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Effect of selected factors on seed storage of Welsh onion (*Allium fistulosum* L.)

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List of publications constituting the dissertation

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List of abbreviations

CAT, catalase
Cv, cultivar
DCFH-DA, fluorogenic dye 2,7-dichlorofluorescein
F_1, hybrid cultivar
GERM, seed germination
ISTA, International Seed Testing Association
IU, international unit
MDA, malondialdehyde
MS, male sterile
Negi, typical fresh use of *Allium fistulosum* in Japan
NUV, near visible ultraviolet
OP, open pollinated cultivar
PCA, principal component analyses
PCR, polymerase chain reaction
PDA, potato dextrose agar
POD, peroxidases
RFU, relative fluorescence unit
RH, relative humidity
ROS, reactive oxygen species
ROS12, reactive oxygen species at 12^{th} month
ROS22, reactive oxygen species at 22^{th} month
SOD, superoxide dismutase
USDA, United States Departemnt of Agriculture

Abstract

Welsh onion (*Allium fistulosum* L.) also known with other names such as Chinese spring onion, Japanese bunching, onion stone leek or Chinese onion, is a vegetable popularly grown in several Asian countries, including Japan, China, Korea, Taiwan, Indonesia, and Malaysia. It has been later introduced and widespread in the western countries, which is more commonly called salad onion, as an alternative to onion (*Allium cepa* L.) cultivars for the production of fresh onion leaves and pseudo-stems. Welsh onion seed storability is one the most critical and key factors of this species, which could have negative effects, if not persevered in ideal conditions. The complicated management of sales forecast and the increasing difficulty of seed production, also due to climate change, make suitable seed storing a critical component to ensure seed availability for the growers, and therefore a stable supply of the fresh Welsh onion into the market. There are different storing procedures that have been proved to be effective keeping seeds for more than 5 years, such as freezing under -18°C or storing under vacuum, however they are expensive and difficult to apply on big seed volumes, and for this reason temperature and relative humidity (RH) control are essential components for ideal storing conditions. The aim of this research was to conduct experiments and evaluate the effect of air temperature and RH on quality parameters of stored Welsh onion (*Allium fistulosum* L.) seed and relate them also to the energy cost needed to maintain such conditions. Consequently, two hypotheses have been elaborated for this dissertation: (I) Welsh onion seeds with moisture content below 10% can be stored under controlled temperature and air humidity for 30 months without a quality loss, (II) the accumulation of reactive oxygen species in Welsh onion seeds stored for 12 and 22 months is limited under controlled temperature and air humidity. The experiments were carried out from April 2018 until February 2021. During this time germination analyzes were carried out following the ISTA, International Seed Testing Association, protocols and conducted every four-month period. Also, mycological analyzes were made, after the first and second year of storage, to test level of infestation by determining the appearance of fungi colonies and their sporulation. In reference to the reactive oxygen species, which have a negative effect on seed ageing, the methodology adopted was the DFCH-DA assay and detected by a fluorescence spectroscopy. The results obtained from this research can be used as a guideline for the storage in climatic and RH controlled chambers of all genotypes belonging to *Allium fistulosum* species, with the aim to maintain its seed high seeds germination related to the duration of the storage and taking into consideration the operating energy costs. Seeds of the tested genotypes kept good germination at 7-8°C and at 10°C. By storing them at 7-8°C and 10°C, the same effect was achieved. In seeds of 3 genotypes (270322, 1240694, 170403214), after storing them for 30 months, their germination was better at the air RH 45% than at 25%. The calculated electricity costs for 30-month seed storage at 10 °C was 11.2% lower than at 7-8 °C. From the company's financial point of view, considering the electricity cost, seed storage at 10 °C is more recommended than at 7-8°C. Generally, the

temperature 7-8°C favoured growth of *Penicillium* spp. and *Phoma* sp. on Welsh onion seeds during 2 years of storage, while in seeds stored at 25°C it increased seed infestation with *Cladosporium* spp. and *Fusarium* spp. In relation to the reactive oxygen species the search for effective indicators of poor seed storability, detected through assays was proved to be a proper strategy, while at the same time, seed technologists should continue their work to improve the current storage protocols, by refining parameters such as temperature, seed moisture content, and gaseous compositions of the storage atmosphere. Seeds from onion and onion-like cultivars still represent a challenge for breeders and seed technologists, due to their storability. Given the complexity of the physiological mechanisms and molecular players involved in seed longevity, a comprehensive evaluation of these dynamics will require extensive studies at a multidisciplinary level. At the same time, efforts should be focused on exploring the impact of the storage protocols on the longevity of primed seeds, by screening hallmarks of seed deterioration such as ROS levels, lipid peroxidation and DNA damage. On the other hand, the environmental conditions during seed maturation of the mother plant dictate the initial quality of seed lots, before storage. Thus, at the company level, data concerning the origin and history of seed lots should also help operators in their management activities.

CHAPTER I. Introduction

Welsh onion is biennial or perennial herb of the genus *Allium* of the *Liliaceae* botanical family, and derived from the wild *Allium altaicum* Pall., which occurs in Siberia and Mongolia. The genus Allium in addition to welsh onion includes a wide number of other species, of which onion (*Allium cepa* L.), leek (*Allium porrum* L.), chive (*Allium schoenoprosum* L.), and garlic (*Allium sativum* L.) are the most important edible onion plants among the total of 850 (FAO, 2006; Pagano *et al*., 2023).

The cultivation of *Allium fistulosum* dates back to at least 200 BC in China and was introduced in Japan before 500 AD, then it spread farther to South-East Asia and consequently in Europe (Borna*,* 1982; Singh and Ramakrishna*,* 2017). In China, it is the most important *Allium* species cultivated and widely used in their traditional cuisine, as commonly bulb onion and leek are used in Europe. While in Japan and in South Korea it is considered the second in importance to the bulb onion (*Allium cepa* L.). Nowadays, the crop is grown throughout the world; although in Europe and in the US Welsh onion is not a traditional species it has been adapted for a different use. The fresh product is commonly named salad onion (or green onion). It has more tender and sweeter pseudo-stems, compared to what is produced in Asia, and long 9-11 cm. It is therefore grown with a different agronomical tecnique which includes a much high plant density per hectare (Boyhan *et al.,* 2009; Padula *et al.,* 2022).

Considering its increasingly importance and widespread in a modern and professional horticulture, germination has become a key quality factor for seed companies and growers, who aim to maximize the percentage of usable plants per seed unit. Seeds can survive and maintain their quality parameters for a considerable time under dry conditions, but during the storage they can lose germination capacity due to chemical reactions, if not properly preserved.

Seed shelf life is a highly variable characteristic, and it is related to several factors, in first place the potential longevity of a seed depends on its initial quality (Zhang *et al.,* 2001; Tang *et al.,* 2011). Seed production technique and location, due to environmental factors, have both a strong effect on seed germination. Air temperature is the parameter which has the biggest affect. 1 °C differences have been shown to have important consequences for seed dormancy in sensitive ranges, consequently restricts the areas and countries in which high-quality seed can be produced for major seed markets (Penfield and MacGregor, 2017). In addition, also the genotype, resulting from an intense breeding activity, has impact on seed germination longevity. Therefore part of modern breeding programmes is to select cultivars with greater potential for storability (Groot, 2014). However, the species or the group of crops they belong to, is the first aspect to be considered in terms of seed shelf life. Brassica, tomato and several cereals seeds are characterized by a relative long shelf life, whereas species such as onion, Welsh onion, lettuce tend to deteriorate more rapidly and consequently lose germination quality relatively fast (Groot, 2014).

The germination loss is related to the seed ageing rate. To reduce this effect and prolong shelf life, it is important to properly store the seeds, under controlled temperatures and relative humidity conditions. In addition to these two parameters, also oxygen has an important role in preserving seed quality due to chemical cell damages that can be caused by oxidation. Moreover, infestation with fungi is a further additional factor which can significantly reduce seed quality (Hourston *et al*., 2020).

When considering seed quality issues, today, there is still poor attention on Welsh onion and strong efforts are needed to overcome the current gap of knowledge and find effective strategies to enhance germination performance and seedling resilience as well as seed viability under storage (Padula *et al*., 2022).

Allium fistulosum L. seeds as all other species from the Liliaceae family, are not characterized by post harvest dormancy period and therefore can easily lose their germination capacity at room temperature, if stored for more than 6-12 months **(**Zhang *et al.*, 2001; Tang *et al.,* 2011; Padula *et al.*, 2022)**.** Poor seed performance after storage has been one of the crucial factors that limits Welsh onion commercial production and development.

Experiments on finding out optimal conditions for longer storage of Welsh onion seeds focused mainly on 2 factors: seed moisture content: from 3.2% to 8.4%, and air temperature of the storing chamber: from -18ºC to 9ºC **(**Yanping *et al.*, 2000; Zhang *et al.*, 2001; Khan *et al.*, 2004; Lazarenko and Bezrukov, 2008). At the same time, much less attention was paid to another important factor when storing the Welsh onion seeds: the relative humidity (RH) of the air during the storage (Padula *et al.*, 2022).

Further experiments have also evidenced a correlation and high sensitivity between *Allium* spp. seed viability and the level of oxygen in the storing chamber, which in combination with a high percentage of seed moisture, resulted in a rapid loss of seed germination (Hourston *et al.*, 2020). Long-term storage under sub-optimal conditions affects seed viability since progressive accumulation of oxidative damage impairs the function of lipid membranes and proteins, also compromising genome integrity. Genotoxic stress resulting from uncontrolled ROS accumulation determines DNA damage that needs to be repaired during the early phase of germination, otherwise the process will fail (Pagano *et al.* 2023). The impact of storage conditions on genome integrity has been investigated in *Allium fistulosum* at the level of chromosomal instability.

In the situation of constant seeds overproduction, it is not common for seed companies to keep Welsh onion seeds in stock for longer than 2-2.5 years. The main reason is to avoid high storing costs associated to a difficult stock management (Padula *et al.*, 2022). Kugbei (2000) pointed out a long list of costs related to storing of sowing material. He classified them into 2 categories: permanent ones, not depending on the number of seeds stored or the period of storage, and variable ones. It was evidenced that out of the latter, most relevant was the expense of electricity (Trail *et al.,* 2019). As a result, one of the short-term goals in a seed company management should be lowering it (Hołubowicz and Bralewski, 2004). No information was available in the literature about possible ways of storing Welsh onion seeds at temperature higher than 4ºC temperatures with the idea to reach the same goal of their storing at lower cost (Padula *et al.*, 2022).

CHAPTER II. Literature review

1. Botanical characteristics and classification

Taxonomically, *A. fistulosum* belongs to the division Mangoliophyta (Flowering plants), class Liliopsida (Monocotyledons), subclass Liliidae, order Liliales, family Liliaceae (lily family), genus *Allium* and species *fistulosum* (USDA, 2018; Kim *et al.,* 2023). Literally, the species *fistulosum* means hollow, named as it possesses hollow leaves and scapes.

Its root system is string-shaped with few and short lateral roots. The stem has a globose or oblate shape and is approximately from 50 to 90 cm tall, with indistinct, ovoid to oblongoid bulb up to 10 cm long. It is surrounded by the base of the leaf sheath, and densely rooted in the lower part (Tendaj and Mysiak, 2007). The young leaves are hidden in the leaf sheath and form a round rod-shaped pseudo-stem with the multi-layer sheaths. The in-soil pseudo-stem is white, and the above-soil part is yellow green (Chen *et al*., 2001; Ma *et al*., 2009). The leaves are long, cylindrical, hollow, green, or dark green, with smooth and waxy surfaces. Each plant has 5-8 leaves, long 10 to 50 cm and are distichously alternate. Its umbels in the developmental stage are hidden in the membranous involucre. The flowers are small and white, bisexual, and insect-pollinated, narrowly campanulated to urceolate shaped, with slender pedicels, long up to 3 cm long (Korohoda, 1974; Małachowski, 1982). They consist of 6 ovate-oblong to oblong white tepals, in 2 whorls and long 6–10 mm, and with greenish midvein; 6 stamens and a 3-celled ovary, each ovary contains 2 seeds (Padula and Hołubowicz*,* 2018).

The inflorescence is a spherical umbel 3–7 cm in diameter, on a long, erect, terete and hollow scape. The flowering starts from the top of the head and follows towards the bottom (Korohoda, 1974; Małachowski, 1982). The fruit is a globular capsule with a 5 mm diameter and once mature it easily opens. The optimal moment for seed harvest is when 5-10% capsules are open, and seeds are black and hard. They are also shield-shaped, with irregular dense wrinklies and sized 3-4 mm x 2,5 mm. Welsh onion seeds are twice smaller than the onion ones (Korohoda, 1974; Kotlińska and Kojima, 2000) with a 1,000 seeds weight between 2.4-3.4 g (Tendaj and Mysiak, 2007). As in other Alliums, the seeds of *A. fistulosum* are also short-lived and their vitality tends to be lost easily during storage

The species is commonly classified into 3 main groups based on the days needed to vernalize (20°C day – 7°C night): **Kaga** Cv which requires more than 60 days to vernalize and grown in the coolest parts of Japan; **Kincho** Cv requires between 50 to

60 days to vernalize; **Kujo** Cv mainly grown in the warmest parts of Japan and mainly for its leaves and vernalizes in 40 days (Yamasaki *et al*., 2000; Tsukazaki *et al.,* 2010).

In Europe, the species is classified in 2 main cultivar groups. single-stemmed cultivars grown in cool climates for their thickened blanched pseudo-stems, and Welsh onion group (subsp. *caespitosum*) multi-stemmed cultivars grown in warm climates for their green leaves (Singh and Ramakrishna*,* 2017)

In Asia, today, more common is to divide Welsh onion cultivars into 3 types based on their length of the pseudo-stem: 1. **Long-white type** (figure 1. a.) - white part over 40 cm. The pseudo-stem length thickness ratio is bigger than 10. This type has high yield, light pungent and sweet taste, high water content, but low resistance to long storage on the shelf. 2. **Short-white type** (figure 1. b.), recognized by thick and a short pseudo-stem with a length to thickness ratio to be below 10. The cultivars of this type are resistant to wind lodging and do not require deep soil cultivation. They give high pseudo-stem yield and can be stored on the shelf longer than the plants from the first type. 3. **Drumstick type** (figure 1. c.), its pseudo-stem length is similar to that of the short-white type, but the base of the pseudo-stem is significantly enlarged (wider) and the upper part is obviously thinner. The fully developed pseudo-stem is of the inverted drumstick shape or garlic shape. The pseudo-stem yield of this type is lower, but the flavour is stronger than in the other two types. Moreover, plants from this type have shown long marketable shelf life (Chen *et al*., 2001; Ma *et al.,* 2009).

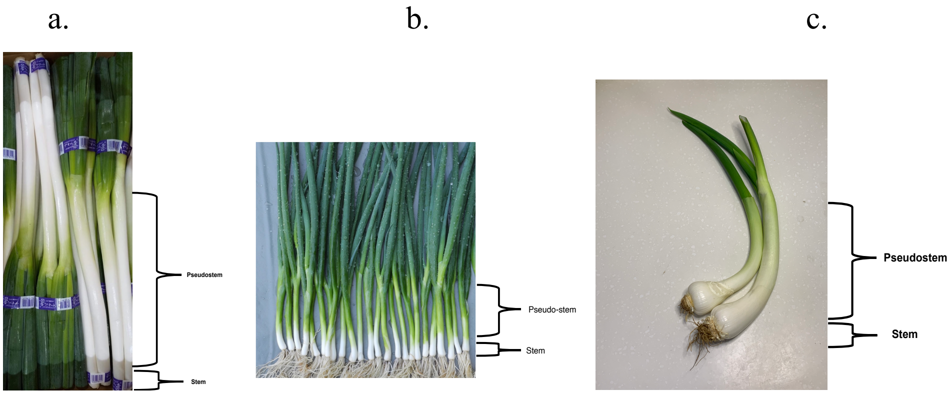

Figure 1. a) Long white type Welsh onion, b) Short white type Welsh onion, c) Drumstick type Welsh onion.

2. Vegetative development and flowering physiology

Welsh onion is propagated through seeds. It has wide adaptability to its growing environment, but in order to achieve high yield and quality, suitable conditions are needed. The plants can survive heavy frosts from −45 to −25°C. The average daily air temperature for effective growth ranges from 7 ◦C to 30 ◦C, and the most suitable daily average air temperature for growth is 13–25 ◦C (Su *et al.,* 2006; Ma *et al.,* 2009; Dong, 2016). The optimum temperatures for different development stages are: for seed germination, 13–20 ◦C; for whole plant dry matter accumulation, 19–25 ◦C; for leaf sheath dry matter accumulation, 13–19 ◦C (higher temperature at this stage will cause decreasing pseudo-stem and leaf quality); for vernalization, 2–7 ◦C; and for reproductive growth, 15–22 ◦C (Ma *et al.,* 2009). This corresponds to the temperature of late spring and mid-autumn in the main growing regions where bunching onion is produced.

The response of Welsh onion development to daylength is neutral. If the light is too strong, the leaves will be aged, which then will affect their edible value. As long as the crop passes through vernalization, no matter how long the daylength is, the plant can still bolt and then flower normally. When special photoselective blue nets were used, they increased the product quality, yield and plant metabolism performance (Gao *et al.,* 2021).

A proper fertilizing with nitrogen, phosphorus and potassium is beneficial to increase the yield and fertilizer use efficiency of the Welsh onion. Among them, nitrogen has the greatest effect on the yield, followed by potassium and phosphorus (Shi *et al.,* 2015). The interaction of nitrogen and sulphur is beneficial to promote the dry matter accumulation and improve the quality of Welsh onion. Among them, nitrogen has the biggest effect and sulphur is beneficial to the accumulation of flavour substances (Kong *et al*., 2013; Majkowska-Gadomska *et al.,* 2016).

The ultrastructure of the typical epicuticular wax layers on leaves is affected by high temperatures, in summer the “Kaga” and “Senju” Cv groups grow well form dendrite type wax, whereas those of “Kujyo” Cv group, which do not grow well at high temperature, form cocoon type wax. While in autumn, most of the wax formed on leaves is the dendrite type, and the difference between groups becomes unclear.

In relation to tillering, each sprout develops from a lateral bud formed in the leaf axil, and the number of tillers produced depends on the genotype and environment. The first tiller develops mostly at the eventh node, regardless of total number of tillers produced (Haruhisa and Tadashi*,* 1990).

Flowering is induced by temperatures below 13°C, when seedlings have at least 11 to 12 leaves, or with a pseudo-stem with a diameter more than 5 to 7 cm, although the temperature and period needed for vernalization is affected also by the cultivar itself (Haruhisa and Tadashi*,* 1990).

Plants are not vernalized when the growing conditions are over 20°C, either under long day or short day, while if grown between 13 or 18°C, however, bolt only in case of short days. So, not only low temperature but also short photoperiods promote floral initiation in the *Allium fistulosum* (Haruhisa and Tadashi*,* 1990).

3. Agronomical practices.

The agrotechniques applied by the farmers depend on the production area or market use of the fresh produce, and this may be blanched pseudo-stems or green salad onion.

- *Allium fistulosum* grown as blanched pseudo-stems

Depending on the cultivars, seeds can be sown in the nursery in early spring for summer and autumn harvest crops, or in autumn for overwintering crops and in this case, cultivars mostly used are characterized by a bolting tolerant trait. Usually, the seed amount used per hectare is variable from 2 to 4 kilograms, but also direct sowing is possible, and the quantity used is much higher from 8 to 16 kg/ha (Haruhisa and Tadashi*,* 1990).

Ideal soils for transplanting are sandy containing high organic matter and well aerated, seedlings are placed in furrows deep 15 cm, consequently the base of the plant is lightly covered by soil. The planting distance between each row is between 55 to 85 cm and on the row from 5 to 15 cm (Haruhisa and Tadashi*,* 1990).

In terms of fertilization, bunching onion prefers ammonium nitrogen to nitrate nitrogen, and in average 200 to 300 kg N are commonly used per hectare. While regarding other nutrients, 100 to 200 kg of P and 150 to 200 kg of K are applied per hectare (Haruhisa and Tadashi*,* 1990).

During the vegetative growth, the base of the plants is covered with soil up to at least 30 cm. This is done gradually in 3 or 4 stages, the first time 50 days after transplanting and the last time 40 to 20 days before harvesting.

At maturation, plants are usually lifted by hand, trimmed and packed into bundles and then placed in boxes. Harvesting by hand is very laborious, in fact it might need 3,000 to 5,000 hours per hectare, therefore mechanized lifters have been developed and nowadays more commonly used, reducing the harvesting time down to 200 to 1,000 hours per hectar (Haruhisa and Tadashi*.,* 1990). An experiment in South Korea for evaluating harvesting performance was performed for the developed Welsh onion. The harvest performance was evaluated at the tractor running speeds of 5.0 cm/s, 11.4 cm/s and 15.8 cm/s, by comparing the operating efficiency, harvest rate, and damage rate of the Welsh onion harvester.

The performance of the harvester was rated as very good, with a 100% harvest rate, regardless of tractor running speed, and the damage rate of harvested Welsh onions seems to increase proportionately from 4.55% to 6.53% and to 11.29% (Hong *et al.,* 2014).

- *Allium fistulosum* grown as green salad onion

The international market also requires a product that is commonly named salad onion (or green onion), with more tender and sweeter pseudo-stems, long 9-11 cm, compared to blanched pseudo-stem type, commonly known as "Long Negi" and very similar to leek.

In Europe, Germany is the country with the largest surface grown of this crop, it is estimated for 1,300-1,400 hectares. However, an important part of the European fresh salad onion production is also located in North Africa, in the countries such as Egypt, with a growing surface of 4,000 hectares, Senegal with 1,200-1,300 hectares and Morocco with 350-400 hectares (Padula and Hołubowicz, 2018).

Green salad onion is not only produced with *Allium fistulosum* but also through an interspecific hybrid obtained between *Allium fistulosum* and *Allium cepa*. This interspecific cross differs for being faster growing, more vigorous and for a slight bulb formation than the two species. Moreover, its leaves are less upright, more similar to *Allium cepa* crops, with a lighter green colour, and also more sensible to downy mildew infestation.

In the USA and Mexico, where the production of green salad onion is also important, the interspecific type is more commonly used instead of *Allium fistulosum* (Sanders, 2001). This is due to the high air temperature that can affect the growing area, therefore producers require faster growing and vigorous cultivars, which are planted in the spring, summer, and fall resulting in nearly a year-round harvest (Smith *et al.,* 2011).

The cultivation is done by direct sowing using from 1,8 to 2 million seeds per hectare, in double rows on beds large 1.,8 to 2 meters, allowing a high plant density of 200 plants per sqm (figure 2). However, growing Welsh onion from transplants, it has been proved to obtain earlier yields. A research conducted in Poland showed that onion grown from seeds ripened four weeks later as compared to the onion grown from sets, and two weeks later than the onion grown from transplants (Gruszecki and Tendaj, 2001; Tendaj and Mysiak, 2011).

Green onion development requires frequent and uniform irrigation. It is done with typically overhead sprinklers. Drip irrigation is not common in green onion production because of the close spacing between the rows. Mild water stress can reduce yield or cause uneven growth patterns in the field. The amount and frequency of irrigation depends on the soil type, weather conditions, and development stage of the crop. The demand for water increases as the plants increase in size.

The crop can be cultivated on all types of soil, however, to facilitate the harvesting stage light to medium-heavy soils are preferred in practice. Although on light soils, water and nutrients supply is more difficult, which deficiencies is a result with yellow leaf tips.

Balanced and continuous water and fertilizer supply is very important for a successful green salad onion fresh production. Fertilization should be based on soil and irrigation water analyses. It is also important to ensure the supply of trace nutrients. A basic fertilization 2 or 3 weeks prior sowing consists in approximately 100 to 120 kg/ha P_2O_5 and 150 to 170 kg/ha K_2O. Regarding nitrogen, the minimum amount is 200 kg N/ha.

High pH levels in the soil can lead to manganese deficiency, which can be remedied by adding manganese chelates during the irrigation. As an alternative, it is possible to spray with manganese sulphate as foliar fertilizer.

Weeds, insects, and diseases can impact commercially grown green onions. Herbicides are used before the onion plants have emerged to kill the faster growing weeds. Other herbicides that target weeds specifically are available that can be applied after the onion plants have emerged. Insects that impact green onion crops include thrips (*Thrips tabaci),* onion fly (*Delia antiqua)*, bulb mites (*Rhizoglyphus spp*.), allium leaf miner (*Phytomyza gymnostoma)* and small mottled willow moth *(Spodoptera exigua).* Soil and foliar pesticides are available that help combat infestations of some of these pests. To avoid pest infestation, growers utilize management practices, such as allowing organic material to completely decompose before planting, that will help reduce the probability of certain pests from becoming established.

There are several bacterial and fungal diseases that can infect green onion crops as well. To avoid severe outbreaks, most commercial growers follow guidelines for sanitation, crop rotation, use of resistant cultivars, and frequent monitoring. Green onions are mostly harvested by hand. The main common method involves undercutting the onions, pulling them up immediately, and gathering them into bunches of ten, which are then tied together with rubber bands. However, harvesting in this way is manpower intensive and expensive, and this is the consequence of a fresh production delocalization in area with lower labour cost. It is a recent practice also the mechanical harvest, which is possible through use of modified leek harvesting machines.

Figure 2. fresh Salad onion production in Germany Pfalz region.

4. Cultivars selection

Its criteria are largely defined by the objective of reducing agronomical costs. For this reason, since harvesting of pseudo-stems is still not a fully mechanised operation and in some case carried out totally by hand, the easy leaf cleaning is a fundamental characteristic required by the producer with the aim to increase plants harvested per unit time. It consists in removing the first and second external leaf directly in the field, before transporting the fresh salad onion to the packing factory.

Mechanical harvesting can be a valid alternative to reduce this cost, however it is important to reduce the product waste cuased by leaf damages during this stage, therefore the selection hybrid cultivars more adaptable fot this operation is highly reccomended.

Plant uniformity is also an important trait that allows a better field management reducing waste and consequently growing cost, as disease resistance against downy mildew (*Peronospora destructor* Caspary), grey leaf blight (*Botrytis squamosa* J.C. Walker) and rust (*Puccinia allii* G. Winter) (Robak and Szwejda, 2008; Tsukazaki *et al.,* 2010; Wako *et al.,* 2016).

When it comes to market needs, the main characters evaluated for cultivar choice are related to the leaf and pseudo-stem quality. Dark green leaves are well requested by consumers, while the pseudo-stem should be white, with a limited green part, uniform, with a diameter between 8 and 12 mm, and no bulbs formation (or very limited) on its bottom (Table 1 and Figure 3).

Table 1. The main commercial cultivar criteria for the Welsh onion (Korohoda*,* 1974; Borna, 1982; Tendaj, 2003; Wako *et al*., 2016; Padula and Hołubowicz*,* 2018)

Agronomical traits	Market traits
Cleanability	Leaf colour
Uniformity	Shaft length
Disease resistance	Shaft diameter
Plant growth habit	No bulbs
Earliness	

Figure 3. Field evaluation of different Salad onion cultivars in Germany Pfalz region.

5. Nutritional value

Welsh onion is a vegetable which can be eaten fresh or cooked. It is a common ingredient in soups, steamed-boils, stir-fries, salads and several other typical cookeries. It is also used in traditional Chinese medicine thanks to its natural chemical compounds which benefit human health. It has been proven to lower the possibility of bacterial infections, occurrences of tumors, high blood pressure, obesity and oxidation. It has a positive effect on the cardiovascular system, as a antiplatelet aggregation, intestinal spasm relief and immune function regulation (Sung *et al.,* 2018; Hirayama *et al*., 2019).

Moreover, the root exudates in soil root-zone have anti-termite, anti-fungal and anti-microbial activities.

The nutritional composition of the pseudo-stems and green leaf blades differs. The green leaf types contain higher levels of β-carotene, vitamin B1 (thiamine), vitamin B2 (riboflavin) and vitamin C (ascorbic acid). The raw green leaves are reported to contain water 90.5 g, energy 34 kcal, protein 1.9 g, fat 0.4 g, carbohydrate 6.5 g, Ca 18 mg, Mg 23 mg, P 49 mg, Fe 1.2 mg, Zn 0.52 mg, vitamin-A 1160 IU, thiamine 0.05 mg, riboflavin 0.09 mg, niacin (vitamin B3) 0.40 mg, folic acid (vitamin B9) 16 µg and ascorbic acid 27 mg in each 100 g edible portions.

Welsh onion leaves contain high levels of quercetin. It is a flavonol compound with potential benefit to human health. It reduces the risk of cardiovascular disease and acts as anti- cancer due to its antiprostanoid and anti-inflammatory responses (Kołota *et al.,* 2012; Singh and Ramakrishna*,* 2017).

Quality and nutritional value of bunching onion is also affected by the growing season and age of plants (Cha *et al.,* 2008). The results of a study conducted in Poland evidenced that the delay of harvest from 60 to 120 days after planting resulted in a depletion of vitamin C, carotenoids, chloophyll a+b, sugars, volatile oils, nitrates, total N, K and Ca content (Kolota *et al.,* 2012).

6. Seeds production

The basic objective of a seed production programme is to supply quality planting material at the right time, and at affordable prices. Low seed quality can potentially decrease the rate of germination and seedling emergence leading to poor stand establishment in the field and consequently yield loss (Kumar *et al.* 2023).

Allium fistulosum belongs to a green plant vernalization type. It means that specific seedling characteristics and sufficient accumulated time at low temperature are indispensable for the completion of its vernalization process. Only, if these conditions for vernalization are fulfilled, the plant will complete the flower bud formation and bolt (Dong *et al.,* 2013). Therefore, seed production of this crop is limited to certain areas that can guarantee a temperature range between 3-10°C, which includes countries located in the temperate zone.

Seed production technique consists in a direct sowing in the beginning of summer; however, it is more common to transplant seedlings. This is realized at the end of the summer or beginning of autumn, and the nursery sowing is realized in spring of the same year.

The field plant density is therefore variable, in case of direct sowing, it can reach more than 400,000 plants/ha, while through transplanting its much lower with 250,000 to 280,000 plants/ha. Despite the lower density, a transplanted field has more vigorous plants with a higher bolting percentage. The production of hybrid cultivars involves an appropriate transplanting ratio between female and male parental lines. Taking into consideration that the breeding technology enabled the obtaining of cytoplasmic male sterility, the most common ratios nowadays adopted are 3 rows of female parental line and 1 row of male parental line (field transplanting scheme 3:1 or 6:2), or 2 rows of female parental line and 1 row of parental male line (field transplanting scheme 2:1 or 4:2).

Flowering is earlier compared to other species of the onion-like crops. In the northern hemisphere it takes place in April of the year following the sowing or transplanting. Seed setting and maturation are characterized by a scalar trend, consequently harvesting is made through more than one step and for this reason it is mainly realized by hand, though the introduction of new mechanical harvesting technique is becoming more common, allowing a lower labour cost. Harvesting consists in collecting the umbels and consequently placing them in specific air dryers with the aim to reduce the seed moisture level down to 7-9%. This phase, usually has a duration of 10 to 15 days, depending on the air moisture. After this phase seeds are ready to be threshed.

7. Seed quality

It refers to the ability of a seed to germinate and includes factors such as germination capacity, viability, vigour and aspects related to dormancy.

Germination capacity is identified in a laboratory test, which is the emergency and development from the seed embryo of essential structures, indicating the ability to develop into a normal plant, under favourable condition in soil (ISTA, 1985; Milivojević *et al.,* 2018).

A seed with a good germination will ensure establishment of an optimum plant stand for a desirable production.

Beside the seeds germination, an important aspect is their vigour, which permits a rapid seed emergency under a wide range of growing conditions.

Germination capacity indicates seed ability to establish seedlings in good field conditions, and vigour is the ability to do so under stressed conditions.

Good seed germination and vigour are considered as key factors for successful yield, granting higher productions per unit area (Hasanuzzaman,
2015)

8. Seed physiology and deterioration process

Welsh onion seeds, as also other onion-like species, e.g. onion, leek and chive, do not go through a dormancy period after harvesting. This is an important seed physiological character as, if there is high air humidity or rain during seed maturation, the seeds may prematurely germinate (sprout) on the umbel. As all other crops of *Allium* species, they exhibit an orthodox storage behaviour, i.e., they store best at low seed moisture contents, low air temperatures and at low relative humidity of the air (George, 1985; Bewley and Black, 1994; Hołubowicz, 2016).

Seeds can be stored for a considerable time under certain temperatures and dry conditions but might lose their germination capacity due to accumulating damage, and there are large variations in shelf life between seeds of different species (Bezrukov and

Lazarenko, 2002). *Allium* seeds lose viability and vigour relatively faster than seeds of most other crops, even if stored under optimum conditions (Lazarenko and Bezrukov, 2008). Poor seed performance is one of the crucial factors that limits Welsh onion production and development, therefore, to reduce the rate of ageing and prolong shelf life of the seeds, it is recommended to store the seeds dry and cool.

Seed ageing is a natural phenomenon, which is the sum of a range of biochemical processes that finally lead to death. The decline in viability of naturally aged seeds are related to the oxidation of macromolecules such as proteins, membranes, DNA, mRNA and lipids. Increased levels of lipid peroxidation decreased levels of antioxidants, and reduced activity of several enzymes involved in hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) accumulation are the result of long-time storage and are directly linked to the seed vigour and germination capacity (Lee *et al.,* 1995; Dong *et al*., 2014; Prokopiev *et al.,* 2014)

Seeds are equipped with oxygen radical detoxifying enzymes, such as dehydrogenase, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) to survive under stressful conditions. Thus, increased antioxidative activities, along with other mechanisms, may contribute to the decreased susceptibility to deterioration of primed seeds (Goel *et al*., 2003; Amjad and Anjum, 2007).

9. Seed storage

Seed storage preserves seed viability and vigour for various periods by reducing the rate of seed deterioration (Groot, 2014; Gebeyehu, 2020). Seed storage is essential for preserving high seed quality, vigour, and viability for future use by farmers or breeders.

Longer seed storage is achieved by preserving the seeds in modified and favourable climatic conditions and an ideal environment. It is necessary to prevent deterioration by providing suitable storage conditions. Low temperature, low seed and air moisture and reduced oxygen activity are the most effective means of maintaining seed quality in storage (Groot, 2014).

Several factors contribute to longer seed storage life. The storage potential of an individual seed is in fact influenced by:

- genotype: some plant species would survive a given set of storage conditions longer than others (Sundareswaran *et al.,* 2023),
- initial seed quality: lots with low vigour and viability tend to deteriorate more rapidly (Dadlani *et al.,* 2023),
- harvesting stage: protection mechanisms are mainly built during the late seed maturation phase; the stage of harvest becomes a critical factor for seed quality and storability (De Vitis *et al.,* 2020; Sripathy *et al*., 2023),

- plant hormones: abscisic acid, auxin and gibberellic acid are reported to be involved in acquisition of seed longevity (Ranganathan *et al.,* 2023),
- preharvest effects: factors during seed development such as temperature, photoperiod, mineral nutrition, and rainfall have an effect on seed storability (Sripathy *et al.,* 2023),
- mechanical damage: seed injury can occur during seed threshing and harvesting,
- and seed moisture content: this is with no doubt the most influential factor affecting seed longevity (De Vitis *et al.,* 2020; Gebeyehu, 2020)

CHAPTER III. Hypothesis and research objectives

Hypothesis

Welsh onion (*Allium fistulosum* L.) seeds with moisture content below 10% can be stored under controlled temperature and air-humidity for 30 month without a quality loss.

Objective

The objective of the research was to determine how germination, seed vigour, fungi development and the accumulation of ROS in Welsh onion seeds change under the following air temperature (T) and RH conditions:

- 7-8°C, 25% RH,
- 7-8°C, 45% RH,
- 10°C, 25% RH,
- 10°C, 45% RH,
- 25°C, 25% RH,
- 25°C, 45% RH,

During the storing phase seeds are subject to natural deterioration making them unsuitable for commercialization, if not stored properly, and this is considered one of the most critical topics in the seed business, especially for onion-like crops which are characterized by one of the shortest shelf life among orthodox seeds.

From this research it was expected the results could provide valuable guidelines on the optimal temperature and air humidity storing conditions for Welsh onion seeds.

CHAPTER IV. Materials and methods

1. Plant material selection

The *Allium fistulosum* seed lots used in this study are listed in **Table 2**. They have been selected taking into consideration two main factors: the breeding background and the reproduction site. The first aspect included different genotypes identified on the seed market as cultivars, hybrids and open pollinated, or inbred breeding lines. The second one was based on seed production location. All the seeds used in the experiments were collected from locations considered in the seed world as optimal for Welsh onion seed multiplication (Hołubowicz, 2016).

Table 2. List and specific features of *A. fistulosum* seed lots used in this study. OP, open pollinated. MS, male sterile. F_1, hybrid cultivar

Seed lot	Seed production site	Breeding site	Genetic background
271227 (BO01a)	Italy	Japan	OP
251051(BO02a)	Chile	Japan	
251533 (BO03a)	Italy	Japan	
250609 (BO04a)	Chile	Japan	
270446 (BO05a)	Chile	Japan	F_1
270341 (BO06a)	Italy	Japan	
261286 (BO07a)	Italy	Japan	
270322 (BO08a)	Chile	Japan	
17TSITGH03 (BO09a)	Italy	Japan	Inbred line (MS)
18STC35 (BO10a)	Chile	Japan	F_1
1240694 (BO11a)	South Africa	Netherlands	OP
170403214(BO12a)	South Africa	Korea	
1240695 (BO13a)	Italy	Netherlands	

2. Description of the selected genotypes

Genotypes 271227, 251051, 250609 and 251533 are open pollinated (OP) cultivars developed by a Japanese breeding company with seed production located in Italy and Chile. Seed production for the two OP cultivars 1240694 and 1240695, originated by a Dutch breeding company, was localized in Italy and South Africa. The seeds of the only genotype bred by a South Korean company were produced in South Africa. All hybrid genotypes have been sourced through Japanese breeding companies and seed production was carried out in Italy and Chile. The 17TSITGH03 seed lot was derived from a parental inbred male sterile (MS) line, used to obtain an hybrid cultivar and produced in Italy under greenhouse conditions. Once reached the final stage of maturity, the seeds of all *A. fistulosum* genotypes were hand-harvested and subjected to artificial drying to reduce seed moisture content at a range of 7-8% on fresh weight basis. The samples showed high germination percentage (> 90%), as confirmed by preliminary germination tests.

3. Seed storing conditions.

Seeds of the genotypes were placed into natural textile sacks, similar to ones used today in standard seed storing conditions. Then, they were stored under 6 different conditions, as shown in **Table 3,** in 6 high-tech Italian designed and built laboratory climatic chambers of the type FDM CB-CS series. Their range of controlled conditions covers from -25°C to + 70°C and an air RH - from 10 to 98% (photo 1). Their electric characteristic (according to the Italian producer) includes the "+++" ikon, i.e., the third (highest) energy saving category. The chamber has been recommended for providing strictly controlled climatic conditions for storing any plant and human live tissues material. The seeds were stored for 30 months under controlled both air temperature and RH. The seed storage parameters kept for each chamber were as follows: 25°C with 25% air RH (chamber 1) and 45% air RH (chamber 2); 10°C with 25% air RH (chamber 3) and 45% air RH (chamber 4); and below 10°C with 25% RH (chamber 5) and 45% air RH (chamber 6).

Table 3. List of storage conditions applied to *A. fistulosum* seed lots used in this study.

Condition	Temperature	Relative Humidity (RH)
A	25 °C	25%
B	25°C	45%
C	10°C	25%
D	10°C	45%
E	7.5 °C	25%
F	7.5 °C	45%

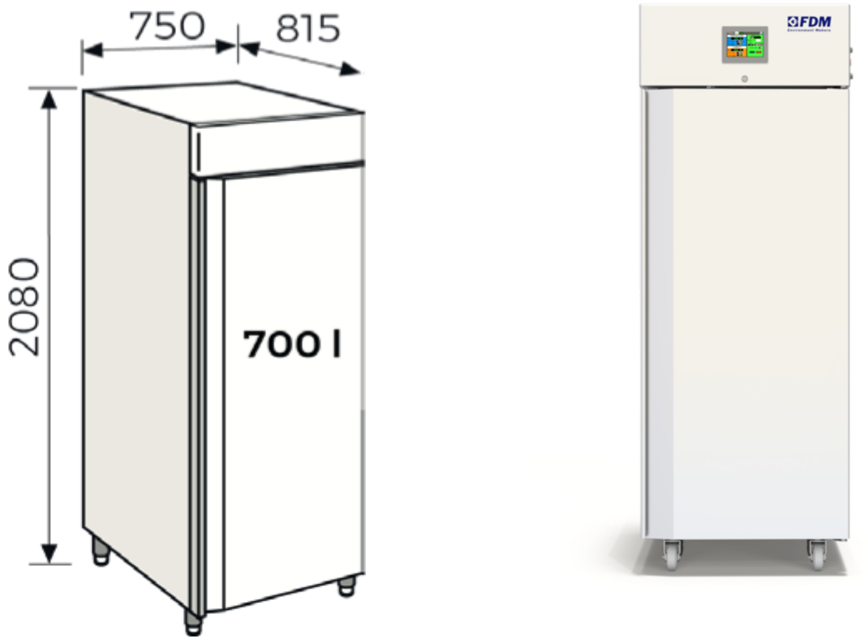

Figure 4. One of the 6 climatic chambers used in the experiments. (source: offered by FDM F.lli Della Marca S.r.l.)

4. Seed germination test

After 10 and 30 months of storage in the chambers, 2 tests of the seed germination were done. They followed the rules for germination test of the International Seed Testing Association (ISTA) (ISTA, 2002-2018). The seeds were previously pre-chilled at 5°C for 4 days. Then, 4 replicates of 50 seeds (each placed in one 9 cm Petri dish) were placed on 6 layers of blotting paper and tested at 20°C with 16 hours light and 8 hours dark phases. The first and the final counts were done after 6 and 12 days, respectively.

(a) (b)

Figure 5. Results of germination test carried out on Welsh onion seeds according to ISTA rules.
a) germination tray, b) Welsh onion seedlings

5. Mycological analysis of the seeds

It started after 1 year and was repeated after 2 years of storage of seeds at temperature 7-8ºC at 25% RH and at 25ºC. It was done only for one genotype: 17TSITGH03. The seeds of this line were produced in the greenhouse with complete insect isolation and optimal conditions for their high health status. For mycological analysis, 400 seeds (4 replications of 100 seeds) were placed on the potato dextrose agar (PDA) medium in 9 cm diameter Petri dishes, 10 seeds per dish, and then incubated at 20°C for 10 days under 12 hours alternating cycle of NUV light and darkness. Streptomycine sulfate at 100 ppm concentration was added to the media to prevent growth of bacteria (Tylkowska and Dorna*,* 1996). Determination of fungi was based on the appearance of their colonies and sporulation (Watanabe, 2002, Mathur and Kongsdal*,* 2003). Additionally, the percentage of the seeds free from fungi was determined.

6. DCFH-DA Assay

The fluorogenic dye 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Milan, Italy) was used to measure ROS levels in seeds. The assay was carried out as described by Pagano and co-workers (Kurek *et al.,* 2019) with the following modifications. About 20 seeds (100 mg) were used for each sample, seed lot, and tested condition. Seed samples were incubated for 15 min. with 100 ml of 10 mM DCFH-DA. Subsequently, the fluorescence sensor (at 517 nm) of the Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia) was used to read the emitted fluorescence, setting the programme for one cycle of 30 s at 25°C. A sample containing only DCFH-DA was used as a negative technical control to subtract the baseline fluorescence. Data was expressed as relative fluorescence units (RFU). All measurements were performed in four technical and biological replicates for each sample/condition.

7. Statistical data evaluation related to germination and fungi test.

For the received data, the variance was calculated. The significant differences were calculated based on the Duncan's test. Means followed by the same letters were not significantly different for $\alpha = 0.05$.

8. Statistical data evaluation related to reactive oxygen species test

Correlation analyses were carried out using ROS measurements (RFU) and germination percentage (%) data. The analyses were performed using the R package (version 3.5.2) (Assaad *et al.,* 2014) available online at https://gentilinidavide.shinyapps.io/correlation/ (URL accessed on 20th December, 2023). The Pearsons r value and significance p values are given for the combination of factors (ROS vs. germination). Moreover, graphical representations of correlation and Principal Component Analyses (PCA) were performed using the OriginPro, Version 2023b, OriginLab Corporation, Northampton, MA, USA. Graphical representation of the different results was produced using Excel graph tools, namely the Combo design to be able to represent all variables in a single graph that includes multiple axes. Germination and ROS data have been analyzed by one-way ANOVA and Tukey–Kramer test using the online software developed by Assaad and co-workers (2014) available at https://houssein-assaad. Shinyapps.io/TableReport (URL accessed on 20th December, 2023).

CHAPTER V. Summary of attached publications

1. Publication nr. 1

Gregorio PADULA, Xianzong XIA, Roman HOŁUBOWICZ. 2022.
Welsh Onion (*Allium fistulosum L.)* Seed Physiology, Breeding, Production and Trade.
DOI: 10.3390/plants11030343

This paper had the purpose to analyze and explore basic information on the origin of *Allium fistulosum* species L., its botanical characteristics, the main breeding directions and achievements, seed production methods and areas, main plant breeding and seed production companies, seed dormancy and the best methods for seed storage, use of the crop and market responses to promotion activities.

Allium fistulosum L. is commonly known as Welsh onion, but also called stone leek, Chinese onion, Chinese spring onion or Japanese bunching onion. It has been domesticated in the neighbourhood of Lake Baikal in Siberia near the Altai Mountains in Russia and in Western or North-Western China (Borna, 1982; Singh and Ramakrishna, 2017).

Later on, in medieval times, the crop was brought to Europe from Russia (Kotlińska and Kojima, 2000). Based on different botanical classification criteria, the Welsh onion includes five botanical cultivars and three cultivar groups. The latter include Chinese, Japanese and Russian cultivars. They all differ in morphological and useful traits, such as number, size and colour of leaves, taste, earliness and overwintering potential.

Welsh onion is a biennial or perennial species of the genus *Allium* and the *Liliaceae* family. Its root system is string-shaped with few and short lateral roots. The stem is short and has a globose or oblate shape. It is surrounded by the base of the leaf sheath, and densely rooted in the lower part (Ma *et al.,* 2009). The young leaves are hidden in the leaf sheath and form a round rod-shaped pseudo-stem with the multilayer sheaths. The underground pseudo-stem is white whereas the above-soil part is yellow green (Chen *et al.,* 2001; Kotlińska *et al.,* 2005). The leaves are long, cylindrical, hollow, green or dark green, with a smooth and waxy surface. Each plant has 5–8 leaves arranged in a fan shape. Its umbels in the developmental stage are hidden in the membranous involucre. The flowers are small and white, bisexual, and insect-pollinated.

The flowers are located on an umbel of the head shape and the flowering starts from the top of the head and follows towards the bottom (Korohoda, 1974; Małachowski, 1982). The fruit, called a capsule, consists of three parts, each with two seeds, which are shield-shaped, with irregular dense wrinkles.

In Asia, it is common to divide Welsh onion cultivars into three types based on their pseudo-stem length (Chen *et al.,* 2001; Ma *et al.,* 2009):

1. Long white type (figure 1a) —white part over 40 cm. The pseudo-stem length-to- thickness ratio is bigger than 10
2. Short white type (figure 1b)—recognized by a thick and short pseudo-stem and with a length-to-thickness ratio smaller than 10.
3. Drumstick type (figure 1c)—the pseudo-stem length is similar to that of the short white type, but the base of the pseudos-tem is significantly enlarged (wider) and the upper part is obviously thinner.

Welsh onion seeds, like seeds of other onion-like species, e.g. onion, leek and chive, do not go through a dormancy period after their harvest. It is an important seed physiological character as, if there is high air humidity or rain during the growing period in the field, the seeds may prematurely germinate (sprout) on the umbel of the seed stalk. As all other crops from the onion-like species, they exhibit an orthodox storage behaviour, i.e. they store best at low seed moisture contents, low air temperatures and, if stored in open containers, at low relative humidity of the air (Selvi and Saraswathy, 2018). If such conditions are kept, they preserve viability for 6–12 months (George, 1985; Bewley and Black, 1994; Hołubowicz, 2016).

Welsh onion is propagated through seeds. It has wide adaptability to its growing environment, but in order to achieve high yield and quality, suitable conditions are needed. The optimum temperatures for different development stages are: for seed germination, 13–20◦C; for whole plant dry matter accumulation, 19–25 ◦C; for leaf sheath dry matter accumulation, 13–19◦C (higher temperature at this stage will cause decreasing pseudo-stem and leaf quality); for vernalization, 2–7 ◦C; and for reproductive growth, 15–22 ◦C (Ma *et al.* 2009).

To achieve high yields and good quality, the soil needs to be well-drained and rich in organic matter, with a pH of 7.0–7.4. Welsh onion has the same requirements for mineral elements as most leafy vegetables and is very sensitive to nitrogen nutrition. *Allium fistulosum* belongs to a green plant vernalization type. i.e. specific seedling characteristics and sufficient accumulated time at low temperature are indispensable for the completion of its vernalization process (Hołubowicz and Bralewski, 2008). Only, if these conditions for vernalization are fulfilled, the plant will complete the flower bud formation and bolt. For this reason, seed production of this crop is limited to certain areas that can guarantee a temperature range in the winter between 3–10 ◦C, which includes countries located in the temperate zone. The following countries meet such growing conditions and have good seed production tradition: Italy, France, Turkey, the US, Chile, Argentina, Australia, South Africa, Russia and China (Shishkina *et al.* 2019, Padula and Hołubowicz, 2018, Hołubowicz and Bralewski, 2008).

Plant breeding companies have nowadays focused their breeding programmes on traits more fitting to the actual growing market and producers needs. They include disease resistance, yield, late bolting, improved consumption qualities (e.g., low pungency or high sugar content), and suitability for mechanized harvesting. As a result, we have high-performing hybrid cultivars adaptable to different climatic conditions, growing areas and season, and with a higher market quality (Wako, 2016).

The breeding technologies to obtain Welsh onion hybrid cultivars are similar to procedures that are used for other onion-like crops. In general, cytoplasmic male sterility (CMS) is indeed an indispensable trait in hybrid (F_1) bunching onion breeding programmes.

China is the leading country in the world in Welsh onion production area with more than 500,000 ha, while South Korea and Japan each have a similar production area—around 25,000 ha. In the EU, Welsh onion is not a traditional species and has been adapted for a different use, the market requires a product that is commonly named salad onion (or green onion), with more tender and sweeter pseudo-stems, long (9–11 cm), and therefore with a different commercial production technology. It provides a much higher plant density, i.e. up to 2 million plants per hectares . Germany in Europe is the country with the biggest area grown with this crop, with an estimated 1,300 – 1,400 hectares.

In US and Mexico, the use of the Welsh onion is very similar to the European fresh product, although it is mostly based on an interspecific hybrid between *Allium fistulosum* and *Allium cepa*.

The cultivar selection criteria are largely defined by the objective of reducing agronomical costs and since harvesting of the pseudo-stems is still carried out by hand, the easy leaf cleaning is a fundamental characteristic required by the producer. Plant uniformity is also an important trait that allows a better field management reducing waste and consequently growing cost. Resistance against downy mildew, grey leaf blight and rust are in demand by the growers, even though the use of chemicals can overcome or limit the spread of the diseases (Robak and Szwejda, 2008).

2. Publication nr. 2.

Gregorio Padula, Xianzong Xia, Dorota Szopińska, Roman Hołubowicz. 2022.
Effect of air temperature and relative humidity on the stored Welsh onion (*Allium fistulosum* L.) seeds.
DOI: 10.15835/NBHA50312956

With this paper I wanted to highlight the changes during the storing of *Allium fistulosum* L. seeds quality parameters, such as germination, seed vigour and fungi development, under different storing air temperatures and relative humidity (RH).

All seed lots at the beginning of storage had high germination over 90%, and moisture content 7-8%. Then, they were stored for 30 months in controlled conditions at 3 different temparatures: 7-8 °C, 10 °C and 25 °C, each with 2 different air RH: 25% or 45%. During the experiment, seed quality was evaluated by germination test according to the ISTA protocol. Additionally, after 1 and 2 years of storing the seed lots were analyzed for their incidence of fungi. In addition, it has been assessed the electricity consumption for each of storing condition on the basis of the techincal details provided by the storing chambers manifacturer.

The carried out experiments showed that in terms of the seed quality, most of the lots used in the experiment maintained an high germination capacity after the storage, only one seed lot clearly suffered low germination figures after 30 months of storage compared to the previous ones. This observation only confirmed earlier findings about serious problems with keeping high quality of Welsh onion seeds on the commercial market (Yanping *et al*., 2000; Khan *et al.*, 2004; Li and Wang, 2005; Li and Wang, 2006; Lazarenko and Bezrukov, 2008; Padula *et al.*, 2022).

The received results of the germination capacities for most all of the samples showed that the seed germination capacities after storing them for 30 months at 7-8°C and at 10°C did not differ. While in relation to the RH after 30 months of storing, quality parameters showed to be higher when seeds were stored at the air RH 45% rather than at the air RH 25%. However, there were also in the results of my tests, cases with no effect of this factor on the seed germination of the seeds. My finding is in agreement with the earlier information that the optimal air RH when storing seeds of onion-like crops was 35% (Doijiode, 2001). Due to high market demand and price of the Welsh onion seeds (Padula and Hołubowicz, 2022; Padula *et al*., 2022), this problem, due to generated costs of storage, needs first further research, then followed by extension advice for plant breeders and seed traders.

The carried out calculations in the seed storage costs savings in these experiments evidenced that the chamber at 10°C used 11.2% less energy compared to the chamber with the storing temperature of 7-8 °C (Table 4). Although, there have been various stable and variable elements of the costs of string sowing material, the easiest way to prove savings caused by temperature of the air is to compare the cost of the used electric current.

Table 4. The comparison (%) of the electric current consumption (kWh) when storing Welsh onion seeds in the climatic chambers of the type FDM CB-CS series at 2 different temperatures: 7-8°C and 10ºC

Temperature for storage	Used energy (kWh)	%
Below 10°C	0.675	100.0
10ºC	0.600	88.8

Referring to the fungi test numerous saprotrophic and pathogenic fungi were identified on the stored Welsh onion seeds, however some of them infested relatively low percentage of seeds. Prolongation of storage, regardless of temperature, resulted in a significant increase in seed infestation with *Alternaria alternata*, *Aurobasidium pullulans*, *Cladosporium* spp. and *Penicillium* spp. Moreover, 2 years of storage at 25°C increased seed infestation with *Fusarium* spp. and *Trichothecium roseum*, while after storage at 7-8°C higher percentage of seeds infested with *Phoma* sp. was observed. Two years of storage, especially at 25ºC, significantly decreased the percentage of seeds infested with *Rhizopus* sp. in comparison with seeds stored for 1 year. Dorna and co-workers (2013) suspected that the reduction of the expansion of one of the microorganisms made possible the growth of the other one. The increase in seed infestation with storage fungi, especially *Penicillium* spp., may resulted in seed deterioration (Maude, 1996). These fungi can tolerate quite wide range of temperatures (Pitt, 1999). Nearly all species from genus *Penicillium* are capable of growing below 5°C, and some even at 0°C. Therefore, it is difficult to prevent spreading of these fungi during storage by temperature control. Moreover, it was found that some species from this genus could be highly pathogenic to the *Allium* spp.(Valdez *et al.*, 2009). On the other hand, at 7-8ºC, the growth of *Fusarium* spp. on the stored seeds was limited. This, in turn, may positively affect growth of the plants in the field, as some of these fungi are seed-transmitted and pathogenic to them (Köycü and Özer, 1997). According to discussed results, despite the used air temperature, seed infestation with both pathogenic and saprotrophic fungi may increase significantly during long term storage. Therefore, it is important to control health of the seeds being prepared for storage to avoid losses of seed viability, and to prevent transmission of seedborne pathogens.

3. Publication nr. 3.

Gregorio Padula, Anca Macovei, Adriano Ravasio, Andrea Pagano, Conrado Jr Dueñas, Xianzong Xia, Roman Hołubowicz, Alma Balestrazzi. 2024.
"Exploring Reactive Oxygen Species Accumulation in *Allium fistulosum* L. Seeds Exposed to Different Storage Conditions."
DOI: 10.3390/seeds3010010

The aim of this research was to investigate the response of Welsh onion seeds of various origins, subjected to different storage conditions, by combining germination with the seed ability to produce ROS. The well documented pivotal role of ROS, both as signaling molecules and toxic agents during early seed imbibition (Bailly *et al.,* 2008) makes them valuable targets to monitor the seed pre-germinative metabolism, from priming to storage (Pagano *et al.,* 2022; Pagano *et al.,* 2023).

In the present study, no seed deterioration was detected during the first year of storage; therefore, ROS analyses were started using seeds stored for 12 months, as "time zero". Then, the comparison was made with seeds stored for 22 months.

Germination of *A. fistulosum* seed lots stored for 12 Months.

The tested seed lots showed a high germination percentage (>90%), as confirmed by preliminary germination tests, carried out before the long-term storage (22 months). The analyses were started using seeds stored for 12 months once they had been fully equilibrated under the indicated RH.

Some of the tested cultivars with the lowest germination percentage showed higher levels of ROS accumulation. This was revealed mainly for the BO07 and BO12 seed lots under storage conditions A (25 °C, 25% RH), B (25 °C, 45% RH), C (10 °C, 25% RH), and D (10 °C, 45%) (Figure 2). Similar responses were also observed under storage conditions E (7.5 °C, 25% RH) and F (7.5 °C, 45% RH). Altogether, seed lots BO07 and BO12 were highly affected by all the different storage conditions taken under consideration.

Subsequently, correlation analyses were performed between the investigated parameters (Table 5). When considering the different cultivars, significant negative correlations were evidenced between ROS and seed germination, as a measure of viability, suggesting that ROS levels are influenced by all of the tested storage conditions.

Table 5. Correlation values grouping the different cultivars for each storage condition, analyzed after 12 months of storage. The Pearson r values are indicated alongside the *p*-values. The *p*-value < 0.05 indicates significant differences, evidenced in bold. Germ, germination percentage; ROS, reactive oxygen species.

Seed Lots	Comparisons	Pearson *r*	*p*-Value
BO01-14A	ROS vs. germ	−0.69	**0.006**
BO01-14B	ROS vs. germ	−0.99	**0.01**
BO01-14C	ROS vs. germ	−0.98	**0.001**
BO01-14D	ROS vs. germ	−0.95	**0.001**
BO01-14E	ROS vs. germ	−0.62	**0.017**
BO01-14F	ROS vs. germ	−0.67	**0.009**

Germination of *A. fistulosum* seed lots stored for 22 Months

A. fistulosum seed germination and ROS levels were then evaluated after 22 months of storage. Similar to the 12-month data, some of the samples characterized by a low germination percentage revealed increased ROS accumulation. Enhanced ROS accumulation was observed for the BO03 samples at all of the tested conditions. Similarly, this was also the case for sample BO14, except for condition F, where the highest values were registered from sample BO07. Specifically, this sample also showed ROS accumulation under storage conditions A (2*5* °C, *25*% RH*),* B (25 °C, 45% RH), C (10 °C, 25% RH)*,* E (7.5 °C, 25% RH) and F (7.5 °C, 45% RH). Similar profiles were observed for the BO12 sample, particularly under storage conditions A, B, C, D, and E. Thus, results of the analyses carried out after 22 months of storage showed that seed lot BO07 was affected by all the different storage conditions taken under consideration. No significant ($p < 0.05$) correlations were observed between germination and ROS levels (Table 6).

Table 6. Correlation values grouping the different cultivars for each storage condition, analyzed after 22 months of storage. The Pearson *r* values are indicated alongside the *p*-values. The *p*-value < 0.05 indicates significant differences, evidenced in bold. Germ, germination percentage; ROS, reactive oxygen species.

Seed lots	Comparisons	Pearson *r*	*p*-Value
BO01-14A	ROS vs. germ	−0.01	0.971
BO01-14B	ROS vs. germ	−0.2	0.487
BO01-14C	ROS vs. germ	−0.04	0.891
BO01-14D	ROS vs. germ	−0.2	0.498
BO01-14E	ROS vs. germ	−0.29	0.309
BO01-14F	ROS vs. germ	−0.25	0.381

To better understand the fluctuations in ROS levels and germination percentage along storage (22 months versus 12 months), data were represented as log2 of the ratio between the values recorded at 22 months and the values recorded at 12 months (figure 6). As for ROS levels, the log2 (ROS22/ROS12) revealed a general increase over time (figure 4a). When considering the different seeds lots, the highest log2 (ROS22/ROS12) values were recorded for BO13 (4.944, storage condition F), BO06 (4.716, storage condition F), and BO14 (4.657, storage condition D) (Figure 4a). Overall, seed lots BO05, BO11, and BO14 showed significantly higher log2 (ROS22/ROS12) in response to all the tested storage conditions. On the other hand, BO02 revealed a significant decrease in log2 (ROS22/ROS12) under storage conditions A, B, and C, as well as BO10 under storage condition A and E (figure 4a). When considering germination percentage, mild decreases occurred. The highest impact in terms of log2 (Germ22/Germ12) was recorded under storage condition A for seed lots BO07 (−1.322), BO12 (−0.585), and BO02 (−0.534) (figure 4b).

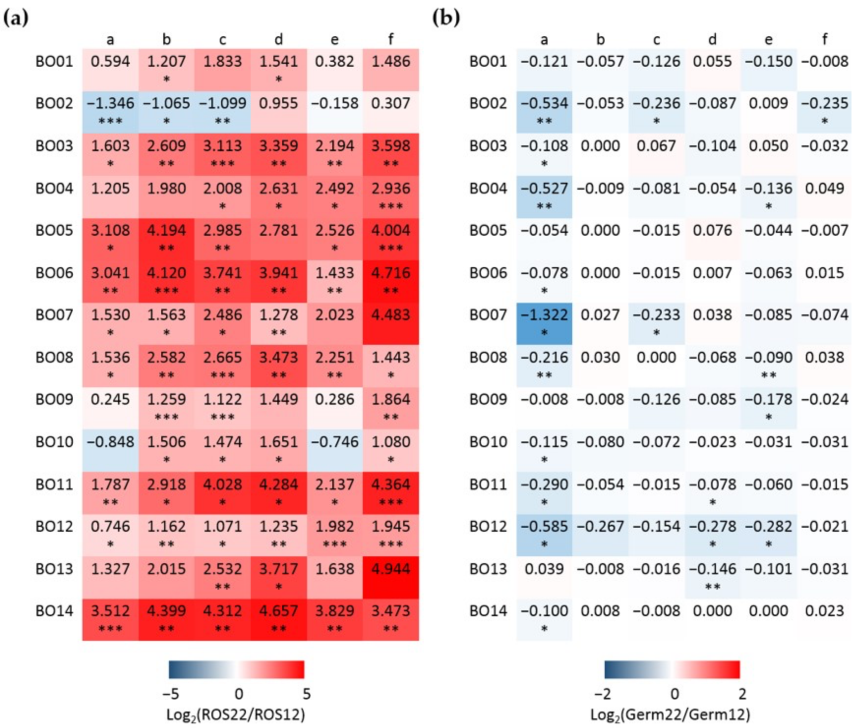

Figure 6. Changes in ROS accumulation levels (**a**) and germination percentage (**b**) observed between 12 and 22 months of storage. ROS12, ROS levels assessed at 12 months of storage. ROS22, ROS levels assessed after 22 months of storage. Germ12, germination percentage assessed at 12 months of storage. Germ22, germination percentage assessed after 22 months of storage. Values are expressed as $\log_2$ of the ratio between the values recorded after 22 months of storage and the values recorded after 12 months of storage. Storage conditions are described in Table 3. Asterisks indicate statistically significant changes as assessed by Students t-test comparing the values recorded at the two time points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

CHAPTER VI. Final remarks

The search for effective indicators of poor seed storability, detected through assays seems like a proper strategy, while at the same time, seed technologists should continue their work to improve the current storage protocols, by refining parameters such as temperature, seed moisture content, and gaseous compositions of the storage atmosphere. Seeds from onion and onion-like crops still represent a challenge for breeders and seed technologists, due to their storability.

However, the results obtained from the research in relation to the effect on seed quality parameters (germination and seed vigour), fungus development and the accumulation of reactive oxygen species can be used, for all genotypes belonging to *Allium fistulosum* species, as a guideline for the seed storage in climatic and RH controlled chambers.

Comparing the results obtained from the germination analyses it is possible to claim that the seeds of the tested genotypes preserved a good quality in the storing chambers with controlled temperatures set at 7.5 °C and at 10°C. While referring to the relative humidity (RH) range, the value that showed a minor negative impact on the seed quality was at RH 45%. In such storing conditions the germination for most of the genotypes never went under 85%, except for the lot 170403214 which quality parameters dropped in all storing conditions since the first control after 10 months

Taking into consideration the electricity costs after 30 months seed storage, it has been calculated that the chamber at 10°C showed a 11.2% lower consumpation compared to the chamber at 7.5°C. Therefor from the seed companies point of view, seed storage at 10°C is more recommended than at 7.5°C.

Generally, the temperature 7-8°C favoured growth of *Penicillium* spp. and *Phoma* sp. on Welsh onion seeds during 2 years of storage, while in seeds stored at 25 °C it increased seed infestation with *Cladosporium* spp. and *Fusarium* spp.

ROS accumulation in Welsh onion dry seeds can provide some interesting information about the way different seed lots respond to different storage conditions and genetic backgrounds. The cultivars with the lowest germination percentage showed a peak in ROS accumulation mostly after 12 months of storage, and their ROS levels were further enhanced after 22 months of storage.

Based on the reported data on ROS accumulation profiles along storage, seed lots BO05, BO06, BO11, BO13, and BO14 showed a significant increase in ROS levels in response to all the tested storage conditions whereas seed lots BO02 and BO10 revealed a significant decrease under certain storage conditions. On the other hand, seed lots BO05, BO06, BO11, BO13, and BO14 appeared to be prone to ROS accumulation, independent of storage conditions. Seed lots BO02 and BO10 were less prone to ROS production, and this was particularly evident for storage conditions A, B, C, and E (25 °C, 25% RH; 25 °C, 45% RH; 10 °C, 25% RH; 7.5 °C, 25% RH).

CHAPTER VII. Conclusions

1. It has been confirmed that the Welsh onion seeds were orthodox. They were able to be effectively stored at their seeds low moisture content, in low air temperature and low relative humidity (RH).
2. The best conditions for storing 14 selected genotypes of Welsh onion were: 7.5°C and 10°C air temperature, seed moisture content 7-8% and 45% air relative humidity (RH).
3. The results of the present study underline the strong heterogenicity of the seed germinability as a result of both environmental and genetic components.
4. The fungi development on the seeds showed different levels of infestation depending on the fungi species. At the temperature of 7-8 °C *Penicillium* spp. and *Phoma* sp. showed favoured conditions for its growth, while at 25 °C the increased seed infestation was related to *Cladosporium* spp. and *Fusarium* spp.
5. Well documented pivotal role of ROS makes the Welsh onion seeds a valuable targets to monitor the seed pre-germinative metabolism and the impact on its longevity. It has been proved a negative association between ROS and germination %, despite correlation analysis also showed that ROS production was influenced by genotype. However specific condition storages (25 °C, 25% RH; 25 °C, 45% RH; 10 °C, 25% RH; 7.5 °C, 25% RH) resulted in a lower impact on seed aging.
6. Electric current consumption is an important parameter to be considered when storing the seeds due to its cost impact. Therefor considering the calculation performed and seed germination at the end of the experiment, it can be recommended to consider storing conditions at the air temperature of 10 °C.

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