concentration went from 3 mg/l to 5 mg/l, a significant decrease in the average height was observed.

Monthly increments with an increase in ABA concentration had a decreasing character. At 18°C±1°C and MS medium after the first month of measurements, the average increase with 1 mg/l ABA was +2.08 cm, 3 mg/l +1.40 cm, and at 5 mg/l +1.02 see. At 5°±1°C, this trend is weak but still present.

Various concentrations of ABA in combination with various nutrient media and temperature regimes did not affect the formation of shoots. The intensity of their

formation was at the level of controls; for example, at 18°C ± 1°C, the average standard deviation was at the level of 1.89 - 3.27, control 2.59 - 2.76.

As for the root system formation, ABA had no effect here. The parameters were at the control level.

The formation of bulbs was of a solitary nature, and the plants did not enter dormant states. With regard to color quality indicators, at 18°C±1°C and ABA concentration of 1mg/l, deviations were observed, and some plants acquired a white-green tint. At elevated concentrations, this phenomenon was observed singly.

At 5°C±1°C and ABA concentration, a change in color to yellow-green was observed in some samples (Figure 28). The level of hyperhydration was at the control level.

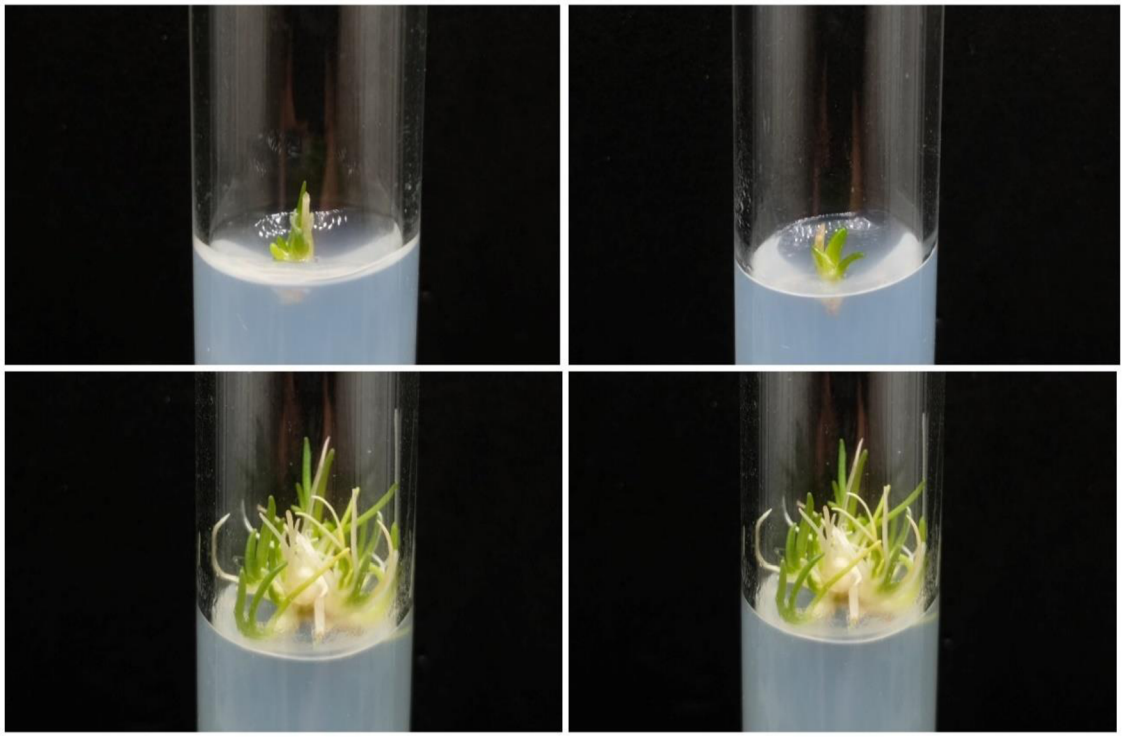

**Figure 26.** Effect of abscisic acid (ABA) combined with full and half composed MS media at different temperature conditions on garlic after 5th months conservation period: 1 – effect of 5 mg/l ABA in combination with 1/2 MS medium at 5°C±1°C, 2 – effect of 5 mg/l ABA in combination with MS medium at 5°C±1°C, 3 – effect of 5 mg/l ABA in combination with 1/2 MS medium at 18°C±1°C, 4 – effect of 5 mg/l ABA in combination with MS medium at 18°C±1°C.

*(Source: Author)*

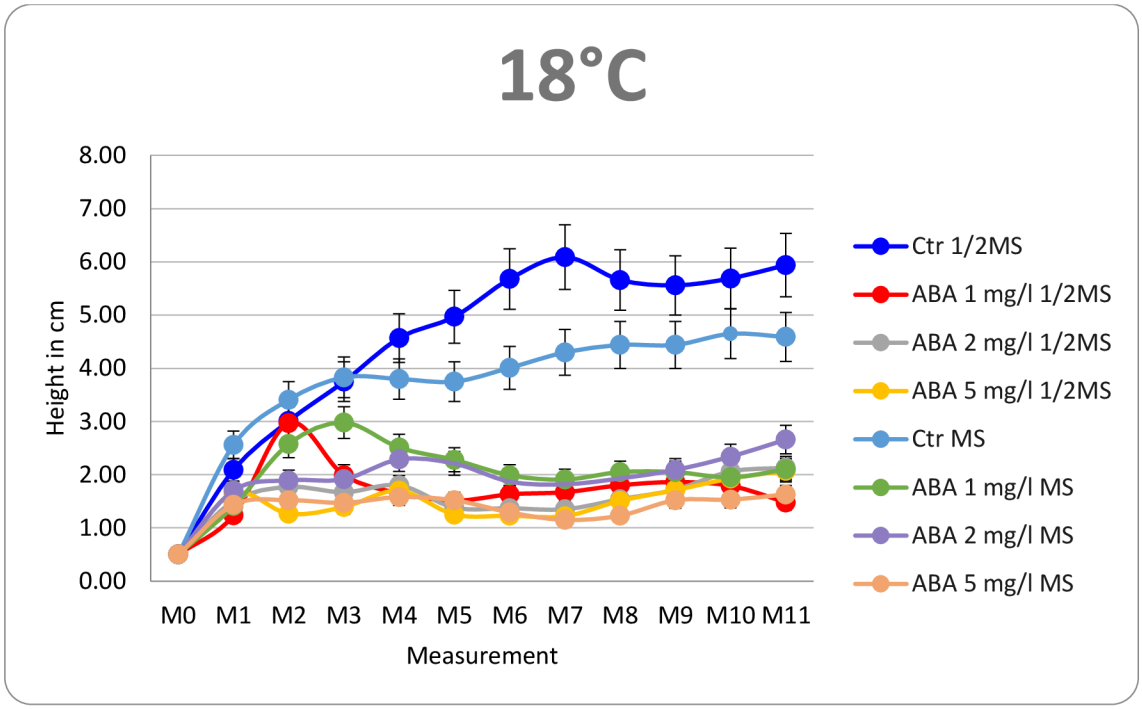


**Figure 27.** Effect of abscisic acid (ABA) combined with full and half composed MS media at 18°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

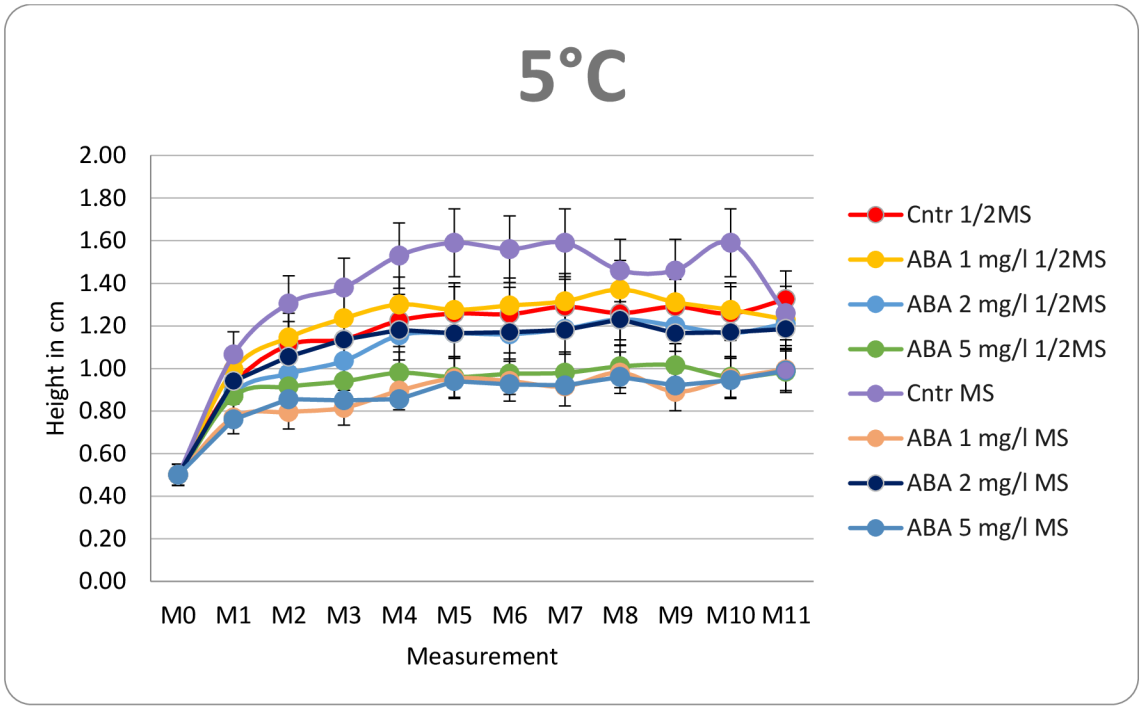


**Figure 28.** Effect of abscisic acid (ABA) combined with full and half composed MS media at 5°C±1°C temperature condition on plant height during a 5-month conservation period.

*(Source: Author)*

## 5.7. Comparison of the best treatments of abscisic acid (ABA), chlormequat chloride (CCC), sucrose, and mannitol

Based on the characteristics of the growth and development of garlic plants on different nutrient media with the addition of various concentrations of sorbitol, mannitol, sucrose, abscisic acid (ABA) and chlormequat chloride (CCC) in different temperature ranges, it was found that the best results for achieving the technique of slow-growth were obtained at 5°C±1°C (Figure 29).

In the case of mannitol, half MS medium in combination with 4% of mannitol at this temperature performed best. At the end of the measurements, the average height of plants was 1.03 cm, which is lower than the control value of 1.15 cm, while the number of shoots formed on average was 1.75 shoots per plant, while the control formed 0.58 shoots medium per plant on the same nutrient medium. However, according to the statistical analysis results, no difference in the formation of shoots was found, p-value of 0.21 (Table 1).

**Table 1. Comparative analysis of the formation of shoots on 1/2 MS medium at 5°C±1°C between control, mannitol 4% and sucrose 10%**

Anova: Single Factor

Summary

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| Shooting control 1/2 MS 5°C±1°C | 20 | 11.66666667 | 0.583333333 | 0.301900585 |
| Shooting mannitol 4% 1/2 MS 5°C±1°C | 20 | 20 | 1 | 2.361111111 |
| Shooting sucrose 10% 1/2 MS 5°C±1°C | 20 | 24.56818182 | 1.228409091 | 1.306106973 |

Anova

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 4.27936 | 2 | 2.139681856 | 1.617247078 | 0.207416481 | 3.158842719 |
| Whithin Groups | 75.4133 | 57 | 1.323039556 | | | |
| | | | | | | |
| Total | 79.6926 | 59 | | | | |

Root formation and bulb formation did not actively occur. The qualitative characteristics of garlic at this concentration also did not cause comments; however, when studying mannitol in different temperature regimes, a high percentage of hyperhydration was found at the same concentration of the chemical. This effect was also observed at concentrations of 2% and 6% but already at a low temperature. Therefore, using this chemical in the slow-growth technique for garlic puts it at high risk.

**Table 2. Comparative analysis of the formation of roots on 1/2 MS medium at 5°C±1°C between control, mannitol 4% and sucrose 10%**

Anova: Single Factor

Summary

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| Rooting control 1/2 MS 5°C±1°C | 20 | 4.08333333 | 0.204166667 | 0.223300439 |
| Rooting mannitol 4% 1/2 MS 5°C±1°C | 20 | 2.91666667 | 0.145833333 | 0.102247807 |
| Rooting sucrose 10% 1/2 MS 5°C±1°C | 20 | 0 | 0 | 0 |

Anova

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 0.44236111 | 2 | 0.221180556 | 2.03822836 | 0.1396462 | 3.15884272 |
| Whithin Groups | 6.18541667 | 57 | 0.108516082 | | | |
| | | | | | | |
| Total | 6.62777778 | 59 | | | | |

In the case of sucrose, the best performance was also achieved in the temperature range of 5°C±1°C against the background of half the nutrient medium and 10% sucrose concentration. The average plant height was 0,85 cm, which is also below the control level. The number of shoots at the end of the measurements was at 1.45 per sample, which is slightly above the control, but according to the results of statistical analysis, no difference in the formation of shoots was found, p-value of 0.21 (Table 1)

An interesting fact is that at this concentration, the formation of the root system did not occur at all (Table 2); bulb formation was also at the control level. The only caveat was the increase in the percentage of hyperhydrated plants and the change in color characteristics in some samples. Color sometimes changed from yellow-green to white.

In the case of sorbitol, the best growth rates were also achieved at a temperature of 5°C±1°C, but with the participation of a complete nutrient medium and its concentration of 4%. At the same time, the average plant height was 0.9 cm. The number of shoots formed was 2.8 pieces per sample, which is 2 times higher than the control indicators, while the standard deviations were within 0.88 and 0.49, respectively. According to the statistical analysis results, no difference in the formation of shoots was found, p-value of 0.37 (Table 3, Table 4). The root system and bulbs were not formed in these samples (Table 6). Quality indicators were also normal.

**Table 3. Comparative analysis of the formation of shoots on MS medium at 5°C±1°C between control, ABA 5 mg/l and sorbitol 4%**

Anova: Single Factor

Summary

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| Shooting control MS 5°C±1°C | 20 | 16 | 0.8 | 0.6998538 |
| Shooting ABA 5 mg/l MS 5°C±1 | 20 | 4 | 0.2 | 0.15672515 |
| Shooting sorbitol 4% MS 5°C±1‘ | 20 | 22.666667 | 1.1333333 | 1.98567251 |

Anova

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 8.948148148 | 2 | 4.4740741 | 4.72239082 | 0.0126561 | 3.158842719 |
| Whithin Groups | 54.00277778 | 57 | 0.9474172 | | | |
| | | | | | | |
| Total | 62.95092593 | 59 | | | | |

**Table 4. Comparison of shoots formation between MS control and MS with 4% sorbitol**

t-Test: Two ssample Assuming Equal Variances

| | *Shooting control MS 5°C±1°C* | *Shooting sorbitol 4% MS 5°C±1°C* |
|---|---|---|
| Mean | 0.8 | 1.133333333 |
| Variance | 0.699853801 | 1.985672515 |
| Observations | 20 | 20 |
| Pooled Variance | 1.342763158 | |
| Hypothesized Mean | 0 | |
| df | 38 | |
| t-Stat | -0.909659872 | |
| P(T<=t) one-tail | 0.184367917 | |
| t Critical one-tail | 1.68595446 | |
| P(T<=t) two-tail | 0.368735833 | |
| t Critical two-tail | 2.024394164 | |

Nutrient media with different concentrations of abscisic acid (ABA) also worked more efficiently against the background of low temperatures, which made it possible to obtain growth and development indicators of garlic below the control ones.

The low temperature of 5°C±1°C against the background of different nutrient media and concentrations of ABA was also the most effective in achieving the goal of slow growth.

The smallest average height achieved using ABA was 0.9 cm at concentrations of 1 mg/l and 5 mg/l against the background of a complete nutrient medium, which is one and a half times lower than the control values. However, if we consider these two

concentrations in terms of qualitative indicators, the color change of plants to yellow-green occurred more at 1 mg/l ABA. Shoots formation also occurred at the same level, and, on average, there were 0.6 shoots per plant per plant at the end of the measurements. Standard deviation at 1 mg/l was 0.37, and at 5 mg/l 0.20. Compared with the control, the formation of shoots was statistically different in favor of the control (Table 3, Table 5). Bulb formation did not occur either.

**Table 5. Comparison of shoots formation between MS control and MS with 5 mg/l ABA**

t-Test: Two ssample Assuming Equal Variances

| | *Shooting control MS 5°C±1°C* | *Shooting ABA 5 mg/l MS 5°C±1°C* |
|---|---|---|
| Mean | 0.8 | 0.2 |
| Variance | 0.699853801 | 0.156725146 |
| Observations | 20 | 20 |
| Pooled Variance | 0.428289474 | |
| Hypothesized Mean | 0 | |
| df | 38 | |
| t-Stat | 2.8992292 | |
| P(T<=t) one-tail | 0.003091731 | |
| t Critical one-tail | 1.68595446 | |
| P(T<=t) two-tail | 0.006183461 | |
| t Critical two-tail | 2.024394164 | |

**Table 6. Comparative analysis of the formation of roots on MS medium at 5°C±1°C between control, ABA 5 mg/l and sorbitol 4%**

Anova: Single Factor

Summary

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| Rooting control MS 5°C±1°C | 20 | 0.583333333 | 0.029166667 | 0.012627924 |
| Rooting ABA 5 mg/l MS 5°C±1°C | 20 | 0 | 0 | 0 |
| Rooting sorbitol 4% MS 5°C±1°C | 20 | 1.166666667 | 0.058333333 | 0.015423977 |

Anova

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 0.03403 | 2 | 0.017013889 | 1.819543974 | 0.171388848 | 3.158842719 |
| Whithin Groups | 0.53299 | 57 | 0.009350634 | | | |
| Total | 0.56701 | 59 | | | | |

But as far as treatments with chlormequat chloride (CCC) are concerned, a low temperature of 5°C±1°C also occurred, the garlic samples had a much lower growth rate and their height compared to 18°C±1°C. However, the effect of CCC on plants is not traced here. Samples with CCC have similar growth and quality parameters as controls.

Based on the above data, the best treatments to achieve the slow-growth technique are abscisic acid (ABA) at a concentration of 5 mg/l in combination with a complete MS medium, sucrose at a concentration of 10% in the background of a half medium and sorbitol at a concentration of 4% in the background complete nutrient medium (Figure 29).

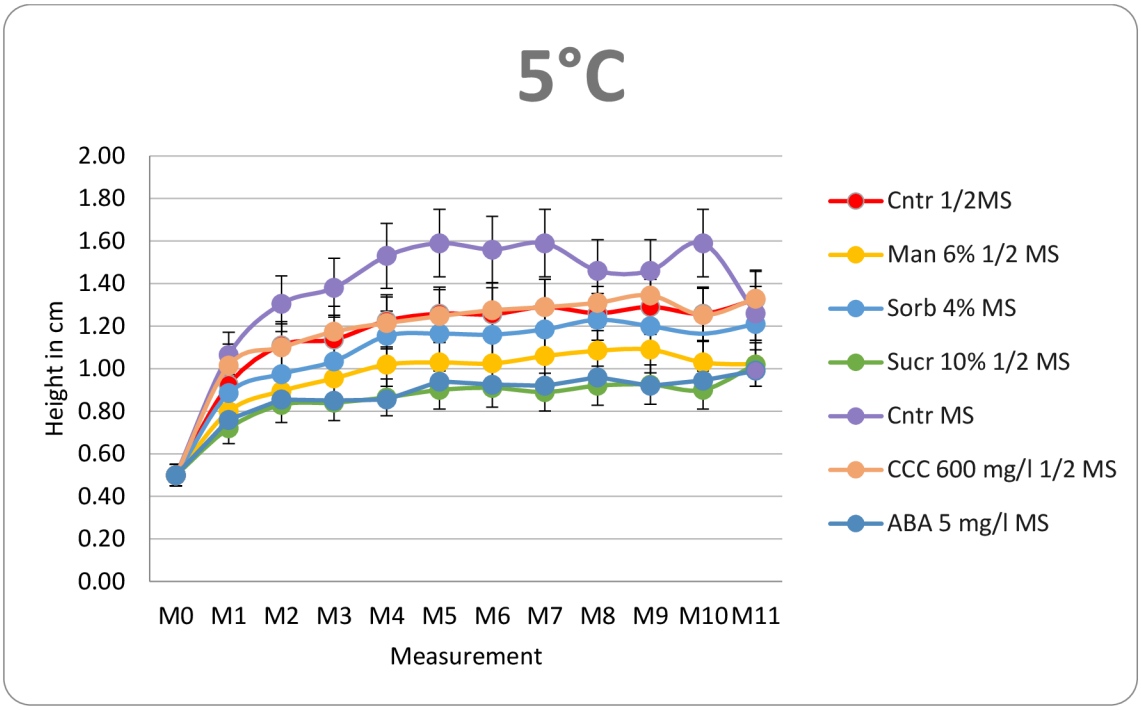


**Figure 29.** Comparison of the best treatments abscisic acid (ABA), chlormequat chloride (CCC), sucrose, mannitol with controls at 5°C ± 1°C.

*(Source: Author)*

## 5.8. Evaluation of the regenerative abilities of garlic after 5 months of slow-grow storage

During an experiment on *in vitro* regrowth of garlic plants after 5 months of storage under slow-growth conditions from the most effective treatment 5°C±1°C (abscisic acid at a concentration of 5 mg/l in combination with a complete MS medium, sucrose at a concentration of 10% in the background of a half medium and sorbitol at a concentration of 4% in the background complete nutrient medium), it was found that garlic plants

cultivated before on 1/2 MS medium with the addition of 10% sucrose had the strongest growth energy. At the end of the measurements, the average height of these plants was 1.56 cm, while the average height of plants cultivated before on the MS medium as control did not exceed 1.28 cm, and on the 1/2 MS as control, 1.12 cm. Plants from other treatments also lagged far behind this height (Figure 30). At the same time, the quality indicators were also at a high level; there were no changes in the color characteristics of the plants, and hyperhydration did not occur at all.

The formation of shoots on such plants also took place at one of the highest levels; after 28 days, on average, there were 14.2 shoots per plant, but plants cultivated as a control for five months on MS medium formed an average of 9.2 shoots per plant, the statistical analysis also confirmed a significant difference, p-value 0.0006 (Table 7).

**Table 7. Comparative analysis of the formation of shoots on MS medium between regenerated plants from MS control and 1/2 MS medium with 10% sucrose.**

Anova: Single Factor

Summary

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| Shooting of regenerated plants from Control MS | 10 | 51 | 5.1 | 7.71111111 |
| Shooting of regenerated plants from sucrose 10% 1/2 MS | 10 | 89 | 8.9 | 0.66944444 |

Anova

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 72.2 | 1 | 72.2 | 17.2303613 | 0.00060021 | 4.41387342 |
| Whithin Groups | 75.425 | 18 | 4.190277778 | | | |
| Total | 147.625 | 19 | | | | |

What about other treatments? Garlic stored on 1/2MS medium supplemented with 6% mannitol showed signs of hyperhydration during regrowth (80% of the samples were hyperhydrated). This indicates the impossibility of using mannitol in the slow-growth technique for garlic at all.

As for the effect of abscisic acid (ABA) on the regenerative capacity of garlic, it was found that the growth was weak; the average plant height at the end of the measurements was only 0.58 cm (control 1.28 cm).

But in the case of the influence of the MS medium with 4% sorbitol, good regrowth results were obtained. At the last measurement, the average plant height was 1.18 cm,

which is 0.5 times less than the plant height after the influence of 10% sucrose against the background of 1/2 MS medium and is almost at the same level of plant height with plants from MS control. Color characteristics were normal; hyperhydration did not appear. The shoots level was high, with an average of 9.1 shoots per plant, which is in line with control plants. Accordingly, we can conclude that plants are easily rehabilitated from the influence of such an osmoregulatory component as sorbitol.

An interesting fact is that the concentration of the nutrient medium also affects the regenerative abilities of plants. The control growing on 1/2MS nutrient medium at 5°C±1°C during the regrowth experiment provoked a high level of hyperhydration; 70% of the samples showed symptoms of this deviation.

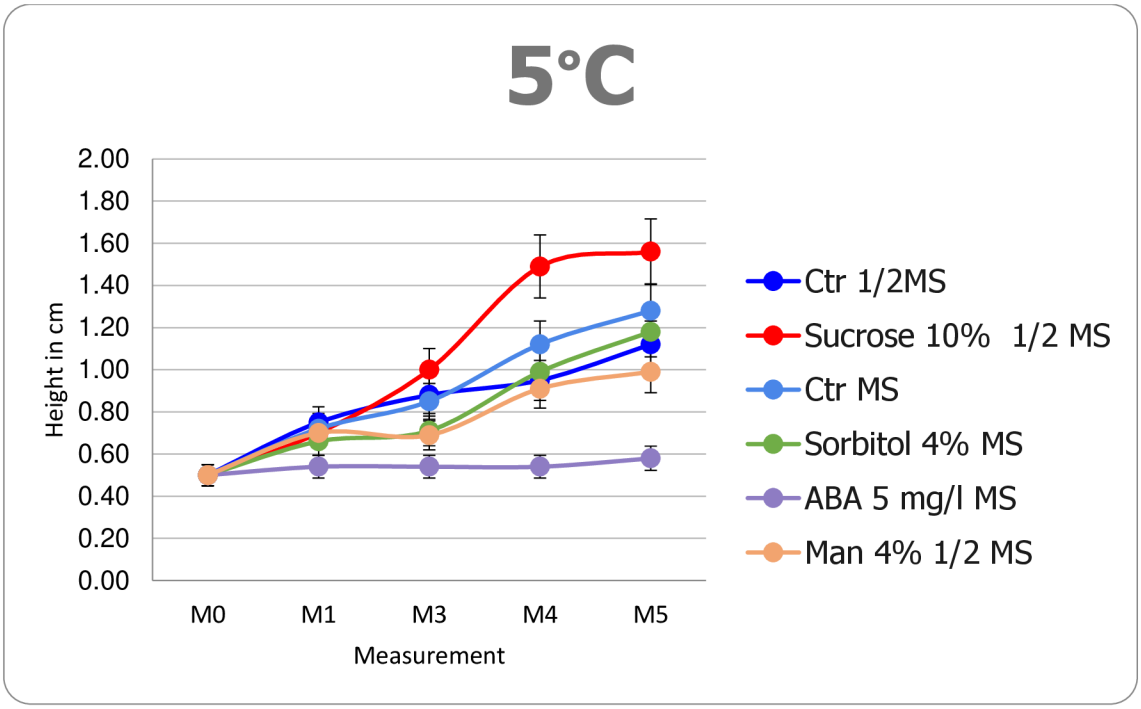


**Figure 30.** Comparative characteristics of the growth of regenerated plants after 5 months of slow-grow storage from the best treatments.

*(Source: Author)*

# 6. Discussion

According to the results of the experiment, it was found that the temperature factor has a severe impact on the growth and development of plants under *in vitro* conditions since it affects the course of all biochemical processes occurring in the plant, especially on respiration and photosynthesis (Kumar & Reddy 2011). In this case, according to our research, the most effective temperature was 5°C±1°C. In all samples placed under this temperature conditions, a significant decrease in growth rates was observed while maintaining quality compared to 18°C±1°C. The results obtained were consistent with our hypothesis that low temperature in addition to media supplements during cultivation will increase the efficiency of the slow-growth conservation protocol of *Allium sativum*. Therefore, Lambardi & Ozudogru (2013) statement that for medium-term conservation, the most commonly used temperature is in the range of 4-5°C can be considered true for slow-growth storage of garlic.

Regarding the quality of the plants, some irreversible changes were seen more often at 18°C±1°C. The most common process was hyperhydration, which partly confirms the words of Ruta et al. (2020) that the temperature regime provokes reversible and irreversible changes in metabolism, membrane composition, and function.

Also, the results showed that MS nutrient medium is a deterrent to the active growth and development of *Alium sativum* at high temperatures. Contrary to our findings, Bonnier & Tuyl (1997) found that the use of half or quarter composition of MS medium leads to a decrease in nutrient absorption, translocation, and utilization, Cha-Um et al. (2007) also states that strength reduction of the culture medium is the first aspect to result in fruitful slow-growth preservation. However, our findings show that a low concentration of nutrients in the 1/2MS medium, on the contrary, provokes rapid growth and development of garlic shoots. The use of 1/2MS and MS culture medium without supplements cannot achieve the effect of slow-growth. Our research showed that these media compositions were not efficient in reducing the plant's growth while maintaining their quality and vitality.

The results obtained in the study of different concentrations of mannitol are explained by the fact that mannitol works as an osmoregulating substance in plants, limiting the amount of available water by osmotic shrinkage through the semi-permeable membranes (Ruta et al. 2020). It follows that these concentrations caused irreversible changes in garlic membranes. But what is more interesting is that this phenomenon began

to occur intensively at 5-6 months of the experiment, which, according to Grigoriadou & Leventakis (2000), may depend on the age of the tissues. They compared *in vitro* shoot proliferation in adult and seedling material of *Myrtus communis* and found that the old plant material showed symptoms of hyperhydricity, while seedling-derived explants did not show signs of this morphological abnormality. The assumptions of E. Benson (2000) may also work here, that hyperhydration can be caused by the created microclimate in the middle of the test tube and the accumulation of ethylene. But if we compare these samples with the control, we found that the above factors play a secondary role since, in control, this phenomenon was found only in 10% of the samples. In this case, the primary reason is the effect of mannitol against a background of higher temperature.

The temperature factor affects the metabolic rate (Ruta et al. 2020), and in this case, a higher temperature contributed to the active penetration and accumulation of mannitol in plant tissues. It is worth noting that at 5°C±1°C, this hyperhydration effect did not occur at all or was at the control level.

As in the case of mannitol, sorbitol was one of the factors that provoked an increased level of hyperhydration of the samples at high temperature compared to the control, but this happened only at a concentration of sorbitol 4% and 6% in 1/2 MS medium. On a half nutrient medium, more active growth occurs, respectively, more active nutrition, which also accelerated the entry of sorbitol into cells and their irreversible changes in the structure of their membranes (Ruta et al. 2020).

The use of sucrose in various concentrations against the background of different nutrient media and temperature regimes showed quite diverse results. This effect on garlic growth can be explained by the fact that sucrose plays a double role, depending on the concentration; first of all, it provides the plant with carbon, but when the concentration increases, it exhibits osmotic properties (Ruta et al. 2020). It should be noted that sucrose is present in almost every nutrient medium (Kumar & Reddy 2011), including the nutrient medium we use, and serves as the main building material for organic compounds. It is true that the prescription concentrations of sucrose and other nutrients in the MS medium induced the maximum growth rates of the controls compared to the increased concentrations of sucrose. At the same time, the heights of the control and elevated sucrose concentrations differed by 0.5-3 times, but this phenomenon was observed only at a temperature of 18°C±1°C, while at 5°C±1°C, the difference was not so significant. This can be explained by the fact that due to the low temperature, all metabolic processes

were slowed down (Kumar & Reddy 2011) and the membrane permeability was not so high, which did not allow the osmotic effect to be manifested in the case of high concentrations of sucrose, and the use of sucrose as a building material in standard concentration, as in the case of the control. After all, the principle of action of sucrose is based on the laws of osmosis (Cha-um 2007).

Very interesting is the statement by Bonnier & Tuyl (1997) that high sugar concentrations of 6-9% lead to the so-called osmotic stress similar to sugar alcohols. We also investigated the effect of sugar alcohols, namely mannitol and sorbitol, on the growth of garlic and the possibility of using them in the slow growth technique and comparing the effect of increased concentration of sucrose and the above chemicals, we can say that their action is similar, as claimed.

Based on the growth and quality characteristics of garlic against the background of various concentrations of sucrose, the best plants were obtained at a temperature of 5°C±1°C on 1/2 MS medium with 10% sucrose concentration, which partially confirms the words of Bonnier and van Tuyl (1997), which successfully stored the *in-vitro* bulblet of *Lilium spp.* for a period of 28 months at 25 °C on ¼-strength MS medium supplemented with 9% (w/v) sucrose. As in our case, in their study, the concentration of substances in the nutrient medium was reduced and the content of sucrose was increased.

But most of all, our results agreed with the statement of Pandey et al. (2015) that sucrose, in combination with low temperature, has also been effective in limiting the growth of garlic (4 °C and 10% sucrose). Much the same, but in our study, better results were obtained on a half nutrient medium compared to a complete one. Regrowth parameters were also among the best.

In the course of the experiment, the hormone ABA proved to be quite good, in all temperature ranges, on all nutrient media, a decrease in growth rates was observed without significant deviations from quality indicators. This confirms the possibility of using this hormone to achieve the minimal growth technique, since ABA hormone plays an important role in the processes of germination and maturation of seeds, in adaptation to environmental conditions, since it contributes to the mechanism of stomatal closure, and also affects gene expression. (Xiong & Zhu 2003).

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using this hormone to achieve the minimal growth technique, since ABA hormone plays an important role in the processes of germination and maturation of seeds, in adaptation to environmental conditions, since it contributes to the mechanism of stomatal closure, and also affects gene expression. (Xiong & Zhu 2003). There are also claims that when applied ABA at concentrations of 0.5, 1, 10, and 100 mg/l to garlic in vitro culture, this can provoke the formation of bulbs, accelerating the entry into a dormant state (Hahn et al. 2003), however during the experiment, it was noticed that the ABA concentrations of 1mg/l, 3mg/l and 5mg/l did not affect the intensive formation of bulbs. The fact that the substance ABA promotes bulb formation was also stated in Abscisic Acid Signal Transduction" (1998) already at concentrations of 0.01, 0.1, 0.5, and 1 mg/l. But again, when using ABA at a concentration of 1 mg / l, such processes were not observed, which may be due to the influence of other factors, such as the varietal characteristics of the garlic used and the factor of the nutrient medium.

The positive effect on slowing down the growth processes of the hormone ABA very well confirms the words of Aitken-Christie et al. (1995) that plant growth regulators are usually the key to control plant growth and development under *in vitro* conditions and that phytohormones can have both a stimulating effect, activating growth processes, and an inhibitory effect, slowing them down (Niazian & Shariatpanahi 2020).

The most unexpected results have been obtained with chlormequat chloride (CCC). As is known, CCC refers to plant growth regulators (PGRs) and inhibits gibberellin metabolism by blocking cyclases copalyl-diphosphate synthase and ent-kaurene synthase and has a proven effect on stem growth, stem elongation, flowering, and somatic embryogenesis (Niazian & Shariatpanahi 2020). And its introduction into the nutrient medium changes the hormonal balance, which in turn changes vital processes in an *in vitro* plant (Espindula et al. 2009). Although Hahn et al. (2003) stated that CCC, like abscisic acid (ABA), when applied to garlic *in vitro* culture, can provoke the formation of bulbs, accelerating the entry into a dormant state, which is an important factor in the application of the slow-growth technique. Their study showed that at all CCC concentrations: 0.5, 1, 10, and 100 mg/l, bulbs were formed, but most of all, at a concentration of 100 mg/l against the background of a consequence of leaf and shoot growth inhibition. We used even higher concentrations of CCC (200 mg/l, 400 mg/l and 600 mg/l) against the background of different nutrient media and temperature ranges, however, no inhibitory effect on plant growth was found. Changes in the intensity of the

formation of shoots, the root system also did not occur. The intensive formation of bulbs, which was mentioned above, was not noticed either.

Possibly different results obtained may depend on the variety of garlic, the method of applying CCC to the nutrient medium and the nature of the chemical itself. Cha-um (2007) stated that introducing growth retardants in the culture medium changes the hormonal balance, but the desired effect is not always achieved. It all depends on the type of hormone and the type of plant on which it is applied, for example, the use of growth retardants like chlormequat chloride (CCC) has proved useful in limited cases.

# 7. Conclusions

The main goal of this research was to optimize the protocol for medium-term *in vitro* conservation of *Allium sativum*. To achieve this goal, the effects of various osmotic agents in various concentrations, such as sorbitol in concentrations 20-60 g/l, mannitol – At 20-60 g/l and sucrose at 30-150 g/l, were assessed. Plant growth regulators such as chlormequat chloride (CCC) at 200-600 mg/l and abscisic acid (ABA) at 1-5 mg/l were also investigated. In addition, 5±1°C and 18±1°C cultivation conditions were used to observe the influence of temperature on the growth of the plants. Full-strength and 1/2 concentrated MS (Murashige and Skoog (1962)) were used as basal culture media.

According to the experiment results, it was found that the temperature factor has a severe impact on the growth and development of plants under *in vitro* conditions. The most effective cultivation temperature proved to be 5°C±1°C. A significant decrease in growth rates was observed in all samples placed under this temperature conditions while maintaining quality compared to 18°C±1°C. The results obtained were consistent with our hypothesis that low temperature in addition to media supplements during cultivation will increase the efficiency of the slow-growth conservation protocol of *Allium sativum.*

Based on the characteristics of the growth and development of garlic plants on different nutrient media with the addition of various osmotic agents and plant growth regulators during 5 months of storage, it was found that the best results for achieving the technique of slow-growth were obtained at 5°C±1°C using one of four treatments either full-strength MS medium supplemented by 5 mg/l ABA, sucrose at a concentration of 10% in half medium, sorbitol at a concentration of 4% in full-strength MS medium or MS medium supplemented by mannitol at a concentration of 6%. In all of these treatments, plant height was below the control level. However, ABA was the most effective in reducing the growth of plantlets. Using this plant growth inhibitor, a minimum plant height of 0.9 cm was achieved. However, according to the results of regrowth after 5 months of storage, it was found that garlic plants stored on 1/2 MS medium with the addition of 10% sucrose had the strongest regrowth capacity. Relatively high regenerative abilities were also shown by plants stored on MS nutrient medium with the addition of 4% sorbitol. As for the plantlets stored on full-strength MS supplemented by 5 mg/l ABA, the regrowth capacity was weak, which shows the need for long-term rehabilitation of garlic plants after the influence of this growth-regulating substance. As for the garlic stored on MS medium with the addition of 6% mannitol, a high level of hyperhydration

appeared during regrowth, making it impossible to use mannitol on garlic in the slow-growth technique.

Concerning the influence of the nutrient medium, it was found that at a temperature of 18°C±1°C and the use of 1/2 MS medium provoked a more active growth of garlic plants, the number of formed shoots did not differ much, plants looked healthy without any deviations, the root system was formed only by single specimens. In contrast, at the temperature of 5°C±1°C, MS medium provoked a more active growth only during the first 4 months; in the formation of shoots, no difference was found, plants also looked healthy without any deviations, the root system did not develop. The results showed that MS nutrient medium is a deterrent to the active growth and development of *Alium sativum* at high temperatures. Using just 1/2MS and MS culture medium with no media supplements will not be able to achieve the effect of slow-growth. Our research showed that these media compositions were not efficient in reducing the plant's growth while maintaining their quality and vitality.

Our observations have also shown that the phenomenon of hyperhydration of garlic occurs more strongly at a temperature of 18°C±1°C and can be provoked by both climatic and chemical influences, as in the case of using mannitol as a culture media supplement at 18°C±1°C, where 90% of the samples at 4 months of storage were hyperhydrated.

All the results obtained during the experiment will be used at developing of an efficient slow-growth method for the *in vitro* storage of garlic *(Allium sativum)*, which will allow storing large garlic collections in sterile laboratory conditions with minimal labor and monetary costs. It will be possible to keep large field collections of several hectares in one small room. Also, this technology will make it possible to obtain garlic plant material free from diseases and viruses at any time of the year, which will greatly simplify research activities and contribute to the conservation of biodiversity, since many local varieties of garlic are highly susceptible to climate change, are being replaced by more intensive varieties or are on the verge of extinction.

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