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# Biotechnological methods to increase and preserve agrobiodiversity in hop

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## Summary

In recent times, we are witnessing to a growing demand of craft beers with original flavours and to an increasing interest for the hop functional compounds. Together with the widening of hop growing area, also the research world is more and more focusing on hop, not only on its propagation, but also on its breeding; other than confering resistance to pests and diseases, breeding programs aim to identify hop genotypes with valuable aroma profiles for craft beer field, and rich in bioactives compounds, with anticabacterial and antiviral properties for herbal and pharmaceutics industry.

However, the process of hop domestication, cultivation and breeding has led to a decreasing of hop intraspecific agrobiodiversity, making these goals more challenging.

In this context, in order to overcome these limitations, it is necessary to valorise the existing hop biodiversity, but also to renew the varietal panorama. Even though the increasing interest in this species, very few are the research going on aiming at valorizing and improving hop, especially through biotechnologies.

Based on these considerations, the general objective of my PhD thesis project was to deep knowledge on hop, following two main research lines, agrobiodiversity valorisation and agrobiodiversity enrichment.

In the framework of **agrobiodiversity valorisation**, in this PhD thesis, the adaptability of International cultivars to the Italian climate was investigated, by comparing hop cone characteristics of cultivar Cascade, grown in different areas of Italy, with those grown in the main producing areas of the United States of America (Oregon and Michigan), Germany (Tettnang region) and Slovenia (Carniola region). Obtained results showed significant differences in bitter acids and xanthohumol content, among the analyzed samples, underlining how geography and climatic conditions may affect hop secondary metabolism.

Further to a recurrent demand for hop plants carrying peculiar features, the depletion of germplasm has been attracting the International attention. In this context, a biotechnological approach to propagate hop plants and to preserve their germplasm has been described. Specifically, organogenesis was induced from petioles of hop,

cultivar Gianni, confirming the possibility of using this kind of hop explants to obtain a great number of plantlets, in limited space and, independently of the season.

Moreover, to overcome the problem of somaclonal variation that may occur during regeneration and the issues connected to conventional germplasm storage methods, for the first time, in our knowledge, hop microcutting encapsulation technology was tested. The high conversion recorded proved the suitability of hop microcuttings to encapsulation and opened new insights to investigate other applications of this technology for hop germplasm propagation and storage.

In the context of **agrobiodiversity enrichment**, the influence of several pre-treatments (timing of chemical scarification, cold stratification, imbibition in water and in gibberellic solution) on *in vitro* hop (cv. Columbus) seed germination was evaluated. The study allowed to set up an efficient protocol for the obtainment of a high number of seedlings, in a relatively short time and out of the natural season, resorting to chemical scarification with sulphuric acid and to the use of gibberellic acid, both in the imbibition solution and in the culture medium. Moreover, by resorting to sex-linked molecular markers it was possible to precocious individuate the seedling gender.

All in the interest of enriching the variability within which will be possible to implement the selection of new genetic combinations, two studies have been carried out to induce new variability, investigating the gamatoclonal and the somaclonal variation. Gametoclonal variation offers the opportunity to discover the natural variability present in plant gametes and to exploit this genetic variability, in order to create new genetic combinations. Since in hop, there is a complete lack of information on gametic embryogenesis, a preliminary study on structure and biology of the hop male flower, microspore and pollen development has been carried out. This study has been carried out in collaboration with the Comav, Polytechnic University of Valencia. The obtained results provided a deep description of male flower, with a peculiar attention to development of microspore and pollen grains. Moreover, it was possible to establish a correlation between microspore/pollen developmental stages and flower bud/anther size, making easier and faster the selection of buds containing microspores at the most appropriate developmental stage in order to obtain androgenesis.

Since, some aspects of the *in vitro* culture environment can led to the occurrence of genetic and epigenetic change, indirect organogenesis was induced from leaves of hop cultivar Gianni, testing the effect of the type and the concentration of several growth regulators in the culture medium and the time in which the explants were maintained in culture on the insorgence of somaclonal variation. Plants obtained from leaves culture have been subjected to cytofluorimetric analysis and to molecular analysis which revealed that 16.8% of regenerants were mutated, some of which were tetraploids.

# FIRST SECTION

## 1. Agrobiodiversity conservation and valorization

Genetic diversity comprises the total genetic variation present in a population or species.

Agricultural biodiversity (often referred to as agrobiodiversity) includes all the components of biological diversity of relevance to food and agriculture, and those that constitute the agro-ecosystem: "the variety and variability of animals, plants and microorganisms at the genetic, species and ecosystem levels, which sustain the functions, structure and processes of the agro-ecosystem" (FAO/PAR, 2010). Agrobiodiversity is a key component of overall biodiversity: it is the result of a selection process operated by inventive developments of farmers, herders and fishers, over millennia; moreover, it plays a critical role in global food production and it is an essential component of any food system. The source of our food is represented by the productive agro-ecosystems, whose sustainability is dependent on the preservation, enrichment and utilization of agrobiodiversity (FAO/PAR, 2010). It is necessary that such use of plant genetic resources is coupled with conservation. Several are the reasons why genetic resources must be preserved. Plant germplasm can be exploited for food and medicine; moreover, it is necessary to keep in mind that plant breeding implies variability, thereby without genetic diversity it is not possible to obtain crop improvement. Although there are many strategies for conservation of biodiversity, the different preservation methods can be divided in two approaches: *in situ* and *ex situ*.

In contrast to *ex situ* conservation*, in situ* conservation allows the conservation of genetic resources within their natural or agricultural habitat, permitting, in this way, the evolutionary processes to take place and to give rise to genetic diversity and adaptability of species to changing environmental conditions. As defined in the Convention on Biological Diversity (1992), "*in situ* conservation means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed

their distinctive properties". Original surroundings are strictly related to species, influencing their quality and characteristics. Indeed, adding value to a particular variety or ecosystem with peculiar characteristics attributable to its geographical origin and different from neighboring ones, allow its maintenance.

*In situ* conservation comprises the conservation in protected areas and on-farm conservation. A protected area is defined as a geographically delimitated area, in which genetic diversity of wild populations is monitored on-farm conservation entails the sustainable management of the genetic variability of locally developed crop varieties, their relatives and the agroecosystems in which they occur (Miller et al., 1995).

*Ex situ* conservation is the conservation of genetic resources outside the natural ecosystem in which they occur; this involves the maintenance of plants at sites such as botanic gardens, field genebanks, genebanks. Botanic gardens are institutions that preserve documented collections of living plants for different purposes including scientific research and conservation (Wyse Jackson, 1999).

The role played by botanic gardens in the conservation of world plant diversity has been vital. Moreover, especially in recent years, many botanic gardens contribute to conservation of plants of importance for food and agriculture. It is estimated that living plant species maintained in botanic gardens from all over the world are around 80,000, meaning almost the 30% of the vascular plant species of the world (Hawkswoth, 1995; Wyse Jackson, 1999). Moreover, many botanic gardens are characterized by the presence of effective seedbanks with the aim of conserving germplasm of wild plant species. The Millennium Seed Bank (MSB) at the Royal Botanic Gardens in UK is one of the most important seedbanks to date (Smith et al., 2007). In field genebanks, plant species that do not produce seeds or produce only recalcitrant seeds are maintained. Genebanks allow the conservation of germplasm that may be stored in the form of pollen, seeds or plant tissue. Several are the strategies that allow the maintenance of germplasm in the most appropriate form by gene bank, including *in vitro* storage and cryopreservation.

Conservation of agrobiodiversity, both *in situ* and *ex situ*, includes all actions that prevent the loss of diversity in agroecosystems. Strictly interrelated with conservation, there is the sustainable use of components of biodiversity, meaning "the use of components of biological diversity in a way and a rate that does not lead to the long-term decline of biological diversity, thereby maintaining its potential to meet the needs and aspirations of present and future generations" (Convention on Biological diversity, 2019).

### *1.1 Valorization of agrobiodiversity: the terroir effect*

Historically, the term *terroir* describes an area or terrain whose characteristics (soil, microclimate) confer peculiar characteristics to food products (Barham, 2003).

*Terroir* effect determines different sensory properties of products, affecting secondary metabolism of the plant (Foroni et al., 2017; Lenglet et al., 2014). Although, initially, the existence of the *terroir* effect was only related to the wine sector, today this concept is extended to other crops, such as coffee, tobacco, chocolate, chili peppers, hops, tomatoes, tea, olive, blueberry, cannabis. Several are the studies carried out in order to determine a correlation between growing areas and specific characteristics of products. Zoratti et al. (2015) studied the effect of altitude on the production of anthocyanins by bilberries and blueberries grown at different altitudes. Their results showed an increasing production of anthocyanins in bilberries along an altitudinal gradient of about 650m. Studies carried out on coffee showed that *terroir* significantly affect its aromatic profile. Oberthur et al. (2011) have investigated the effect of pedoclimatic conditions of different Colombia regions on sensory properties of green coffee, demonstrating the occurrence of differences in biochemical and sensory properties, between coffee produced in Cauca and Nariño regions. Olives of the same cultivar produce oils characterized by different aromatic profile on the basis of the region of growth (Kalua et al., 2007).

Over the years, there has been an increased awareness that the territory is strictly connected with the product quality; the same relationship is remarkably observed in

hop. Green et al. (1997) evidenced that hops of the same cultivars grown in different regions of USA and Australia, showed different phytochemical profile.

### *1.2* In vitro *tissue culture techniques for agrobiodiversity conservation*

Germplasm of crops that are propagated vegetatively is generally stored in vegetative forms, such as tubers, rhizomes, ecc... However, the maintenance of these forms in field gene banks results to be expensive, due to high labor costs, necessity of wide spaces and vulnerability to environmental factors. Moreover, pests and pathogens can represent an additional issue for the preservation of valuable germplasm. *In vitro* conservation of germplasm is complementary to field gene banks and represents a valid alternative for conservation of vegetatively propagated species (Bhat et al., 1995; Fay, 1994; Sharma and Chandel, 1996).

Plant tissue culture is defined as the process in which plant cells, tissue and organs are maintained and grown in aseptic and controlled conditions (Scowcroft, 1984).

In this context, micropropagation may be considered as one of the first strategies for the obtainment of rapid mass propagation of plant species and their germplasm preservation. Micropropagation, through the use of existing meristems (characterized by undifferentiated cells), such as axillary buds, or non-meristematic tissues, in which meristems are developed starting from leaves, petioles, roots, internodes, allows the production of plants that are phenotypically and genotypically identical to the starting material. However, since, the longer is the time in which the explants remain in culture, the greater is the risk of somaclonal variation, which can affect the genetic fidelity of the obtained material, micropropagation cannot be considered as a method of long-term preservation (Bonga and von Aderkas, 1992).

Therefore, once plant material has been established *in vitro*, it can be stored for long term, resorting to slow growth and cryopreservation (Kartha, 1985).

Slow growth methods entail the reduction of the growth rate of plant material *in vitro* (Engelmann 1990, 1991; Withers 1990 a,b, 1991). Several are the strategies that can be utilized to achieve this goal: temperature reduction, growth inhibitors addition, culture media with reduced nutrient content utilization, *etcetera*.

Even though slow growth is widely used (Engelmann, 1990; Roca, 1989), this method also shows limiting factors related to the stress imposed on plants. An alternative to slow growth is represented by cryopreservation: it entails the storage of material at very low temperatures (-196°C), using liquid nitrogen that arrests the process of cell division and metabolic activities; samples remain viable and can be stored without time limit (Benson, 2008; Harding, 2004; Wang et al., 2014).

One of the main applications of plant tissue culture is the technology of synthetic seed that combines the advantages of clonal propagation with those of seed propagation, such as easy handling, storability, easy of transport and resistance to pest and diseases (Lambardi et al., 2006; Micheli et al., 2007). To obtain a synthetic seed, somatic embryos are enclosed in a bead, an artificial endosperm, with trophic and protective functions. This technology is usually combined with procedures of cryopreservation, such as encapsulation/dehydration. With this method, explants are first encapsulated in calcium alginate beads, and then precultured in a medium with high sucrose concentration, desiccated and immersed in liquid nitrogen. Sucrose acts as cryoprotector, induces osmotic dehydration and preserves the maintenance of plasma and membrane integrity during the process of dehydration and freezing (Plessis et al., 1993).

## 2. Agrobiodiversity enrichment

Agriculture is the deliberate planting and harvesting of plants, while plant breeding is a branch of agriculture that aims to develop plants endowed with new and improved characteristics that are permanent and heritable. Plant breeding is a process started about 10 millennia ago, when hunthers became sedentary producers of plants and animals (Gepts et al., 2012). This was a gradual process, during which the transformation of wild species into domesticated crops took place (Abbo et al., 2014). Moreover, it was during this time that most basic plant breeding techniques were discovered: people started to select, among biological variability present in a population, the variants showing the desirable traits (Bettinger, 2012; Gepts et al., 2012). Indeed, to make selection it may be necessary to have variability. At first, only

naturally present variability in crop species was exploited; then, traditional breeding methods and finally, thanks to the advance of science and technology, biotechnologies were used to artificially generate new variability and to obtain targeted and directional changes in the nature of the plants (Vaughan et al., 2007).

### *2.1 Traditional breeding techniques*

Traditional plant breeding allows to obtain or improve cultivars, resorting to conservative instruments for manipulating plant genome, exploiting the natural properties of the species (Al-Khayri et al., 2015).

A breeding program comprises several general steps including determination of breeding goals, assembling genetic variability, recombination of variation, selection of recombinants, evaluation of selections. Before starting a breeding program, it is necessary to have or create new heritable variability and to define an ideotype, that is 'a biological model which is expected to perform or behave in a predictable manner within a defined environment' (Donald, 1968), in order to achieve targeted modifications in plants. Generally, breeders aim to obtain plants resistant to pests and diseases, or produce varieties characterized by a higher yield, or with higher nutritional value (Acquaah, 2012).

Generally, creation of variability is obtained by resorting to hybridization or crossing of two different plants. By crossing selected parentals, it is possible to produce genotypes endowed with a combination of desirable traits of both parents (Acquaah, 2012). Moreover, during the process that leads to the formation of reproductive cells, rearrangements can occur in DNA that can be present in descendants. However, the success of crossing is strictly related to the species and the effect of this action may differ from one specie to another. In self-pollinated species, where fertilization is made between gametes of the same flower, crossing is more difficult. Generally, in these cases, all male organs are removed by the process of *emasculation*. Crossing is followed by a series of selections, in which discrimination among biological variation present in the population is made, in order to identify variants with desirable traits (Dudley and Lambert, 1992).

Seeds are an important tool for breeding programs, thanks to the enormous variability due to cross pollination; within this obtained variability a selection program could be carry out (Stajner et al., 2008).

Although creation of variability and selecting within the obtained variation represent the two key activities of plant breeding, developing a new variety resorting to conventional approaches includes several disadvantages. First of all, it is time-consuming and may need over ten years for cultivar release. Indeed, it is not possible to know in advance all information that will be transmitted, including undesirable traits. To overcome this problem, several subsequent selections could be needed. Undesirable traits can be eliminated crossing again the crossing products with parents that do not show these characteristics (backcrossing). In addition, the methods used to discriminate within the large obtained variation is not completely accurate and reliable. Another weakness of traditional breeding is that it is restricted to sexually reproducing species that are compatible.

### *2.2 Modern breeding techniques*

The breeding process has been revolutionized by the development of the genetic science and technology that made it more efficient (Acquaah, 2012).

Through biotechnologies it is possible to overcome the natural biological boundaries of the species, create new variability, manipulate plant genetics and obtain new varieties with desirable traits (Jakowitsch et al., 1999).

Plant biotechnologies can be divided in ***cellular biotechnologies*** and ***molecular biotechnologies***.

#### *2.2.1 Cellular biotechnologies*

Research carried out in plant tissue culture has led to development of technologies and techniques that are used worldwide to quickly multiply several plant species and to enhance their characteristics. Every single cell, thanks to its genetic potential, can be cultured *in vitro*, to develop into full plants (totipotency) (Acquaah, 2012). It was Gottleib Haberlandt (1854-1945) who, for the first time, proposed the concept of the

totipotency of plant cells, an idea of worldwide importance and that has marked the actual beginning of tissue culture (Laimer and Ucker, 2003). Over the years, the technology allowed to make several steps forward in the knowledge of breeding, and specifically in the technology of recombinant DNA. Plant tissue culture provides several tools and techniques that can be used to complement traditional breeding methods (Khan 2009; Takeda and Matsuoka, 2008; Thakur et al., 2012).

Regeneration entails the preventive de-differentiation of single cells of tissue or organ that, under endogenous and exogenous conditions and thanks to its meristematic activity, is made to start cell division. New meristematic areas can be radical or apical and can lead to the formation of new organs (organogenesis) or embryos (embryogenesis). Both organogenesis and embryogenesis occur by one of two pathways, indirect or direct. The indirect pathway goes through the formation of callus (a mass of dedifferentiated cells); from callus adventitious meristems are induced and plant regeneration can start. The direct pathway involves regeneration from de-differentiated cells, bypassing callus formation.

Several are the applications to plant breeding of *in vitro* culture that allow to overcome limitations imposed by traditional breeding techniques.

With embryo culture or embryo rescue it is possible to recover embryos that might fail to develop into a complete plant, especially if they are obtained by crosses between genetically distant plant species; moreover, this technique can be used to overcome seed intraspecific dormancy: indeed, isolating the embryo from the other seed tissues (teguments and endosperm) cancel the influence of dormancy factors, speeding embryo germination (Al-Khayri et al., 2015). This technique, firstly set up by Tukey, in 1933, is now widely applied for numerous plant species such as *Lilium* (Chi, 2002; Proseviçius and Strikulyte, 2004), *Malus* (Dantas et al., 2006), *Prunus* (Kukharchyk and Kastrickaya, 2006), numerous fruit crops (Fathi and Jahani, 2012) and *Capsicum* (Debbarama et al., 2013).

In protoplast fusion. protoplasts, cells without cell wall, are fused in order to obtain a new somatic hybrid plant with the characteristics of both parentals (Acquaah, 2012). Protoplast fusion technology allows to transfer desirable traits from one specie to

another, bypassing fertilization, offering the possibility of improving commercially important crops, also as support of traditional breeding (Taji et al., 2002). Numerous are the valuable traits that are successfully transferred to hybrids resorting to protoplast fusion, such as increased biomass and yield using *Brassica napus (+) B. rapa* (Qian et al., 2003), resistance to bacterial and fungal wilts in *Solanum melongena (+) S. sisymbrifolium* (Collonnier et al., 2003), improved bio-control efficiency against rice weeds using *Curvularia lunata* (+) *Helminthosporium gramineum* (Zhang et al., 2007 ).

By resorting to ovary/ovule culture or anther/microspore culture is possible to produce complete homozygous lines in only one generation. On the contrary, several years of multiple selections in segregating popolutations, followed by inbreeding and other selections are required in traditional breeding (Al-Khayri et al., 2015).

The variability within seeds is not easy exploitable, resorting to traditional nursery activity; indeed, more around ten years are necessary to individuate a new cultivar. The use of biotechnology and specifically the *in vitro* seed culture can represent a way to speed up the selection process, obtaining seedlings, in controlled conditions, overcoming dormancy factors and irrespectively of the season and the weather, in a relatively short time.

#### *2.2.1.1 Plant breeding through gametoclonal and somaclonal variation*

Plant breeders resort to traditional and biotechnological methods in order to create new variability and to select new varieties endowed with interesting characteristics. *In vitro* tissue culture, through the exploitation of genetic variation that occurs in the regeneration process, can be recognized as a powerful tool of crop improvement. Specifically, studies have highlighted the insurgence of variability from the culture of gametic cells, that as been referred as gametoclonal variation (Evans et al., 1984; Morrison and Evans, 1987), and from culture of cells, tissues and organs that has been referred to as somaclonal variation (Larkin and Scowcroft, 1981).

*Gametoclonal variation*

The term haploid refers to a sporophyte carrying only one set of chromosomes. Haploids can occur spontaneously; Blakeslee et al. (1922) described spontaneous development of haploid plant in *Datura stramonium*. However, spontaneous haploid occurrence is a rare phenomenon, while it can be artificially obtained through modified *in vivo* pollination methods (chromosome elimination, wide crosses...) or by *in vitro* culture of anther/isolated microspore (androgenesis) or of ovary/ovule (gynogenesis). Among plants regenerated from gametic cells, it is possible to observe variation that has been referred to as gametoclonal variation (Evans et al., 1984; Morrison and Evans, 1987). Thanks to gametoclonal variation, it is possible to exploit the variability within gametes to individuate genotypes carrying interesting characteres. Since there is only one embryo sac per ovary, while in each anther are contained thousand of microspores, androgenesis is considered to be more efficient than gynogenesis (Acquaah, 2012).

The response of gametes in culture depends on several factors, both endogenous and exogenous, including genotype, starting material, stage of pollen development (Atanassov et al., 1995; Datta, 2005; Smykal, 2000; Wang et al., 2000). Indeed, since healthy conditions of mother plant significantly affect the androgenic response, it is really important to use stress-free mother plants to harvest flower buds (Szarejko, 2003). Success of the process largely depends on the developmental stage of the gamete; even though the developmental window of embryogenic competence may differ among the species (Seguì-Simarro, 2010). In order to increase the efficiency of the process, flower buds can be subjected to several types of stress treatments. Cistué et al. (2003) reported the positive effect of cold shock and carbohydrate starvation in stimulating androgenesis in barley.

Generally, haploid plants do not represent the final goal of gametic embryogenesis. Haploids may be subjected to chromosome doubling, in order to obtain double haploid individuals (DHs) (Kasha and Maluszynski, 2003). In this way, it is possible to produce complete homozygous lines in only one generation. On the contrary, several years of multiple selections, followed by inbreeding and other selections are required in

traditional breeding to obtain the same results (Al-Khayri et al., 2015). The potentialities of DHs in breeding programs dates back to 1964, with the production of haploid plants of *Datura*, starting from anther culture (Guha and Maheshwari, 1964). Since then, the technology has developed considerably and nowadays, double haploid technology has been successfully used in breeding programs of several species. in which efficient methods of haploids generation and doubling systems have been set up (Acquaah, 2012). Moreover, doubled haploids can be further employed in plant breeding, as they represent a valuable tool to uncover recessive traits, to establish marker association and to construct genetic maps (Maluszynski et al., 2003).

*Somaclonal variation*

As a rule, plants regenerated from culture of somatic cells are clones of the genotype from which they derived; however, tissue culture can produce material that is not an exact *replica* of the starting plant. Such variation that can arise through the *in vitro* culture of plant cells, tissues and organs (somatic tissues) and which does not result from meiosis, is referred to as somaclonal variation (Larkin and Scowcroft, 1981). Several are the mechanisms that are involved in this phenomenon; although the molecular bases that affect somaclonal variation are not precisely known, both genetic and epigenetic factors seem to be involved (Jiang et al., 2011). There are many factors that play a role in the occurrence of somaclonal variation; much of this variation may be related to the presence of plant growth regulators that can act as mutagens (Gao et al., 2010); moreover, the type of tissue, explants source, plant genotype, time in which the explants are maintained in culture can affect the genetic stability of the *in vitro* cultured plant material; generally, more differentiated tissues such as roots, leaves, stems give rise to a greater variation, compared to buds or other apical meristems explants with existing meristems. (Duncan 1997; Pijut et al., 2012).

The presence of genetic diversity represents an important factor that could be exploited in plant breeding. However, genetic variation is not always present in the desirable or right combination. Jain et al. (1998) suggest that such induced somaclonal variation can be used in combination with traditional breeding tools in order to obtain

crops endowed with desirable characteristics. Different types of genetic alterations may be associated to *in vitro* tissue culture and somaclonal variation can be evidenced by changes in chromosome number (polyploidy, aneuploidy) or in chromosome structure (such as translocations, delections, insertions) and DNA methylation (Nwauzoma and Jaja, 2013; Rodriguez-Enriquez et al., 2011). Variation in chromosomal number has been observed in potato, wheat and other crops (Bayliss, 1980; Nwauzoma and Jaia, 2013).

Thus, the chance of success in applying somaclonal variation is strictly related to genetic stability of selected somaclonal mutants. Several are the strategies that can be used to discover the presence of somaclonal variation, among them there are technologies based on cytogenetic analysis and molecular or biochemical genetic markers (Tan et al., 2013).

### *2.2.2 Molecular biotechnology*

Molecular biotechnologies play an important role in plant breeding, both in the genetic engineering field and in assessment of genetic variability, through the use of molecular markers.

#### *Genetic engineering*

In molecular plant breeding the DNA is manipulated directly, resorting to the use of different biotechnological tools. Although genetic transformation is a phenomenon that naturally occurs in plants (Hooykaas and Schilperoort, 1992), the possibility of transferring one or more selected traits into a specific plant, derives from the development of recombinant DNA technology, towards the end of the 1970 with the first transformation experiments in tobacco (Barton et al., 1983; Deblock et al., 1984; Herrera-Estrella et al., 1983; Horsch et al., 1984). Since then this technology has progressed considerably. Several are the methods that can be used to modify genetically plants which are divided into two types: direct and indirect.

Direct DNA introduction into plant cells is achieved resorting to direct gene transfer into cells (Songstad et al., 1995), through microprojectile bombardment (biolistic

method), through electroporation and through microinjection. Unfortunately, the transformation with direct methods involves further disadvantages, since usually plant cells transformed show insertion of undesirable DNA, in multiple and fragmented copies.

Among indirect methods there is *Agrobacterium*-mediated transformation that entails the use of *Agrobacterium tumefaciens* to introduce selected DNA fragments into cells (Gelvin, 1990; Kado, 1991; van Wordragen and Dons, 1992; Zupan et al., 2000). This strategy represents an attractive and efficient system, since it is easier than direct methods, and has reduced equipment costs.

*Molecular markers technology*

The use of genetic markers has revolutionised the plant breeding process; indeed, they can be used to assess genetic diversity and consequently to help and improve the selection process, making it more efficient and effective (Acquaah, 2012).

A genetic marker is a fragment of DNA that has a specific position on a chromosome and can facilitate the individuation of other genes of interest. In breeding programs, it can be useful to know the association (linkage) between the marker and the gene of interest codifying for desirable characteristics. Therefore, since the identification of a marker is faster and easier, the identification of the desirable trait is made by indirectly selecting for the marker. Genetic markers can be classified in morphological markers and molecular markers (biochemical markers and DNA markers), since they can be detected both at morphological level or at molecular level (Acquaah, 2012).

Morphological markers derive from the interaction of genes controlling traits and environment and are related to visible traits of the plants, such as seed shape and size, colour of the flower, etc... However, morphological markers, unlike from molecular markers, can be affected by environmental conditions and for this reason they are underused in plant breeding.

Molecular markers are used to pursue several breeding purposes. One of the main uses of genetic markers is to assist the selection process, making it more practical,

productive (Acquaah, 2012); this application of molecular markers in plant breeding has referred to as Marker Assisted Selection (MAS).

Biochemical markers (enzyme-based markers) are considered to be the earliest type of molecular marker and are based on modifications in the sequence of aminoacids within a protein; whilst, DNA based marker, detect the variation in DNA sequence (Al-Khayri et al., 2015).

Several are the uses and the applications of molecular markers in plant breeding. However, to be useful, markers have to be characterized by a set of desirable properties, such as a high degree of polymorphism, they have to be frequent and random distributed in the genome, to have low rate of mutations and easy to manage and to asses (Collard et al., 2005). The types of DNA-based markers existing include restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs) marker, amplified fragment length polymorphisms (AFLPs), microsatellites (SSR) and single nucleotide polymorphisms (SNPs).

# SECOND SECTION

## *Humulus lupus* L.: an ancient plant with a bright future

## 1 Introduction

The plant of *Humulus lupulus* L. is well known all over the world for its use in the brewing industry and in the cosmetic and pharmaceutical field (Zanoli and Zavatti, 2008).

Its name, "*lupulus"*, derives from the Latin word *lupus*, a wolf climbing on a sheep (Grieve, 1971) and reflects the plant's habit of climbing. At first, hop plants were harvested in the wild, then its cultivation started in Germany since mid ninth century, hence it spread to other regions of central Europe. Wilson (1975) describes the diffusion of hop in Europe and its use in brewing industry. Originally, *Humulus lupulus* L. was used as an alternative to *Myrica gale*, as a beer additive; afterwards, from the eighteenth century, probably due to its better preservative property, the use of hop has replaced completely that of *Myrica gale* (Behre, 1999). As a result of its antimicrobial proprieties, at first it was used to preserve the durability of the beer and only later to impart bitterness and fragrance to beer (Moir, 2000). However, how hops become an essential ingredient for beer production is unknown; hop shows its stability properties only after one hour and a half of boiling, and nobody knows how and who discovered this property. Arnold (2005) reports the utilization of hops, inherent of beer production by Benedictine monks from a monastery close to Amiens (France), but it is not clear if hop was used as a stabilizing or aromatizing agent. The naturalist Pline the Elder mentioned hop in his "Natural History" and, particularly, he described the use of hop shoots as a vegetable in Roman diet (Grieve, 1971). At first the leaves and flowers of hops were used to generate a great brown dye, while the stems were used due to their fibrous nature to produce a kind of fabric (Grieve, 1971). Moreover, Blumenthal (1998) reported the use of hop for medical purposes to treat sleeplessness and nervousness in native American tribes. As in the past, nowadays hop secondary metabolites continue to be used, due to their positive effects in the treatment of a wide range of disturbances (Zanoli and Zavatti, 2008).

In the recent past, in a conference organized by Bayern Innovativ (Barth and Haas, 2010), in Wolnzach, the delegates from Germany, Austria and Switzerland focused on functional compounds in plants, among which hop. They have pointed out the growing importance of hop plant in food, pharmaceutical and cosmetic field.

In recent years, the consumer increasing interest for natural and products, has led to a greater demand for functional compounds derived from plants (secondary metabolites), particularly in food, pharmaceuticals and cosmetics industries. This is partially due to progress in biosciences that has made possible to understand the mechanisms of action of many compounds more clearly and to discover and identify new physiologically interesting substances. In line with this growing interest in the health benefits of plants*, Humulus lupulus* L. has received particular attention and alternative uses for hops have been the subject of research for some years now (Zanoli and Zavatti, 2008). Hop female flowers (cones) accumulate secondary metabolites, and they could be used as treatment for hormonal complaints, diabetes, skin diseases, allergies, insomnia, cancer prevention, etc... (Blumenthal, 1998; Grieve, 1971). Tyler (1987) reports that the traditional use of hop as a mild sedative, dates back into the past and comes from the observation of sleepiness and fatigue observed in hop-pickers; probably due to the transfer of hop resin from their hands to their mouth. The use of hops for the treatment of “mood disturbances, such as restlessness and anxiety, sleep disturbances” has been approved by the German Commission E and by the European Scientific Cooperative on Phytotherapy (Blumenthal, 1998). Moreover, according to Schulz et al. (2001), the plant (hop dried extract) improves digestion and exercises diuretic effects. Its secondary metabolites have been described as potent antimicrobial agents against a wide range of microorganism (Olšovská et al., 2016) and, already in the past, hops were used and added to beer for its antimicrobial activity as a preservative, classifying this plant as a natural source of compounds with biological effects (Simpson and Smith, 1992). In recent years, hop has been studied considering its estrogenic properties and its potential cancer chemopreventive activities. Particularly, some hop active compounds, such as xanthohumol, are thought to be involved in cancer-inhibiting mechanisms (Zanoli and Zavatti, 2008).

Although some hop secondary metabolites, produced by female flowers, are used in the pharmaceutical field, their main application is in the brewing industry, because essential oil and resins provide flavour, bitterness and aroma to beer (Zanoli and Zavatti, 2008).

The world area harvested with hop is of 58,739 ha with a production of 118,401 tonnes, showing an increase of 6% in the last two years (56,137 ha with a production of 112,125 tonnes) (Bart and Haas report, 2017-2018), of which 1,952 mhl are destined to beer production. The major world hop producer country is the USA with a world market share of 40%; next comes Germany with 35%. The primacy of the USA is also shown in the high number of different hop varieties produced: International Hop Growers's Convention reports that, in USA, 87 of the 271 hop varieties present all over the world are cultivated, while in Germany "only" 41 are grown.

Depending on their content in essential oils and resins, the brewing industry resorts to two main classes of hop cultivars: aroma and bitter; aroma hop varieties show an alpha-acids content of up to 8%, while bitter varieties, possess an alpha-acid content of up to 18%.

During the last years, the increased interest of consumers for craft beer, and the necessity of diversifying the beer offer, led brewers and hop growers to widen the varietal panorama, opening more to aroma varieties, rather than bitter varieties (Bart and Haas, 2017). Indeed, the area cultivated with aroma varieties expanded by 3.1% in USA and by 5.3% in Germany. As a whole the hop industry is growing; one of the reasons of this development is the rise, worldwide, of craft beer demand and production; it used to represent only the 2.2 % of total world beer production, while now it absorbs some 20 to 25% of the entire world hop crop (Bart and Haas, 2017-2018). In Italy, together with the consumption, also beer production has increased, but craft breweries have to purchase the hop cones they need from abroad, because the Italian hop cultivation did not grow as fast (Assobirra, 2018). In recent years, fortunately, hop cultivation has become more widespread; indeed 88 are the commercial hop-poles recorded along the Italian peninsula, corresponding to almost 56 cultivated hectares (Amoriello, 2019).

## 2. Phytogeography and botany

The genus *Humulus,* belonging to the *Cannabaceae* family, includes three species, *H. japonicus*, *H. yunnanensis*, *H. lupulus* L.

It is believed that hop is native of China, because all *Humulus* species were found in this area, from where it diffused to Japan, America and Europe (Neve 1991; Small, 1978). *H. japonicus* Sieb. & Zucc is widespread throughout China and Japan and it is mostly used as an ornamental plant; *H. yunnanensis* grows at high altitude in Southern China and remarkably little is known about it. *Humulus lupulus* L. is naturalized in central Europe and it is widely cultivated in areas comprised between 34 and 66° latitude, thus in Europe, North America, Japan, Asia, Australia and New Zeeland, Argentina and South Africa.

*Humulus lupulus* L. is almost ubiquitous in temperate climates; wild hops grow in the wet areas close to rivers and in places where it can find a support for its stem (Rybacek, 1991). Actually, this species shows a great adaptability to a wide variety of conditions, including semiarid, maritime, humid continental and sub-tropical regions; indeed, hop stands are integral part of the countryside around USA, Europe, South Africa, Australia, Germany, China and Czech Republic. From a commercial standpoint, hop cultivation provides better results in regions roughly comprised between 45 and 55 degrees north or south in latitude, while the correct altitude for its growing is between 250 and 800 meters (Mez, 1969), an area that includes also Italy, where hop is endemic.

Hop is a dioecious plant (with female and male flowers on separate plants); however, it is possible to find individual monoecious plants that are often originated by seeds and that are infertile (Burgess, 1964). Feminine and masculine plants cannot be distinguished before the flower comparison: female inflorescences are cones (called strobiles), characterized by the presence of bracts, while male flowers are long racemes. At the base of cone bracts, it is possible to find yellow glandular trichomes that secrete lupulin, a resinous substance. Lupulin glands are also present on the abaxial side of hop leaves. The cone harvesting is carried on when their color turns from greenish-yellow to yellow-brown. Generally, in order to maintain the genetic

uniformity of products, in hop growing areas, only female individuals are present (Neve, 1991). Males, however, are essential for breeding new hop varieties.

*Humulus lupulus* L. is a perennial plant; the organs above soil die every year at the beginning of winter and the plant regrows each spring from the rhizomes of an underground rhizome; during its dormant stage it can survive even in extremely cold temperatures (Burgess, 1964). In Europe, the vegetative stage begins in spring; flowering stage takes place between July and August.

## 3. Hop biodiversity and agrobiodiversity

Hop is almost ubiquitous; indeed, thanks to a high degree of intraspecific genetic variability, it is able to adapt itself to different conditions, both climatic and ecological (Patzak et al., 2010a). A great degree of diversity has been individuated among the secondary metabolites of *Humulus lupulus* L. of different populations (Eri et al., 2000; Patzak et al., 2010a). Since the quality and the value of hop cones depends on the phytochemical profile of their secondary metabolites (Kralj et al., 1991), hop breeding has been traditionally on the obtaining of varieties characterized by a peculiar phytochemical profile, particular flavors and tastes, as well as on yield and cone size. Hence, this particular attention for hops endowed with specific characteristics has led, during the breeding process, to a shrinking of intraspecific agrobiodiversity and an impoverishment of the genetic basis (Jarvis and Hodgkin, 1999). According to FAO report (2019) on the status of biodiversity, over the last years, a loss of a high number of staple food species for human diet was observed. The impoverishment of agrobiodiversity is taking the UE to stimulate the public opinion and, most of all, the research world to find ways to evaluate and to enrich the existing biodiversity. Several are the studies carried out on hop biodiversity by authors from different countries, exploiting diverse approaches. Henning et al. (2004) identified hop germplasm diversity classifying 129 hop accessions from the USDA-ARS hop germplasm field collection, on the basis of morphological and chemical data. Other studies have discussed hop genetic variation resorting to molecular methods. Suštar-Vozlič and Javornik (1999) used both random amplified polymorphic DNA (RAPDs) and hop cone

essential oil composition to analyze 65 world hop cultivars. Patzak et al (1999) studied 32 hop cultivars from a world collection, using RAPDs. Solberg et al. (2014) employed DNA fingerprinting using amplified length polymorphisms (AFLPs) to detect genetic relationship and variability among 62 Danish and 34 Norwegian clones. The AFLP analyses resulted in 41 polymorfic bands and allowed to separate the majority of the accessions. Rodolfi et al. (2018) characterized genetic diversity present in wild Italian hop cultivars and compared them with European and American hop cultivars, through the use of microsatellite markers. This study has underlined the high biodiversity existing in Italy and the presence of considerable differences among Italian, European and American hops. Results obtained in these studies suggest the potential use for agrobiodiversity valorization and for breeding programs of cultivated varieties.

## 4. Hop propagation and breeding through biotechnologies

According to Wilson (1975), at first, hops were collected into the wild; only from the later 19$^{th}$ century, hop cultivation started in Germany. In a short time, the interest in improving and selecting new hop variants increased notably (Moir, 2000); indeed, in this period, hop breeding started with clonal selection from adapted wild hops. Differently from the past, new modern hop varieties are, instead, derived from hybridization, which allows the introduction of new combinations. Current breeding technologies aim to improve several aspects of the hop plant, including disease resistance (Verticillium wilt, downy mildew, and powdery mildew), increase the content in resins and oils and improve the yield (Stajner et al., 2008).

Usually, hop plants are vegetatively propagated by layering or by rooting soft-wood cuttings (Neve, 1991); biotechnologies and, specifically, the *in vitro* tissue culture techniques provide a valuable alternative to traditional multiplication. *In vitro* micropropagation of hop occurs starting from different kinds of explants, such as node cuttings, apical tips (Roy et al., 2001), meristems (Adam, 1975), shoots, leaves, roots (Batista et al., 1996). Roy et al. (2001) studied the effect of plant growth regulators and of different culture media composition on shoot multiplication efficiency, starting from nodal explants of hop variety H138.

As a rule, clonal propagation ensures genetic fidelity of the obtained plants; however, due to the occurrence of genetic and epigenetic changes that can be caused by some *in vitro* culture conditions, regenerated plants could be different from the starting material (Kaeppler and Phillips, 1993). In literature, several are the references to molecular techniques to evaluate the appearance of mutations in *in vitro* obtained plants. In hop, Peredo et al. (2006) described the use of amplified fragment-length polymorphism (AFLP) markers to detect genetic variation in hop plants, obtained from organogenic calli.

Among the different *in vitro* tissue culture techniques, mainly used in agricultural biotechnology, the culture of meristem and shoot tip has been notably exploited, due to its advantages, such as virus elimination, rapid clonal multiplication, germplasm preservation (Nehra and Kartha, 1994). Vine and Jones (1969) reported the first application of tissue culture for hop virus-free plants at East Malling Research Station in the United Kingdom. Since then, the technology has developed notably, and recently this approach has been employed to produce virus–free stock plants in the Czech Republic (Svoboda, 1992) and Slovakia (Faragó and Nešťáková, 1998). In recent years, as a result of a growing interest in the health benefits of plants, hop has been receiving particular attention due to the pharmaceutical importance of its secondary metabolites including flavanones, chalcones, and phloroglucinol derivatives (Zanoli and Zavatti, 2008). Therefore *in vitro* systems, based on establishment of cell suspensions, have been set up in order to *in vitro* produce interesting metabolites (Pšenáková et al., 2009).

More than, tissue culture techniques play a crucial role in regeneration of transgenic plants (Faragó et al., 2008). In hop, genetic transformation through *Agrobacterium tumefaciens* is considered to be a hopefully approach, considering that hop is one of the natural hosts of this bacterium (De Cleene and De Ley, 1976). However, few are the reports concerning the genetic transformation of hop (Becker, 2000). Horlemann et al. (2003) reported the first successful transformation and regeneration of hop transgenic plant cv. Tettnanger.

## Aim of the PhD project

All over the world, the interest in hop cultivation is addressed to its female flowers (cones) in which are accumulated secondary metabolites. Some of these compounds, especially essential oils and resins, have particular relevance in the pharmaceutical field since they can be used to treat various types of disturbances, as well as in the brewing industry, since they provide flavour, bitterness and aroma to beer (Zanoli and Zavatti, 2008). Unluckily, during its domestication, cultivation and breeding process, hop suffered from a shrinkage of intraspecific agrobiodiversity (Patzak et al., 2010a,b).

In this context, my PhD thesis project is set in order to valorise and enrich existing hop agrobiodiversity, exploiting the potential of this species.

In the frame of hop **agrobiodiversity valorization**, two research were carried. The first research aimed at studying the adaptability of International hop cultivars to the Italian climate, evaluating the qualitative aspects of hop varieties. Original surroundings are strictly related to species, influencing their quality and characteristics; therefore, geographical indications can represent a tool able to enhance the value of agrobiodiversity. Indeed, adding value to a particular variety or genotype with peculiar characteristics, attributable to its geographical origin and different from neighboring areas, permits its maintenance.

The second research activity was carried out to find a solution for a problem raised by hop growers; indeed, due to the increasing interest of growers in hop cultivation, there is a constant demand of hop plants that is not satisfied by nursery activity. Resorting to *in vitro* hop propagation, through petiole regeneration and microcutting encapsulation, not only represents a valuable tool to overcome these issues and to support the nursery sector activity, but it also ensures the valorization and storage of hop germplasm.

With the aim of **enriching the existing agrobiodiversity**, traditional and biotechnological methods may be used to speed up the selection of new genotypes.

Seeds represent a valuable resource for breeding studies; indeed, they represent a high source of variability, within which it is possible to make selection (Stajner et al., 2008). Biotechnologies, and specifically, *in vitro* seed culture could be a way to speed

up the selection process, but there is a lack in literature, that needs to be filled. In order to fill this gap, in this Ph.D. thesis a study aimed at setting up a valid protocol to maximize hop seed germination was carried out.

Among biotechnological breeding methods, several are the studies carried out in hop to exploit somaclonal variation, whilst, in our knowledge, nothing is in the literature about gametoclonal variation. Gametoclonal variation can be reached through gametic embryogenesis. This technology could be used to produce haploids, and, resorting to chromosome doubling, doubled haploids (DHs). DHs are pure lines, obtained faster and easier, in comparison to traditional breeding, making gametic embryogenesis a valuable tool to speed up hop breeding. However, to our knowledge, there is a lack of information in literature concerning DH production in hop. For this reason, a research aimed at studying the different phenological phases of flowering in hop and the corresponding developmental stages of microspores/ pollen grains contained therein.

Other than gametic embryogenesis, variation can be obtained through somatic organogenesis. Actually, passing through callus phase in the organogenic process, but also the use of growth regulators and the time explants are subjected to *in vitro* culture conditions, can induce the occurrence of genetic, chromosomic and genomic variations. Among them, genomic mutations can lead to the formation of tetraploids, particularly important in hop breeding, because they can be crossed with diploid plants to obtain triploids, considered superior to both diploids and tetraploids. In this thesis, with the aim of evaluating the possible somaclonal variation occurrence, hop leaf culture has been carried out, considering the influence of time of leaf portion culture and growth regulator type and concentration.

*Hop agrobiodiversity valorization*

## Hop agrobiodiversity valorization

**Study 1:** Changes in chemical profile of Cascade hop cones according to the growing area.

**Study 2:** Evaluation of two *Humulus lupulus* (L.) *in vitro* propagation methods: petiole regeneration and encapsulation technology.

# Study 1 - Changes in chemical profile of Cascade hop cones according to the growing area

Running head: influence of growing area in Cascade hop cones

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**Abstract**

*BACKGROUND*

The growing area has a substantial effect on plants, affecting secondary metabolism. In hops, different authors studied the effect of growing area on the chemical composition of cones with the aim of verifying and understanding the changes in hop characters. Despite the scant literature the subject receives an increasing attention by brewers and hop growers. The present study aimed to characterize with gas chromatography-mass spectrometry (GC-MS), and HPLC-UV, cones of hop (*Humulus lupulus* L.) cultivar Cascade. Plant material was obtained from 9 different areas of Italy and compared with Cascade samples grown in USA, Germany and Slovenia.

*RESULTS*

Differences in bitter acids and xanthohumol content were observed. Nevertheless, no correlation between bitter acids and xanthohumol production, on one side, and rainfall, temperatures and latitude, on the other, were observed in our samples. The Slovenia samples were richer in molecules that confer hoppy, woody and flower notes; USA2 samples were more characterized by woody, earthy, grassy and floral aroma, quite different characters if compared to USA1, which had the lowest presence of grassy aromatic compounds. In the Italian samples, TRENTINO was the genotype most characterized by limonene presence.

*CONCLUSION*

The results of this study are indicative of the importance for hop users to know and characterize hops coming from different growing regions. The study pays special attention to the characterization of the differences in chemical characters of Cascade hop in Italy, where hop cultivation has developed only recently, but is in continuous expansion.

**Keywords:** *Humulus lupulus* L., bitter acids, oil yield, xanthohumol, oil composition

## 1. Introduction

Related to regional identity is the *terroir* concept; initially associated only to the wine sector, the *terroir* indication involves a number of biotic and abiotic factors, strictly related to a defined territory. The *terroir* may be determined by a distinctive soil structure, composition, altitude, position and by the precipitation regime; all these aspects, typical of a given growing zone, unavoidably affect the properties of the final products (Barham, 2003; Foroni et al., 2017; van Leeuwen, 2010). In the wine sector researchers studied the different aspects of *terroirs*, such as the effect of water and nitrogen on wine quality and polyphenolic content (Choné et al., 2001) the differences in microbial communities in musts and on entire grape plants (Knight et al., 2015), the impact of microbial populations on the regional identity of wines (Gilbert et al., 2014), or the *terroir* influence on stilbene production in grapes (Fernández et al., 2013).

Nowadays, the *terroir* concept is applied to a number of agricultural products, particularly vegetables and, in a derivate mode, to cheeses and cold cuts (Petruzzellis and Winer, 2016; Siqueira et al., 2011; Turbes et al., 2016). Zoratti and coworkers (2015) studied bilberries and blueberries grown at different altitudes in the Alps of Northern Italy and observed that anthocyanins production was directly correlated to altitude. In coffee production, the *terroir* has a marked relevance and several studies were made on the interactions between cultivation area and aromatic profile (Avelino et al., 2005; Oberthur et al., 2011). Oberthur and coworkers (2011), showed how specific pedoclimatic conditions of different Colombia regions affected green coffee sensory properties. Data analysis allowed the individuation of biochemical and sensory differences in the coffees produced in Cauca and Nariño regions, to the extent that it was possible to attribute a commercial quality recognition. In processed foods, it is possible to record *terroir* effects in olive oils. Olives of the same cultivar, grown in different regions, produce oils characterized by distinctive aromatic profiles, due to intrinsic characteristics of the cultivation areas (Kalua et al., 2007). It is therefore acknowledged that the *terroir* has a substantial effect on plants, affecting secondary metabolism and inducing differences in the final sensory characters of the products (Foroni et al., 2015; Lenglet, 2014). Related to *terroir*, is territory branding of

agricultural products, that is becoming a key factor in marketing, and consumers are increasingly aware of the relationship between territorial certification and product quality. This connection with territory is observable in hops coming from different areas. Hops are used in brewing process to contribute to the final aroma and flavor of beers. Each cultivar possesses distinctive characters (Stenroos and Siebert, 1984) and provides different flavors to beers. In the brewing process, hops can be used at different steps: during boiling to add bitterness, or at the end of the process to finalize the aromatic structure.

Differences in hops characters connected to the growing areas had already been observed by Stenroos and Siebert, in 1984, in a study focused on hops varietal identification; they studied the aromatic profile of 148 hop varieties from Europe and North America, and from data analysis they found varietal, but also regional correlations. More recent research in different countries was carried out to verify and understand the possible changes in hop varieties aroma (Forster et al., 2002; Green, 1997; Van Holle et al., 2017), but also to valorize a particular region of production (Capper and Darby, 2014). Green (1997) compared different fine aroma hops from Europe with the same culivars grown in the USA and Australia.

From data analysis on bitter acids and oil composition, differences were highlighted; in Tettnanger samples, for example, there were differences in the value of the farnesene-caryophyllene and humulene-caryophyllene ratios, and in the essential oils content. Other important differences related to territory were found by Forster and Gahr (2014) and Forster and coworkers (2002) between Cascade and Comet plants grown in Yakima (Oregon) and in Hallertau (Germany), especially with reference to the polyphenolic fraction. Further differences were observed in the distinctive presence of aromatic molecules like linalool (more present in Yakima Cascade) and isobutyil isobutyrate and 2-methyl-butyl 2-methylpropanoate (characteristic of Hallertau Cascade) (Forster et al., 2002; Forster and Gahr, 2014). Olšovská and coworkers (2013), in a study on proanthocyanidins in Czech hops, highlighted the dependence of the proanthocyanidin profile on hop varieties, as well as to the geographical origin. Capper and Darby (2014) studied the British *terroir* for some English hop varieties: in the

study, the importance of the country of origin of hops was considered, and it was shown that English hop varieties grown in England produced high quantities of oils, if compared to English hops grown in other countries. Moreover, they explained the unique and distinctive aroma characters of British hops grown in England, which produce delicate and complex flavors, with the peculiar environment that determining low levels of monoterpenes.

Despite the scant literature available on the study of hops *terroir* and/or the influence of the growing area on the final product, the subject receives an increasing attention by brewers and hop growers. The distinction and characterization of the properties of hop cultivars coming from different growing regions could be very important for brewers in the selection of a specific hop to utilize. In Italy, where the brewing sector is in a rapid expansion, brewers are particularly interested in hops able to characterize and differentiate their production with new scents and flavor and are constantly looking for new ways to improve their product and to tell and promote, both in Italy and abroad, their regional identities. Moreover, in Italy, where hop cultivation is dawning, the knowledge of the effect of the territory on the final product is a necessity. As a matter of fact, Italy, for its heterogeneous pedoclimatic conditions, could allow a considerable differentiation also among hops grown in the same region. The characterization of hop regional identities could be a thrust for this rising industry in Italy. The aim of this paper was therefore the evaluation of the chemical composition variation (bitter acids, oil yield and aromatic profile) in Cascade hop cones grown in different areas of Italy, in comparison to Cascade hops grown in the main hop producing areas of the United States of America (Oregon and Michigan), Germany (Tettnang region) and Slovenia (upper Carniola region). Cascade is a variety characterized by 4-7% of α – acids, 4.8 – 7% of β – acids, 30 - 40 % of cohumulone (on the total of α-acids), 0.7 -1.4 % of total oil and a content of myrcene in the amount of 40 – 60% of total oil (Haas, 2014). This cultivar is described as an aroma variety and is distinguished by a prevalent citrus and fruity aroma (Haas, 2014).

## 2. Materials and methods

### *2.1 Plant material*

For the experimental plan, Cascade hops were obtained from 9 Regions of Italy, from Germany, Slovenia and from two production areas of the USA (Table 1). All hop cones used in the study were picked from 3 years old plants, and hop cones were sampled for two years. Hop plants were cultivated with similar spacings, trellis height, irrigation system and fertilization plan. Temperature and rainfall data of the different areas, from March to September, were collected by temperature data logger (HOBO Pendant® Temperature/Light 64K Data Logger) and rainfall data logger (HOBO Pendant® Event Data Logger) (Tables 2 and 3) for the two years of study.

### *2.2 Chemicals and solvents*

Dichloromethane and anhydrous sodium sulfate were purchased from Sigma-Aldrich (Milan, Italy). Toluene was purchased from Carlo Erba (Milan, Italy). Ultra-pure water was in house produced by using a Milli-Q-System (Millipore, Bedford, MA, USA). Standards of caryophyllene, myrcene, humulene, and (+)-linalool were purchased from Sigma-Aldrich (Milan, Italy). Methanol (HPLC grade) was purchased from Sigma-Aldrich (Milan, Italy). Bitter acids mixture standard (international calibration extract, ICE-3) was from Labor Veritas Co. (Zürich, Switzerland). The mixture standard contained α-acids with 13.88% of cohumulone and 30.76% of *n*-humulone + adhumulone, and β-acids with 13.44% of colupulone and 10.84% of *n*-lupulone + adlupulone. Xanthohumol from hop (*Humulus lupulus*) ≥ 96% HPLC grade standard was purchased from Sigma-Aldrich (Milan, Italy).

### *2.3 Bitter acids extraction*

Dried cones (0.5 g) were extracted with 20 ml of methanol in a flask and homogenized with Ultra Turrax mixer (IKA®, T18 Basic) for two 20 sec periods, to avoid sample overheating (Farag et al., 2012). Extracts were stirred for 3 hours at room temperature in the dark to prevent degradation and photo-oxidation. Subsequently the extracts were centrifuged at 1800 xg at 20°C for 20 min. Supernatant was then

transferred to a volumetric flask (50 ml). The exhausted matrix was re-extracted with methanol (15 ml), centrifuged as above and the supernatant was pooled with the first extract; the volumetric flask was then filled to the final volume with methanol. After filtration through 0.45 µm PTFE filter, the sample (10 µl) was analyzed by HPLC-UV.

*2.4 HPLC-UV analysis*

All samples were analysed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a 2487 Dual λ Absorbance Detector (Waters Co., Milford, MA, USA). HPLC was equipped with Jupiter C18 column (3u, 300A, 150 x 2.00 mm) (Phenomenex® Castel Maggiore, Bologna, Italy). Solvent A ($H_2O$ + 0.2 % $CH_3COOH$) and solvent B ($CH_3CN$ + 0.1 % $CH_3COOH$) were used for mobile phase. Chromatographic conditions were set as follows: the flow rate was 0.2 ml/min; the column temperature was set at 30°C; the injection volume was 10 µl; gradient elution was performed using eluent A and eluent B: initial condition at 50% A, 0–5 min. isocratic step, 5–12 min. linear gradient to 70% B, 12–19 min. isocratic step at 70%B, 19–20 min. linear gradient to 85% B, 20-24 min. isocratic step at 85% B, 24-25 min. linear gradient to 90% B, 25-35 min. isocratic step at 90% B, 35-36 min. linear gradient to 50% B and requilibration step at 50% B for 14 min. (total analysis time: 50 min.). Chromatograms were acquired at 326 nm. Three injections were performed for each sample. For the quantification of α- and β- acids, a calibration curve was obtained from dilution of ICE-3 standard, according to the official method (Analytica-EBC, section 7, method 7.7).

*2.5 Essential oil extraction and sample preparation for GC-MS analysis*

Prior the analysis, essential oils were extracted by steam distillation with a Clevenger apparatus for four hours. The essential oils were diluted in $CH_2Cl_2$ (1:200 v/v) in a vial and added of a small amount of anhydrous sodium sulfate.

*2.6 GC/MS analysis*

All samples were analysed with a Thermo Scientific (San Jose, CA, USA) TRACE 1300 gas-chromatograph coupled to a Thermo Scientific ISQ™ Single Quadrupole mass

spectrometer. The gas-chromatograph was equipped with Supelcowax 10 (30 m x 0.25 mm, f.t. 0.25 µm) (Supelco, CITTA, PAESE) capillary columns and helium was used as carrier gas (1 ml $min^{-1}$). GC/MS oven temperature gradient started from 50°C, this condition was maintained for 3 minutes, then the temperature was raised to 200°C (5°C/minute). The final temperature was maintained for 18 minutes. The injector was maintained at 230°C operating in split modality, ratio 1:20. The mass spectrometer was equipped with an electron impact source (EI, 70 eV) and the acquisition mode was full scan (from 40 m/z to 500 m/z). A solvent delay time of 4 minutes was applied. The main volatile compounds were identified on the basis of their mass spectra compared with the reference mass spectra libraries (WILEY275, NBS75K, Adams, 2001) and of their calculated Retention Indexes through the application of the Kovats' formula (KI) compared with those reported in the literature. When it was not possible to find the KI in the literature, a tentative identification was obtained by matching with mass spectra libraries data: a match quality of 98% minimum was used as a criterion. In order to determine the RI of the components, a mixture of alkanes (C8-C20) was injected in the GC-MS equipment and analysed under the same conditions described above. The gas-chromatographic signals were manually integrated and the resulting peak areas were compared with the total sum of area and expressed in percentage. The number of volatile compounds identified were the larger peaks and the most suitable to be used to discriminate the Cascade samples grow in different areas.

*2.7 Statistical analysis*

All data obtained were evaluated by the XLSTAT software (Addinsoft SARL, NY, USA). The collected data were analyzed for mean and standard deviation (SD) and also by using univariate analysis of variance (ANOVA). One-way analysis of variance was carried out by applying Tukey's post hoc test ($p \leq 0.05$). Principal component analysis (PCA) has been used as an alternative way to represent inter-individual relationships using phytochemicals data (Mongelli et al.,2015; 2016).

## 3. Results and discussion

As indicated by the European Regulation (CE) N. 1850/2006, concerning row hop and transformed hop products certification, raw hop (dried and not pelletized) must have a humidity content below 14%. In this study the water content of the samples varied between 8.39% (TRENTINO) and 14.76% (VENETO) (data not shown).

### *3.1 HPLC analysis*

From the HPLC analysis, it was possible to highlight significant differences between the studied samples, showing *terroir* influence on the bitter component of hops (Table 4). Differences in the bitter acids content are present in the American samples, where USA 1 (Oregon sample) had higher quantities of α-acids (67.3±3.2 g $kg^{-1}$) than USA 2 (Michigan sample) (47.4±0.3 g $kg^{-1}$), whereas β-acids are more present in the USA 2 sample. A study related to the *terroir* of the cv Amarillo (Van Holle et al.,2017), showed differences in the bitter acids content, and the Washington samples had higher values of α-acids if compared to the sample from Idaho.

Comparing USA1 sample with German and Slovenian Cascade samples, α-acids are significantly higher in USA1 than in Cascade from Slovenia (31.6±0.4 g $kg^{-1}$), whereas German Cascade showed similar α-acids content (Table 4). A similar result was obtained by Forster and Gahr (2014), where α-acids content of the Hallertau Cascade sample were higher than the Yakima Cascade. In our results, USA1 samples showed higher cohumulone content (39.02% of the total α-acids), compared with the German sample (34.98%). Cohumulone content is important as an indicator for the bitterness that hops give to beers, as it has more bittering power than the other bitter acids; this means that cv. Cascade from Oregon, possesses a higher bittering potential.

Slovenian and German Cascade are characterized by higher content of β-acids compared to the reference sample USA1 (Table 4). This could be due to greater mean monthly rainfall recorded in Germany and Slovenia compared to USA1 sample (Table 3). These data confirm the influence of the growing area on bitter acids production.

Regarding Italian Cascade samples, the bitter acids content is variable and is not correlated to latitude (Table 4). α-Acids values range from 26.5 g $kg^{-1}$ for VENETO to

69.3 g $kg^{-1}$ for APULIA Cascade samples (Table 4) and this means that hops from different areas possess different bittering power in beer. The β-acids ranged from 49.0 g $kg^{-1}$ for LIGURIA to 74.7 g $kg^{-1}$ for ABRUZZO samples (Table 4). The majority of samples have higher β-acids content than a-acids, with the exception of TRENTINO, LIGURIA and APULIA samples (Table 4). The highest percentage of cohumulone on the total α-acids is found in TRENTINO sample, with 38.16%, while the sample with the lowest presence of cohumulone on total α-acids is LAZIO (29.3%) (Table 4). However, all samples have a value of cohumulone on total α-acids which is consistent with Cascade cultivar standard values (Haas, 2014).

Xanthohumol, the major hop polyphenol with potent antioxidant activity, is significantly higher in Cascade from Germany (3.8 g $kg^{-1}$), if compared to the other samples (Table 5a,b). This data is in agreement with the study of Forster and Gahr (2014), where Comet and Cascade varieties, collected in USA and Hallertau, were evaluated; the authors highlighted the highest polyphenols content in Hallertau samples. In Italian hop samples xanthohumol contents vary from region to region, but the differences are not correlated with latitude, as TRENTINO (Northern Italy) and APULIA (southern Italy) samples showed similar xanthohumol contents (Table 4).

Meteorological data of the different growing areas do not explain the differences in the amount of xanthohumol and bitter acids. Rybacek (1991) affirms that β-acids content depends on rainfall, but in our study this correlation was not observed. In the study of Kučera and Krofta (2009), a mathematical model for the prediction of α - acids in Saaz variety was developed. The authors assert that the temperature in the stage of flowering (July) affect most of the α - acids production in hops. However, in our samples, a correlation between α - acids content and temperature is not present (Tables 2 and 4). In other studies (Fandiño et al.,2015; Srečec et al.,2013), an influence of rainfall on α- acids content was found, but such data are not confirmed in our study, where no such trend is highlighted (Tables 3 and 4). Although no correlation was found in our study, bitter acids and xanthohumol analysis showed significant differences among Cascade samples, thus confirming the important role of the geographical origin

on the production of these compounds, even if the differences are not related to the parameters taken into consideration.

*3.2 Essential oils yields*

Oil distillation shows differences in yields (Table 5a,b). From a first comparison between the two American samples, USA1 sample shows significantly higher oil yield value (1.34±0.030) than USA2 (0.87±0.054) (Table 5a,b). Similar results were obtained by Van Holle et al., (2017) on cones of the cultivar Amarillo grown in Washington State and Idaho; the samples showed different oil yield, and the greatest amount of oil was obtained in Washington samples.

Data analysis showed no statistical difference in oil content between USA1 (1.34±0.030) and GERMANY (1.39±0.098) samples, while statistical differences were observed when compared with SLOVENIA sample (1.00±0.102) (Table 5a,b). Forster and Gahr (2014) and Forster et al. (2002) compared the brewing characteristics of Comet and Cascade varieties grown in Yakima (USA) and Hallertau (Germany), finding out discordant results of oil content in the two crop years under study.

Italian Cascade samples have an oil content between 0.94±0.063% for APULIA sample and 2.15±0.059% for LIGURIA sample (Table 5a,b). All samples have an oil content superior to the minimum varietal oil content (0.7%) (Haas, 2014) and some Italian Cascade (PIEDMONT, VENETO, LIGURIA and CAMPANIA) have an oil yield higher than the maximum varietal limit (>1.4%) (Haas, 2014), in particular, LIGURIA sample has an oil yield beyond 2.0%. Only APULIA and ABRUZZO samples show oil contents significantly lower than the USA1 Cascade reference. These results show the high adaptability of cultivar Cascade to the Italian environments, and the varied suitability of the studied areas. Capper and Darby (2014) studied the British *terroir*, and the essential oil production of British cultivars grown in England and in other countries, like USA and New Zealand, was evaluated; they observed a lower oil production in such cultivars grown in other countries, and the explanation was that British varieties were less adaptive in new growing regions. In our study, it is instead proven the good

adaptability of the cultivar Cascade, in most of the Italian growing regions. No correlation was instead found between essential oil yield and climate data.

*3.3 GC/MS analysis*

From the GC-MS analyses 19 molecules present at relatively high levels and characterizing hop flavour, were identified (Table 5a,b). Differences between the analyzed samples occur; α-pinene, limonene, perillene, β-copaene, β-caryophyllene, trans-β-farnesene, muurolene, β-selinene, α-selinene, τ-cadinene and geraniol are responsible of significant differences among the provenances (Table 5a,b). Regarding the two USA samples, there are significant differences as to β-selinene, α-selinene and geraniol contents (Table 5a,b), these molecules being more abundant in the USA2 sample. Selinene molecules cause a grassy aroma, geraniol instead gives floral notes (Eyres et al., 2008). Differences in aroma compounds were also found by Van Holle et al., (2017) in Amarillo cultivar in northwestern USA. The Authors concluded that it is important to know the *terroir* in which a given hop is grown to obtain the desired hoppy aroma in brewing.

Similar results were obtained in the comparison between USA1 sample and Germany and Slovenia samples; in the latter countries selinene (Slovenia) and geraniol (Germany and Slovenia) represent the molecules that characterize more hop cones, which means that beers obtained using these hops will be most likely characterized by fresh and flower notes.

In the Italian samples, a high variability can be noticed (Table 5a,b). Significant statistical differences were detected for α-pinene, limonene, perillene, β-copaene, β-caryophyllene, trans-β-farnesene, muurolene, β-selinene, α-selinene, τ-cadinene and geraniol. The highest amount of α-pinene is found in PIEDMONT sample, while the lowest levels are in APULIA and ABRUZZO samples. The limonene aroma characterized TRENTINO Cascade with citrusy notes, whereas it is less present in LAZIO sample. Perillene, with fruity aroma, is more present in LIGURIA sample; copaene, with woody characters, is more present in USA2. Significant differences are also highlighted for β-caryophyllene, more present in USA2 sample and scarce in TRENTINO Cascade. Trans-

β-farnesene is higher in SLOVENIA sample and lower in EMILIA ROMAGNA Cascade. Also, muurolene is higher in SLOVENIA and USA2 samples, and less present in the TRENTINO sample. Regarding the molecules giving grassy and fresh aroma to hops and beers, USA2 sample has the highest amount of β-selinene, α-selinene and τ-cadinene, and the least amount for these molecules is present in TRENTINO Cascade and in USA1 for α-selinene and in EMILIA ROMAGNA and LIGURIA samples for τ-cadinene. Geraniol seems to characterize Italian samples due to its scarcity (Table 5a,b), which means a likely lower floral flavor in the beers obtained using these hops. The humulene/caryophyllene ratio (Table 5a,b), does not evidence statistical differences among the analyzed samples, showing stability in the biosynthesis of these compounds, which seems to be mostly related to varietal characters. These data do not agree with those of Green (1997).

In summary, the SLOVENIA sample seems to be richer in molecules that give hoppy, woody and flower notes; the USA2 sample is instead more characterized by woody, earthy, grassy and floral aroma, quite different characters if compared to USA1, which has the least presence of grassy aromatic compounds. In the Italian samples, TRENTINO seems to be the most characterized by limonene presence.

*3.4 PCA analysis*

With the aim of better understanding differences among the studied samples, PCA analyses were made using bitter acids, xanthohumol, and essential oil content and composition. From the PCA analysis (Figure 1), it is not possible to distinguish groups differing by provenance, but all samples differ from each other. It is however possible to observe 4 clusters, where USA1 reference sample is grouped together with CAMPANIA, VENETO, PIEDMONT and LIGURIA samples, in contrast to USA2 sample, that is instead positioned on the opposite side of the graph near SLOVENIA. APULIA and ABRUZZO, grouped in a single cluster, showing similarities in chemical composition (Figure 1). Another group, formed by GERMANY and LAZIO samples, indicate connection between these samples, despite the geographic differences. The last

cluster is made by two Italian samples, TRENTINO and EMILIA ROMAGNA, from the north of Italy.

The data show the incidence of environmental conditions in Cascade cultivar, in fact every Cascade sample is unique and characterized by peculiar characters that allow a differentiation among the several zones of origin. These results appear to agree with Henning (in Hieronymus, 2012): environment and epigenetics work together to give unique characteristics to hop in a given region; differences in soil composition, temperature and rainfall can influence the DNA methylation process, turning on or off specific genes, so that although DNA is the same, the methylation model changes.

**4. Conclusions**

In this study no clear correlations among samples, with reference to geography and some climatic conditions were found, cone composition resulting remarkably varied in each growing area; this means that these aspects have a strong effect on hop secondary metabolism. In our results, Cascade hops from different provenances possess distinctive characters, related to bitter acids production and essential oils profiles. This indicates that brewers must pay attention to hop provenance when planning a beer, since it is proven that hop cultivars grown in different locations display quite varied compositions, and these differences are likely to influence the final result in beers.

For a better understanding of the phenomenon further research is needed, including more cultivars and soil analysis of the different growing areas, an information that was not possible to obtain from this study.

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**References**

Avelino J., Barboza B., Araya J.C., Fonseca C., Davrieux F., Guyot B., Cilas C. (2005) Effects of slope exposure, altitude and yield on coffee quality in two altitude *terroirs* of Costa Rica, Orosi and Santa Maria de Dota. J Sci Food Agric 85:1869–1876. https://doi.org/10.1002/jsfa.2188.

Barham E. (2003) Translating terroir: the global challenge of French AOC labeling. J Rural Stud 19: 127–138. https://doi.org/10.1016/S0743-0167(02)00052-9.

Capper A, Darby P (2014) What makes British Hops Unique in the world of Hop Growing? British Hop Association. IBD Asia Pacific Convention, 24th March 2014.

Choné X., Van Leeuwen C., Chery P.H., Ribéreau-Gayon P. (2001) *Terroir* influence on water status and nitrogen status of non-irrigated Cabernet Sauvignon (*Vitis vinifera*). Vegetative development, must and wine composition (example of a Medoc top estate vineyard, Saint Julien area, Bordeaux, 1997) S Afr J Enol Vitic 22(1):8-15.

Eyres G., Dufour J.P. (2008) Hop essential oil: Analysis, chemical composition and odor characteristics, in: Beer in Health and Disease Prevention. Academic Press San Diego, CA, 22:239-254. https://doi.org/10.1016/B978-0-12-373891-2.00022-5.

Fandiño M., Olmedo J.L., Martínez E.M., Valladares J., Paredes P., Rey B.J., Mota M., Cancela J.J., Pereira L.S. (2015) Assessing and modelling water use and the partition of evapotranspiration of irrigated hop (*Humulus lupulus*), and relations of transpiration with hops yield and alpha-acids. Ind Crop Prod 77:204-217. https://doi.org/10.1016/j.indcrop.2015.08.042.

Farag M.A., PorzelJürgen A., Ludger S., Wessjohann A. (2012) Metabolite profiling and fingerprinting of commercial cultivars of *Humulus lupulus* L. (hop): a comparison of MS and NMR methods in metabolomics. Metabolomics 8(3):492–507. https://doi.org/10.1007/s11306-011-0335-y.

Fernández-Marín M.I., Guerrero R.F., García-Parrilla M.C., Puertas B., Ramírez P., Cantos-Villar E. (2013)Terroir and variety: Two key factors for obtaining stilbene-enriched grapes. J Food Compos Anal 31(2):191-198. https://doi.org/10.1016/j.jfca.2013.05.009.

Foroni F., Vignando M., Aiello M., Parma V., Paoletti M.G., Squartini A., Rumiati R.I. (2017) The smell of terroir! Olfactory discrimination between wines of different grape variety and different terroir. Food Qual Prefer 58:18–23.

Forster A., Beck B., Schmidt R., Jansen C., Mellenthin A. (2002) Über die Zusammensetzung von niedermolekularen Polyphenolen in verschiedenen Hopfensorten und zwei Anbaugebieten. Monatsschr f Brauwissenshaft 55(5/6):98–108.

Forster A., Gahr A.A. (2014) Comparison of the analytical and brewing characteristics of Cascade and Comet hop varieties as grown in Yakima (USA) and Hallertau (Germany). Brew Sci, 67:137–148.

Gilbert J.A., van der Lelie D. (2014) Zarraonaindia I. Microbial *terroir* for wine grapes. PNAS 111(1): 5-6. https://doi.org/10.1073/pnas.1320471110.

Green C.P. (1997) Comparison of Tettnanger, Saaz, Hallertau and Fuggle hops grown in the USA, Australia and Europe. J Inst Brew 103:239-243. https://doi.org/10.1002/j.2050-0416.1997.tb00951.x.

Haas I.J. (2014) Barth-Haas hops companion. A guide to the varieties of hops and hop products. Ed Barth-Haas hops companion. Second edition. Yakima, Washington USA.

Hieronymus S. Growing hops, in For the Love of Hops. Brewers Association, Boulder, CO (2012).

Kalua C.M., Allen M.S., Bedgood Jr D.R., Bishop A.G., Prenzler P.D., Robards K. (2007) Olive oil volatile compounds, flavour development and quality: A critical review. Food Chem 100:273–286. https://doi.org/10.1016/j.foodchem.2005.09.059.

Knight S., Klaere S., Fedrizzi B., Goddard M.R. (2015) Regional microbial signatures positively correlate with differential wine phenotypes: evidence for a microbial aspect to *terroir*. Sci Rep 5:14233. https://doi.org/10.1038/srep14233.

Kučera J., Krofta, K. (2009) Mathematical model for prediction of alpha acid contents from meteorological data for 'Saaz' aroma variety. In: II International Humulus Symposium Acta Hortic 848:131-140. https://doi.org/10.17660/.

Lenglet F. (2014), Influence of terroir products meaning on consumer's expectations and likings. Food Qual Prefer 32:264-270. https://doi.org/10.1016/j.foodqual.2013.09.003.

Mongelli A., Rodolfi M., Ganino T., Marieschi M., Caligiani A., Dall'Asta C., Bruni R. (2016) Are *Humulus lupulus* L. ecotypes and cultivars suitable for the cultivation of aromatic hop in Italy? A phytochemical approach. Ind Crop Prod 83:693–700. https://doi.org/10.1016/j.indcrop.2015.12.046

Mongelli A., Rodolfi M., Ganino T., Marieschi M., Dall'Asta C., Bruni R. (2015) Italian hop germplasm: characterization of wild *Humulus lupulus* L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis. Ind Crop Res 70:16–27. https://doi.org/10.1016/j.indcrop.2015.02.036

Oberthür T., Läderach P., Posada H., Fisher J.M., Samper L.F., Illera J., Collet L., Moreno E., Alarcón R., Villegas A., Usma H., Perez C., Jarvis A. (2011) Regional relationships between inherent coffee quality and growing environment for denomination of origin labels in Nariño and Cauca, Colombia. Food Policy 36:783-794. https://doi.org/10.1016/j.foodpol.2011.07.005.

Olšovská J., Kameník Z., Čejka P., Jurková M., Mikyška A. (2013) Ultra-high-preformance liquid chromatography profiling method for chemical screening of proanthocyanidins in Czech hops. Talanta 116:919-926. https://doi.org/10.1016/j.talanta.2013.07.067.

Petruzzellis L., Winer R.S. (2016) Rediscovering the essentiality of marketing. Proceedings of the 2015 Academy of Marketing Science (AMS) World Marketing Congress, Springer. 518.

Rybacek V. (1991) Hop production. Ed. Elsevier and Faculty of Agronomy, Agricultural University, Prague, Checz Republic.

Siqueira A.D., Eduardo S., Brondizio E.S. (2011) Local food preference and global markets. Perspectives on açai fruit as *terroir* and a Geographic Indicator product. Appetite 56(2):544-544. https://doi.org/10.1016/j.appet.2010.11.261.

Srečec S., Čeh B., Ciler T.S., Rus A.F. (2013) Empiric mathematical model for predicting the content of alpha-acids in hop (*Humulus lupulus* L.) cv. Aurora. Springer Plus 2(1):59.

Stenroos L.E., Siebert K.J. (1984) Application of pattern-recognition techniques to the essential oil of hops. J Am Soc Brew Chem 42:54–61. https://doi.org/10.1094/ASBCJ-42-0054.

Turbes G., Linscott T.D., Tomasino E., Waite-Cusic J., Lim J. (2016) Meunier-Goddik L., Evidence of *terroir* in milk sourcing and its influence on Cheddar cheese. J Dairy Sci 99:1–11. https://doi.org/10.3168/jds.2015-10287.

Van Holle A., Van Landschoot A., Roldán-Ruiz I., Naudts D., De Keukeleire D. (2017) The brewing value of Amarillo hops (*Humulus Lupulus* L.) grown in northwestern USA: A preliminary study of *terroir* significance. J Brew Distilling 123:312–318. https://doi.org/10.1002/jib.433.

van Leeuwen C. (2010) Soils and terroir expression in wines. Soil and Culture Springer Netherlands 453-465. https://doi.org/10.1007/978-90-481-2960-7_28.

Zoratti L., Jaakola L., Haggman H., Giongo L. (2015) Anthocyanin Profile in Berries of Wild and Cultivated Vaccinium spp. along Altitudinal Gradients in the Alps. J Agri Food Chem 63(39):8641–8650. https://doi.org/10.1021/acs.jafc.5b02833.

**Tables**

Table 1. List and origin of the 13 Cascade hops utilized in the research.

| PROVINCE AND REGION | STATE | ID |
|---|---|---|
| PADOVA (VENETO) | ITALY | VENETO |
| TERAMO (ABRUZZO) | ITALY | ABRUZZO |
| CUNEO (PIEDMONT) | ITALY | PIEDMONT |
| SANZENO (TRENTINO) | ITALY | TRENTINO |
| ROMA (LAZIO) | ITALY | LAZIO |
| BENEVENTO (CAMPANIA) | ITALY | CAMPANIA |
| MODENA (EMILIA ROMAGNA) | ITALY | EMILIA ROMAGNA |
| SAVONA (LIGURIA) | ITALY | LIGURIA |
| FOGGIA (AULIA) | ITALY | APULIA |
| TETTNANG | GERMANY | GERMANY |
| BREZOVICA | SLOVENIA | SLOVENIA |
| OREGON | USA | USA 1 |
| MICHIGAN | USA | USA 2 |

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Table 2. Mean of maximum, minimum and mean (express in °C) monthly temperatures in field collections of the different Cascade growing areas. Temperature data were collected in field by using a temperature data logger (HOBO Pendant® Temperature/Light 64K Data Logger).

| SAMPLE | MARCH | APRIL | MAY | JUNE | JULY | AUGUST | SEPTEMBER |
|---|---|---|---|---|---|---|---|
| Trentino | T MAX 16.0 | T MAX 20.8 | T MAX 22.9 | T MAX 28.0 | T MAX 30.7 | T MAX 29.8 | T MAX 27.3 |
| | T MEAN 11.3 | T MEAN 16.2 | T MEAN 18.2 | T MEAN 22.9 | T MEAN 25.8 | T MEAN 24.7 | T MEAN 22.2 |
| | T MIN 4.1 | T MIN 9.2 | T MIN 10.9 | T MIN 15.9 | T MIN 18.6 | T MIN 16.6 | T MIN 14.7 |
| Piedmont | T MAX 12.6 | T MAX 18.1 | T MAX 18.1 | T MAX 25.4 | T MAX 28.6 | T MAX 27.7 | T MAX 25.1 |
| | T MEAN 8.6 | T MEAN 14.3 | T MEAN 6.9 | T MEAN 21.7 | T MEAN 24.8 | T MEAN 24.0 | T MEAN 20.8 |
| | T MIN 1.9 | T MIN 7.1 | T MIN 10.2 | T MIN 15.4 | T MIN 17.4 | T MIN 16.4 | T MIN 13.7 |
| Veneto | T MAX 14.0 | T MAX 19.4 | T MAX 22.1 | T MAX 26.2 | T MAX 30.2 | T MAX 23.3 | T MAX 19.4 |
| | T MEAN 10.0 | T MEAN 15.2 | T MEAN 18.3 | T MEAN 23.1 | T MEAN 26.6 | T MEAN 23.5 | T MEAN 18.2 |
| | T MIN 8.0 | T MIN 12.3 | T MIN 15.4 | T MIN 20.0 | T MIN 23.1 | T MIN 20.1 | T MIN 17.1 |
| Emilia Romagna | T MAX 14.3 | T MAX 20.2 | T MAX 22.8 | T MAX 27.6 | T MAX 32.2 | T MAX 30.3 | T MAX 27.6 |
| | T MEAN 9.9 | T MEAN 15 | T MEAN 17.7 | T MEAN 22.3 | T MEAN 26.4 | T MEAN 24.3 | T MEAN 21.6 |
| | T MIN 5.3 | T MIN 9.6 | T MIN 12.3 | T MIN 16.9 | T MIN 20.6 | T MIN 18.2 | T MIN 16.2 |
| Liguria | T MAX 14.9 | T MAX 19.2 | T MAX 21.2 | T MAX 24.8 | T MAX 28.9 | T MAX 29.7 | T MAX 27.7 |
| | T MEAN 13.4 | T MEAN 16.8 | T MEAN 19.1 | T MEAN 22.9 | T MEAN 26.7 | T MEAN 27.3 | T MEAN 24.5 |
| | T MIN 11.2 | T MIN 11.2 | T MIN 14.2 | T MIN 18.3 | T MIN 21.2 | T MIN 20.4 | T MIN 16.7 |
| Lazio | T MAX 16.6 | T MAX 22.4 | T MAX 23.0 | T MAX 27.5 | T MAX 32.4 | T MAX 31.4 | T MAX 27.4 |
| | T MEAN 13.1 | T MEAN 18.3 | T MEAN 20.1 | T MEAN 24.4 | T MEAN 28.6 | T MEAN 27.7 | T MEAN 23.6 |
| | T MIN 7.0 | T MIN 10.3 | T MIN 13.1 | T MIN 17.0 | T MIN 19.0 | T MIN 19.2 | T MIN 16.5 |
| Abruzzo | T MAX 14.7 | T MAX 19.3 | T MAX 22.2 | T MAX 27.0 | T MAX 30.1 | T MAX 28.2 | T MAX 26.0 |
| | T MEAN 10.4 | T MEAN 14.5 | T MEAN 17.6 | T MEAN 22.3 | T MEAN 25.5 | T MEAN 23.5 | T MEAN 20.7 |
| | T MIN 6.1 | T MIN 9.8 | T MIN 11.9 | T MIN 16.6 | T MIN 19.7 | T MIN 17.8 | T MIN 15.8 |
| Campania | T MAX 16.0 | T MAX 22.1 | T MAX 23.6 | T MAX 27.1 | T MAX 30.1 | T MAX 30.1 | T MAX 27.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | T MEAN 11.0 | T MEAN 16.5 | T MEAN 18.4 | T MEAN 23.7 | T MEAN 25.3 | T MEAN 24.6 | T MEAN 22.6 |
| | T MIN 6.0 | T MIN 10.1 | T MIN 12.2 | T MIN 18.5 | T MIN 20.8 | T MIN 16.1 | T MIN 16.7 |
| Apulia | T MAX 14.3 | T MAX 21.6 | T MAX 23.3 | T MAX 30.2 | T MAX 33.4 | T MAX 31.6 | T MAX 25.8 |
| | T MEAN 10.9 | T MEAN 16.8 | T MEAN 19.4 | T MEAN 25.6 | T MEAN 28.8 | T MEAN 27.0 | T MEAN 21.6 |
| | T MIN 6.9 | T MIN 10.1 | T MIN 12.7 | T MIN 12.7 | T MIN 20.9 | T MIN 20.2 | T MIN 15.8 |
| Germany | T MAX 9.0 | T MAX 13.3 | T MAX 18.2 | T MAX 21.5 | T MAX 23.3 | T MAX 22.5 | T MAX 19.3 |
| | T MEAN 4.5 | T MEAN 8.3 | T MEAN 12.9 | T MEAN 16.3 | T MEAN 18.1 | T MEAN 17.4 | T MEAN 14.5 |
| | T MIN 0.0 | T MIN 3.4 | T MIN 7.6 | T MIN 11.1 | T MIN 13.0 | T MIN 12.4 | T MIN 9.7 |
| Slovenia | T MAX 18.2 | T MAX 24.9 | T MAX 28.1 | T MAX 32.3 | T MAX 34.2 | T MAX 31.9 | T MAX 30.3 |
| | T MEDIA 13.5 | T MEDIA 13.4 | T MEDIA 15.5 | T MEDIA 20.1 | T MEDIA 23.8 | T MEDIA 21.7 | T MEDIA 18.0 |
| | T MIN -1.7 | T MIN 0.1 | T MIN 5.4 | T MIN 11.8 | T MIN 14.2 | T MIN 9.3 | T MIN 7.7 |
| Usa 1 | T MAX 15.1 | T MAX 23.5 | T MAX 25.8 | T MAX 29.4 | T MAX 31.8 | T MAX 32.8 | T MAX 26.2 |
| | T MEAN 8.2 | T MEAN 14.65 | T MEAN 17.3 | T MEAN 20.4 | T MEAN 23.2 | T MEAN 22.7 | T MEAN 17.0 |
| | T MIN 1.3 | T MIN 5.8 | T MIN 8.8 | T MIN 11.3 | T MIN 14.5 | T MIN 12.6 | T MIN 7.8 |
| Usa 2 | T MAX 11.8 | T MAX 15.2 | T MAX 22 | T MAX 27.6 | T MAX 29.3 | T MAX 29.4 | T MAX 25.2 |
| | T MEAN 6.2 | T MEAN 8.9 | T MEAN 15.2 | T MEAN 20.2 | T MEAN 22.7 | T MEAN 22.6 | T MEAN 19.0 |
| | T MIN 0.5 | T MIN 2.5 | T MIN 8.3 | T MIN 12.8 | T MIN 16.1 | T MIN 15.8 | T MIN 12.8 |

Table 3. Monthly average rainfall (millimeters) in the different geographic areas. Rainfall data were collected in field by using a rainfall data logger (HOBO Pendant® Event Data Logger).

| SAMPLE | MARCH | APRIL | MAY | JUNE | JULY | AUGUST | SEPTEMBER |
|---|---|---|---|---|---|---|---|
| TRENTINO | 45 | 53 | 82 | 81 | 93 | 92 | 67 |
| PIEDMONT | 45 | 53 | 82 | 81 | 93 | 92 | 67 |
| VENETO | 49 | 54 | 52 | 55 | 48 | 60 | 52 |
| EMILIA ROMAGNA | 60 | 67 | 65 | 53 | 43 | 58 | 61 |
| LIGURIA | 90 | 82 | 76 | 38 | 21 | 43 | 55 |
| LAZIO | 77 | 77 | 48 | 36 | 24 | 42 | 80 |
| ABRUZZO | 63 | 55 | 35 | 44 | 34 | 54 | 61 |
| CAMPANIA | 91 | 81 | 52 | 38 | 29 | 35 | 84 |
| APULIA | 43 | 36 | 37 | 36 | 26 | 27 | 46 |
| GERMANY | 59 | 83 | 102 | 127 | 131 | 124 | 93 |
| SLOVENIA | 106 | 91 | 146 | 142 | 72 | 95 | 54 |
| USA 1 | 46 | 37 | 26 | 15 | 26 | 40 | 40 |
| USA 2 | 113 | 181 | 107 | 18 | 94 | 180 | 94 |

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Table 4. Quantitative bitter acids and xanthohumol composition (express in g $kg^{-1}$ DW) of Italian hop germplasm determined by HPLC-UV. In columns, different letters indicate statistically significant differences at 0.95 confidence by Tukey's test.

| SAMPLE | TOT. α ACIDS | TOT. β ACIDS | XANTHOHUMOL | % COH α TOT |
|---|---|---|---|---|
| TRENTINO | $56.3\pm2.5^{abc}$ | $49.6\pm1.9^{c}$ | $2.9\pm0.1^{b}$ | $38.15\pm0.51^{ab}$ |
| PIEDMONT | $37.7\pm1.6^{def}$ | $61.4\pm1.3^{abc}$ | $2.0\pm0.03^{cd}$ | $35.02\pm0.10^{cde}$ |
| VENETO | $26.5\pm1.2^{f}$ | $52.3\pm2.9^{bc}$ | $1.6\pm0.1^{def}$ | $31.33\pm1.25^{fgh}$ |
| EMILIA ROMAGNA | $38.7\pm2.8^{def}$ | $62.7\pm0.5^{abc}$ | $2.1\pm0.1^{cd}$ | $30.61\pm0.68^{gh}$ |
| LIGURIA | $68.9\pm7.4^{a}$ | $49.0\pm4.4^{c}$ | $2.3\pm0.3^{bcd}$ | $306.31\pm0.98^{bc}$ |
| LAZIO | $46.6\pm4.0^{cde}$ | $63.0\pm6.3^{abc}$ | $1.0\pm0.1^{f}$ | $29.39\pm0.20^{h}$ |
| ABRUZZO | $50.9\pm4.3^{bcd}$ | $74.7\pm9.7^{a}$ | $1.9\pm0.2^{cde}$ | $33.32\pm0.13^{def}$ |
| CAMPANIA | $45.1\pm5.1^{cde}$ | $51.9\pm5.4^{bc}$ | $1.2\pm0.2^{ef}$ | $32.00\pm1.00^{fg}$ |
| APULIA | $69.3\pm7.9^{a}$ | $67.1\pm7.7^{abc}$ | $2.9\pm0.4^{b}$ | $32.48\pm0.63^{efg}$ |
| GERMANY | $68.3\pm3.8^{a}$ | $69.1\pm2.6^{ab}$ | $3.8\pm0.2^{a}$ | $34.98\pm0.52^{cde}$ |
| SLOVENIA | $31.6\pm0.4^{ef}$ | $58.2\pm1.2^{abc}$ | $2.0\pm0.1^{cd}$ | $32.56\pm0.62^{cd}$ |
| USA 1 | $67.3\pm3.2^{ab}$ | $51.0\pm2.0^{bc}$ | $2.5\pm0.1^{bc}$ | $39.02\pm0.35^{a}$ |
| USA 2 | $47.4\pm0.3^{cde}$ | $63.8\pm0.3^{abc}$ | $2.4\pm0.1^{bc}$ | $29.92\pm0.01^{gh}$ |

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Table 5a. Mean (±DS) of the essential oil yields and relative content of essential oil constituent, determined by GC–MS, of Cascade hop genotypes. Different letters in each row indicate statistically significant differences mean at 0.95 confidence by Tukey's test.

| | TRENTINO | PIEDMONT | VENETO | EMILIA ROMAGNA | LIGURIA | LAZIO | ABRUZZO |
|---|---|---|---|---|---|---|---|
| **OIL YIELD** | 1.58±0.082$^{c}$ | 1.97±0.039$^{ab}$ | 1.52±0.106$^{c}$ | 1.40±0.179$^{c}$ | 2.15±0.059$^{a}$ | 1.69±0.17$^{bc}$ | 0.95±0.015$^{e}$ |
| **α-PINENE** | 0.70±0.427$^{ab}$ | 1.52±0.274$^{a}$ | 1.04±0.322$^{ab}$ | 0.76±0.411$^{ab}$ | 0.94±0.067$^{ab}$ | 0.77±0.091$^{ab}$ | 0.36±0.089$^{b}$ |
| **MYRCENE** | 27.68±12.528$^{a}$ | 38.98±1.565$^{a}$ | 39.45±2.215$^{a}$ | 28.03±15.416$^{a}$ | 34.88±2.459$^{a}$ | 37.42±0.514$^{a}$ | 30.31±7.096$^{a}$ |
| **LIMONENE** | 1.58±0.797$^{a}$ | 1.22±0.438$^{abc}$ | 0.56±0.066$^{abc}$ | 0.51±0.121$^{abc}$ | 1.18±0.269$^{abc}$ | 0.28±0.027$^{c}$ | 0.40±0.034$^{bc}$ |
| **ALFA PHELLANDRENE** | 0.13±0.061$^{a}$ | 0.51±0.158$^{a}$ | 0.35±0.067$^{a}$ | 0.21±0.099$^{a}$ | 0.15±0.003$^{a}$ | 0.23±0.07$^{a}$ | 0.10±0.045$^{a}$ |
| **PERILLENE** | 0.20±0.167$^{b}$ | 0.26±0.087$^{b}$ | 0.14±0.02$^{b}$ | 0.38±0.303$^{ab}$ | 0.81±0.256$^{a}$ | 0.07±0.019$^{b}$ | 0.20±0.038$^{b}$ |
| **COPAENE** | 0.13±0.05$^{c}$ | 0.21±0.063$^{abc}$ | 0.17±0.005$^{bc}$ | 0.15±0.093$^{c}$ | 0.20±0.025$^{bc}$ | 0.24±0.007$^{abc}$ | 0.26±0.028$^{abc}$ |
| **LINALOOL** | 0.40±0.178$^{a}$ | 0.67±0.147$^{a}$ | 0.53±0.101$^{a}$ | 0.28±0.148$^{a}$ | 0.76±0.034$^{a}$ | 0.37±0.095$^{a}$ | 0.37±0.065$^{a}$ |
| **β-CARYOPHYLLENE** | 3.48±1.66$^{b}$ | 6.54±0.831$^{ab}$ | 6.49±0.254$^{ab}$ | 4.49±3.306$^{ab}$ | 5.03±0.163$^{ab}$ | 8.58±0.460$^{ab}$ | 7.44±0.662$^{ab}$ |
| **TRANS-β-FARNESENE** | 6.49±2.108$^{ab}$ | 8.45±1.584$^{ab}$ | 6.68±0.269$^{ab}$ | 3.82±2.231$^{b}$ | 5.83±0.742$^{ab}$ | 6.26±0.235$^{ab}$ | 7.69±2.499$^{ab}$ |
| **α-HUMULENE** | 7.96±4.354$^{a}$ | 11.84±0.946$^{a}$ | 13.8±0.933$^{a}$ | 10.56±7.169$^{a}$ | 10.16±0.167$^{a}$ | 17.08±0.269$^{a}$ | 18.21±1.678$^{a}$ |
| **α-MUUROLENE** | 0.44±0.249$^{b}$ | 0.80±0.172$^{ab}$ | 0.76±0.061$^{ab}$ | 0.56±0.385$^{ab}$ | 0.81±0.020$^{ab}$ | 0.68±0.004$^{ab}$ | 0.66±0.222$^{ab}$ |
| **β-SELINENE** | 0.63±0.303$^{d}$ | 0.91±0.173$^{bcd}$ | 1.12±0.086$^{bcd}$ | 0.82±0.483$^{cd}$ | 0.86±0.111$^{bcd}$ | 1.22±0.048$^{bcd}$ | 1.76±0.400$^{ab}$ |
| **α-SELINENE** | 0.72±0.19$^{c}$ | 1.08±0.453$^{bc}$ | 1.18±0.176$^{bc}$ | 0.80±0.518$^{bc}$ | 0.95±0.235$^{bc}$ | 1.34±0.113$^{bc}$ | 1.89±0.362$^{ab}$ |
| **τ-CADINENE** | 0.58±0.294$^{b}$ | 1.02±0.239$^{ab}$ | 0.96±0.010$^{ab}$ | 0.57±0.665$^{b}$ | 0.36±0.041$^{b}$ | 1.44±0.088$^{ab}$ | 1.09±0.273$^{ab}$ |
| **GERANIL ACETATE** | 1.51±0.753$^{a}$ | 2.47±0.411$^{a}$ | 2.20±0.192$^{a}$ | 1.08±0.474$^{a}$ | 2.16±0.203$^{a}$ | 1.45±0.155$^{a}$ | 1.48±0.338$^{a}$ |
| **GERANIL ISOBUTIRRATE** | 1.46±0.781$^{a}$ | 1.81±0.401$^{a}$ | 1.40±0.051$^{a}$ | 0.57±0.262$^{a}$ | 1.34±0.127$^{a}$ | 0.73±0.011$^{a}$ | 1.04±0.521$^{a}$ |
| **GERANIL PROPIONATE** | 0.82±0.473$^{a}$ | 1.08±0.223$^{a}$ | 1.06±0.046 | 0.45±0.221$^{a}$ | 0.90±0.070$^{a}$ | 0.51±0.030$^{a}$ | 0.60±0.306$^{a}$ |
| **GERANIOL** | 0.21±0.133$^{bc}$ | 0.48±0.083$^{b}$ | 0.08±0.005$^{c}$ | 0.05±0.012$^{c}$ | 0.24±0.019$^{bc}$ | 0.12±0.036$^{c}$ | 0.15±0.006$^{bc}$ |
| **CARYOPHYLLENE OXIDE** | 1.00±0.62$^{a}$ | 0.67±0.097$^{a}$ | 0.73±0.076$^{a}$ | 1.29±0.217$^{a}$ | 2.22±0.339$^{a}$ | 0.61±0.064$^{a}$ | 1.62±0.464$^{a}$ |
| **Others** | 43.88±26.127$^{a}$ | 19.48±5.02$^{a}$ | 21.3±2.081$^{a}$ | 44.61±11.495$^{a}$ | 30.21±0.435$^{a}$ | 20.61±0.983$^{a}$ | 24.38±2.845$^{a}$ |
| **Humulene/Caryophyillene ratio** | 2.24±0.180$^{a}$ | 1.87±0.080$^{a}$ | 2.11±0.220$^{a}$ | 2.03±0.180$^{a}$ | 2.02±0.030$^{a}$ | 1.81±0.070$^{a}$ | 2.49±0.440$^{a}$ |

Table 5b. Mean (±DS) of the essential oil yields and relative content of essential oil constituent, determined by GC–MS, of Cascade hop genotypes. Different letters in each row indicate statistically significant differences mean at 0.95 confidence by Tukey's test.

| | CAMPANIA | APULIA | GERMANY | SLOVENIA | USA 1 | USA 2 |
|---|---|---|---|---|---|---|
| **OIL YIELD** | 1.97±0.041$^{ab}$ | 0.94±0.063$^{e}$ | 1.39±0.098$^{c}$ | 1.00±0.102$^{e}$ | 1.34±0.030$^{c}$ | 0.87±0.054$^{e}$ |
| **α-PINENE** | 1.15±0.230$^{ab}$ | 0.57±0.265$^{b}$ | 0.88±0.018$^{ab}$ | 0.47±0.138$^{b}$ | 0.69±0.035$^{ab}$ | 0.48±0.065$^{b}$ |
| **MYRCENE** | 43.72±5.205$^{a}$ | 24.67±14.49$^{a}$ | 36.66±5.204$^{a}$ | 15.06±1.435$^{a}$ | 29.02±9.368$^{a}$ | 16.73±1.412$^{a}$ |
| **LIMONENE** | 0.61±0.112$^{abc}$ | 0.63±0.057$^{abc}$ | 1.46±0.152$^{ab}$ | 0.46±0.263$^{abc}$ | 1.08±0.231$^{abc}$ | 0.78±0.066$^{abc}$ |
| **ALFA PHELLANDRENE** | 0.38±0.083$^{a}$ | 0.31±0.024$^{a}$ | 0.25±0.012$^{a}$ | 0.36±0.46$^{a}$ | 0.12±0.024$^{a}$ | 0.03±0.005$^{a}$ |
| **PERILLENE** | 0.13±0.003$^{b}$ | 0.41±0.148$^{ab}$ | 0.11±0.023$^{b}$ | 0.39±0.15$^{ab}$ | 0.27±0.055$^{ab}$ | 0.38±0.013$^{ab}$ |
| **COPAENE** | 0.22±0.061$^{abc}$ | 0.29±0.057$^{abc}$ | 0.27±0.017$^{abc}$ | 0.34±0.024$^{ab}$ | 0.21±0.053$^{abc}$ | 0.38±0.012$^{a}$ |
| **LINALOOL** | 0.57±0.153$^{a}$ | 0.41±0.217$^{a}$ | 0.28±0.361$^{a}$ | 0.55±0.015$^{a}$ | 0.66±0.115$^{a}$ | 0.73±0.019$^{a}$ |
| **β-CARYOPHYLLENE** | 7.42±1.499$^{ab}$ | 7.21±0.965$^{ab}$ | 6.51±0.114$^{ab}$ | 7.48±1.582$^{ab}$ | 4.70±0.994$^{ab}$ | 8.74±0.044$^{a}$ |
| **TRANS-β-FARNESENE** | 7.66±1.794$^{ab}$ | 8.03±2.311$^{ab}$ | 9.06±0.214$^{ab}$ | 9.90±0.685$^{a}$ | 4.86±0.820$^{ab}$ | 7.92±0.729$^{ab}$ |
| **α-HUMULENE** | 12.73±0.993$^{a}$ | 15.25±2.659$^{a}$ | 12.9±1.239$^{a}$ | 14.96±2.757$^{a}$ | 9.48±3.242$^{a}$ | 16.29±1.58$^{a}$ |
| **α-MUUROLENE** | 0.61±0.148$^{ab}$ | 1.05±0.317$^{ab}$ | 0.68±0.115$^{ab}$ | 1.27±0.042$^{a}$ | 0.58±0.145$^{ab}$ | 1.21±0.159$^{a}$ |
| **β-SELINENE** | 1.00±0.304$^{bcd}$ | 1.49±0.238$^{abcd}$ | 1.51±0.103$^{abcd}$ | 1.61±0.012$^{abc}$ | 0.62±0.068$^{d}$ | 2.40±0.089$^{a}$ |
| **α-SELINENE** | 1.19±0.407$^{abc}$ | 1.63±0.241$^{abc}$ | 1.81±0.044$^{abc}$ | 1.93±0.246$^{ab}$ | 0.70±0.003$^{c}$ | 2.71±0.124$^{a}$ |
| **τ-CADINENE** | 1.23±0.354$^{ab}$ | 0.97±0.326$^{ab}$ | 1.26±0.089$^{ab}$ | 1.03±0.433$^{ab}$ | 0.81±0.100$^{ab}$ | 1.92±0.072$^{a}$ |
| **GERANIL ACETATE** | 2.12±0.400$^{a}$ | 1.85±0.791$^{a}$ | 1.74±0.089$^{a}$ | 2.24±0.145$^{a}$ | 2.54±0.376$^{a}$ | 2.30±0.130$^{a}$ |
| **GERANIL ISOBUTIRRATE** | 1.27±0.282$^{a}$ | 1.70±1.623$^{a}$ | 2.01±0.249$^{a}$ | 2.66±0.154$^{a}$ | 1.73±0.026$^{a}$ | 2.63±0.223$^{a}$ |
| **GERANIL PROPIONATE** | 0.86±0.251$^{a}$ | 0.51±0.224$^{a}$ | 0.65±0.09$^{a}$ | 0.61±0.066$^{a}$ | 1.11±0.120$^{a}$ | 0.88±0.063$^{a}$ |
| **GERANIOL** | 0.12±0.058$^{c}$ | 0.24±0.224$^{bc}$ | 0.9±0.021$^{a}$ | 1.18±0.074$^{a}$ | 0.31±0.046$^{bc}$ | 1.15±0.097$^{a}$ |
| **CARYOPHYLLENE OXIDE** | 0.71±0.240$^{a}$ | 2.76±2.559$^{a}$ | 0.59±0.076$^{a}$ | 3.71±1.042$^{a}$ | 1.03±0.197$^{a}$ | 2.32±0.431$^{a}$ |
| **Others** | 16.3±0.178$^{a}$ | 30.05±10.121$^{a}$ | 20.48±5.923$^{a}$ | 33.79±5.139$^{a}$ | 39.48±5.292$^{a}$ | 30.02±0.691$^{a}$ |
| **Humulene/Caryophyillene ratio** | 1.64±0.490$^{a}$ | 2.11±0.080$^{a}$ | 1.98±0.220$^{a}$ | 1.99±0.060$^{a}$ | 1.87±0.170$^{a}$ | 2.18±0.27$^{a}$ |

**Figures**

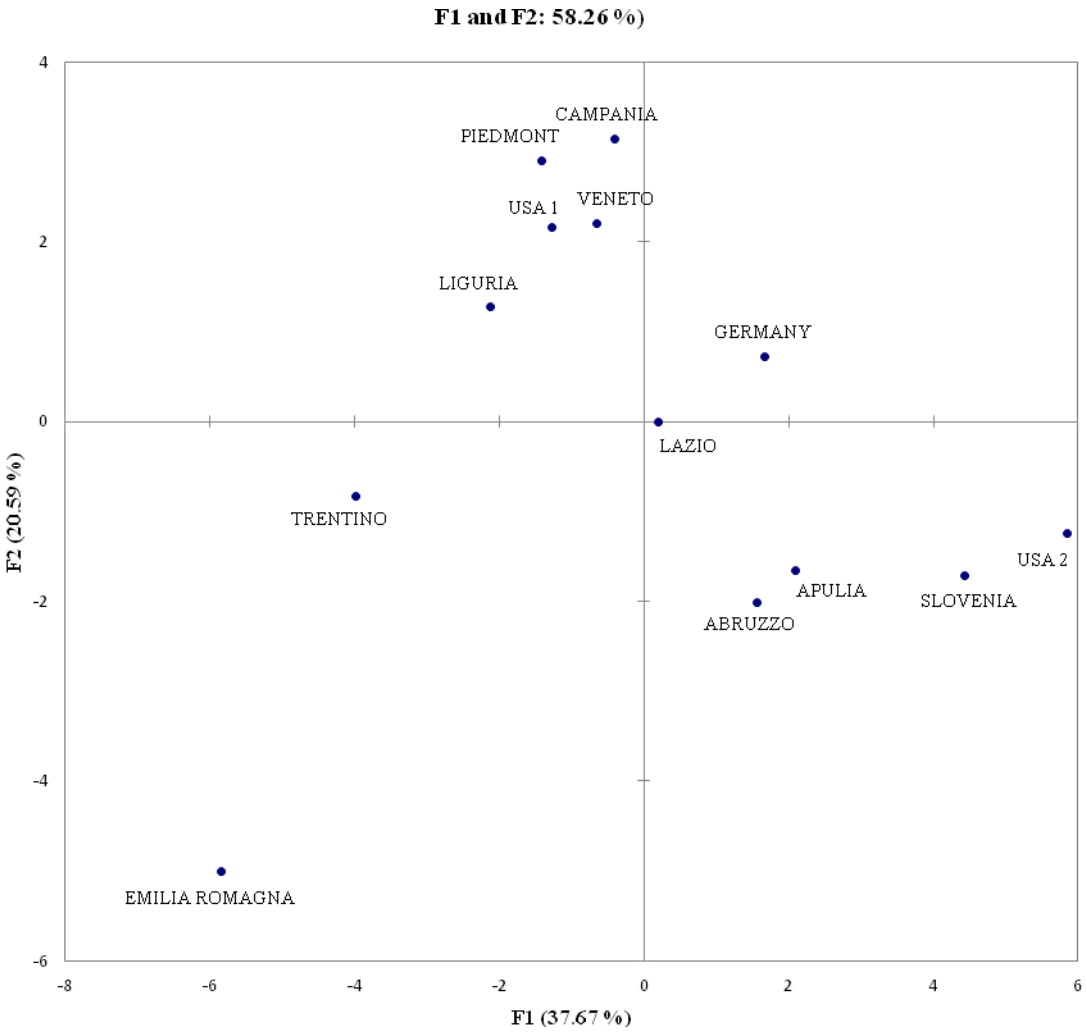


Fig. 1. Score plots obtained from the PCA of hop cones chemical composition (bitter acids, xanthohumol, oil yield, and oil composition) from *H. lupulus* L. cv. Cascade samples from different part of the world.

# Study 2 - Evaluation of two *Humulus lupulus* (L.) *in vitro* propagation methods: petiole regeneration and encapsulation technology

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**Abstract**

Due to the increasing interest of growers in hop cultivation, there is a constant hop plant demand that is not enough satisfied by nursery activity. Biotechnological methods and, specifically, *in vitro* propagation, could offer new opportunities to overcome these limitations, allowing the production of a great number of plantlets, in reduced space and, independently of the season. In this study regeneration from petioles and, for the first time in our knowledge, encapsulation technology have been evaluated to deep the knowledge on hop micropropagation and to try to satisfy a hop nursery need. Results obtained confirmed the possibility of resorting to petiole regeration as a propagation method, only if a precise and continuous check of somaclonal variation is carried out, due to the recording of a small percentage of tetraploids individuated. Moreover, hop microcuttings was demonstrated to be a suitable starting material to encapsulation; indeed, in less than one month, it was possible to obtain a multiplication rate and a very high conversion (up to 82%). These results represent a first step towards the application of this technology to hop for other purposes, such as cryopreservation.

## 1. Introduction

Hop (*Humulus lupulus* L.) is a dioecious perennial species belonging to the *Cannabaceae* family. All over the world, the commercial value of hop is in their lupulin glands, located predominantly on female inflorescences, and in which are accumulated essential oils and resins that confer flavour, bitterness and aroma to beer (Zanoli and Zavatti, 2008).

In line with consumer growing interest for craft beers and for hops endowed with particular phytochemical profiles (Barth and Hass, 2016), in Italy, the culture of hop has recently been attracting the curiosity of growers and researchers. But, the interest of growers in increasing areas dedicated to hop has not gone hand to hand to the hop plant nursery activity, so there are not enough plants to satisfy the grower request (Cherubini and Carbone, 2016). Moreover, the problem of hop plant lack is, ulteriorly, exacerbated by the plant growing slowness and by the season-dependency of traditional propagation material, such as dormant rhizomes and soft wood cuttings (Neve, 1991). To support the nursery sector, researchers are studying different biotechnological methods to speed up hop propagation; specifically, micropropagation can represent a valuable alternative to hop traditional multiplication; indeed, through *in vitro* techniques, it could be possible to obtain a high number of true-to-type plants out of the natural

season, in a relatively short time and in limited space (Barlass and Skene, 1982). *In vitro* plant tissue culture can be performed starting from different types of explants: meristems, characterized by the presence of undifferentiated cells, and differentiated tissues, such as leaf portions, stems, petioles and buds (Smith et al., 1998).

Numerous are the published studies carried out exploiting all the potentialities of hop *in vitro* tissue culture. Most of them report the use of micropropagation to eradicate virus, resorting to meristem culture and cloning (Adams, 1975; Vine and Jones, 1969; Samyn and Welvaert, 1983); Batista et al. (1996) report the successful plant regeneration from hop petioles and stems; Liberatore et al. (submitted) induced organogenesis from leaf portions of hop, cv. Gianni, studying the effect of different types and concentrations of growth regulators and of the time in which the explants were maintained in culture. The same paper reports that the 16.8% of obtained regenerants from leaf culture were mutated. Also, Šuštar-Vozlič et al. (1999) studied the phenomenon of somaclonal variation in hop plants, regenerated from petioles; their results showed that 4 of 46 obtained regenerants were tetraploids. Indeed, although, generally, micropropagation led to the obtainment of plants true to the starting material, sometimes, due to several conditions of *in vitro* tissue culture and, most of all, to the induction of de- and redifferentiation processes (Piccioni et al. 1997; Standardi et al., 1999), various type of changes, known as somaclonal variation, may occur (Larkin and Scowcroft, 1981). If the somaclonal variation occurrence can be a resource for breeding programs, it represents a big problem in nursery activity, because it invalidates the concept itself of micropropagation.

To overcome this problem, it would be possible to resort, as starting material, to vegetative propagules (bulbs, tubers, corms) or shoot cuttings and buds, exploiting the natural ability of the plant species to produce already organized meristematic tissues (Standardi, 1999). Actually, in this way, the de- re-differentiation phase will be avoided, highly reducing the risks of somaclonal variation. Encapsulation technology, resorting to non-embryogenic (unipolar) plant propagules, represents one of the solutions to overcome somaclonal variation and, moreover, presents numerous advantages in the plant specie micropropagation. Specifically, artificial seed or synthetic seed defined as “artificially encapsulated somatic embryos, shoots or other tissues which can be used for sowing under *in vitro* or *ex vitro* conditions” (Aitken-Christie et al., 1995), combines the advantages of clonal propagation (high efficiency production, genetic uniformity of plant material, sanitary plant conditions, perfect reduced spaces requirements) with those of zygotic seeds (easy handling and transportability, storability, reduced dimensions, mechanization potentiality)

(Standardi et al., 1999). Moreover, encapsulation technology represents a valuable tool to facilitate the exchange of sterile plant material among different laboratories, thanks to the reduced dimensions of propagules and to the easiness of transportability (Gray et al., 1995; Piccioni and Standardi, 1995; Redenbaugh, 1993). The germplasm of hop is generally stored in vegetative forms in field gene banks; however, this method of preservation results to be expensive due to high labor costs, necessity of wide spaces, vulnerability to pests and environmental factors. Encapsulation technology has been used as a powerful alternative to traditional methods and ensures long term preservation of plant material (Bouafia et al., 1995; Fabre and Dereuddre, 1990; Micheli et al., 2003; Na and Kondo, 1996; Niino et al., 1992); indeed, this technology is sometimes combined with cryopreservation, such as encapsulation/dehydration that allows the conservation of material without time limit (Benson, 2008; Harding, 2004; Wang et al., 2014).

Several are the studies reporting the use of encapsulation technology in different plant species, resorting to *in vitro* derived unipolar explants, including *Actinidia deliciosa* Liang & Ferguson (Piccioni and Standardi, 1995), *Malus* spp. (Piccioni, 1997), *Morus* indica L. (Bapat et al., 1987), *Olea europaea* L. (Micheli et al., 1998), Carrizo citrange (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.) (Germanà et al., 2011). However, to the best of our knowledge, nothing has been published about the use of encapsulation technology applied to hop propagation.

The aim of this study was to evaluate the possibility of propagating hop, resorting to two *in vitro* tissue culture techniques, and, specifically, petiole regeneration and microcutting encapsulation. With this aim, different types of growth regulators, at different concentrations, were applied, studying their effect on vegetative performance of the different explant types tested. Since, previous reports on organogenesis (Duncan, 1997; Krishna et al., 2016) indicated a certain somaclonal variation incidence among regenerants, a ploidy analysis was carried out within shoots obtained from petiole in vitro culture.

## 2. Material and methods

### *2.1 Plant material*

Petioles, for the regeneration experiment, and microcuttings, for encapsulation, were isolated from two-month-old plantlets of hop, cv. “Gianni”, maintained, *in vitro*, in a growth chamber, at 25±1°C and light intensity of 20 µmol $m^{-2}$ $s^{-1}$, under 16 h photoperiod.

*2.2 In vitro petiole regeneration*

Isolated petioles were cut in sections (0.5 mm) and put in culture, in sterile Petri dishes (ten explants per each Petri dish, ten Petri dishes per treatment). In order to evaluate the influence, on hop petiole regeneration, of type and concentration of growth regulators in the culture medium, six culture media, with the following composition, were tested: (i) **MS-HF:** Murashige and Skoog (MS) salt mixture (Murashige and Skoog, 1962), MS vitamin mixture (1x) (Murashige and Skoog, 1962), 30 g $L^{-1}$ of sucrose, 8 g $L^{-1}$ of agar; (ii) **MS-BAP0:** MS-HF culture medium supplemented with 0.1 µM 1-Naphthaleneacetic acid (NAA); **MS-BAP2**: MS-BAP0 culture medium supplemented with 8.88 µM 6-Benzylaminopurine (BAP); **MS-BAP4**: MS-BAP0 culture medium supplemented with 17.77 µM BAP; **MS-BAP6**: MS-BAP0 culture medium supplemented with 26.66 µM BAP; **MS-BAP8**: MS-BAP0 culture medium supplemented with 35.55 µM BAP. Petri dishes were then sealed and placed in growth chamber, at 25±1°C and light intensity of 20 µmol $m^{-2}$ $s^{-1}$, under 16 h photoperiod. Culture media, after adjusting the pH to 5.8, were sterilized in autoclave for 20 min at 121 °C. Cultures were placed in a growth chamber, at 25±1°C and light intensity of 20 µmol $m^{-2}$ $s^{-1}$, under 16 h photoperiod.

*2.3 Flow cytometry analysis of petiole regenerants*

The ploidy level of regenerants obtained from petiole culture was evaluated using a NovoCyte (Acea Biociences) and compared with that of the mother plant. Each regenerant was analysed three times. About 0.5$cm^2$ of sample (a young leaf of regenerated shoot or of mother plant, cv Gianni) was chopped using a razor blade in a Petri dish containing 0.5mL of extraction buffer (Partec CyStain PI Absolute P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany). The suspension was filtred through a 30-µm filter filter into a 3.5mL plastic tube, to which was then added 2.0mL of Partec CyStain PI Absolute P Staining Buffer, containing 12 µl of Propidium Iodide Solution and 6 µl RNase. Samples were maintained in the dark for 30 min and then analized by flow cytometry. At least 5000 nuclei were analysed in each sample, Acea NovoExpress v.1.25 software was used.

*2.4 Microcutting encapsulation*

Starting from *in vitro* proliferated shoots, uninodal microcuttings (3-4mm long), without leaves and with two axillary buds, were excised and, subsequently, subjected to encapsulation with the following procedure: single microcuttings were immersed in artificial endosperm (AE), composed by half-strength MS medium (Murashige and Skoog, 1962) supplemented with 50 g $l^{-1}$ sucrose and

0.1 µM NAA, enriched with sodium alginate (alginic acid sodium salt, medium viscosity; code 366551 Carlo Erba) solution (2.5%, w/v). Then the alginate coated propagules were subjected to complexation procedure for 35 min in AE enriched with $CaCl_2$ (1.1% w/v) After complexation, the capsules were rinsed three times (15 min each time) with sterile AE (Micheli and Standardi, 2005).

In order to test the effect of type and concentration of growth regulators on hop encapsulated microcuttings vegetative parameters, the following thesis were assessed: **EMC-0**: microcuttings encapsulated Artificial Endosperm (AE); **EMC-0.5**: microcuttings encapsulated in Artificial Endosperm (AE) with 2.22 µM BAP; **EMC-1.0**: microcuttings encapsulated in AE with 4.44 µM BAP; **EMC-1.5**: microcuttings encapsulated in AE with 6.66 µM with BAP; **EMC-2.0:** microcuttings encapsulated in AE with 8.88 µM. Ten capsules were placed in each sterile Petri dishes, containing **MS-HF** culture medium; ten Petri dishes were prepared per each thesis. As control, 100 microcuttings not encapsulated (naked, **MC**) were put in culture on the same culture medium (ten microcuttings per Petri dish).

*2.5 Statistical analysis of data*

In the "petiole regeneration" experiment, the following parameters were measured every week for 14 weeks: the number of viable explants (explants with a green or light brown appearance, without necrosis or yellowing), the number of callus producing explants, the number of root producing explants, the number of shoot producing explants, the number of roots per each explant, the length of roots, the number of shoots per each explant, the length of shoots. Data of viable explants, explants with callus, roots and shoots were used to calculate percentage. At the end of the experiment, Mean Regeneration Time (MRT) and Regeneration Energy (RE) were calculated; the formulae used were the following: MRT = $\sum f*x/\sum f$ (f=number of explants with shoots at day x); RE = (number of leaf portions with shoot regeneration before the peak*100)/total number cultured leaves.

In the "encapsulation" experiment, the following parameters were measured every week for 4 weeks (at the end of this period, no more changes were observed in the cultured explants): viability (percentage of encapsulated explants with a green appearance, without necrosis or yellowing), regrowth (percentage of encapsulated explants producing shoots >4 mm), conversion (percentage of explants with extrusion of shoots and 4 mm long roots), number and length of shoots per explant, and number and length of roots per explant. At the end of the experiment, Mean Regrowth Time (MRET), Regrowth Energy (REE), Mean Conversion Time (MCT) and

Conversion Energy (CE) were calculated; the formulae used were the following: MRET = ∑f*x/∑f (f=number of explants with shoots at day x); REE = (number of explants with shoot regeneration before the peak*100)/total number cultured explants; MCT = ∑f*x/∑f (f=number of explants with roots at day x); RC = (number of explants with root regeneration before the peak*100)/total number cultured explants. Data were used to calculate means.

For both experiments, one-way ANOVA was used to calculate the differences among treatments per each parameter considered; Tukey's test ($p \leq 0.05$) was used for mean separation; when data were not normally distributed, non parametric tests (Kruskal-Wallis' test, $p \leq 0.05$) were carried out and Dwass-Steel-Critchlow-Fligner'test was used for mean separation (SYSTAT 13.1, Systat Software, Inc; Pint Richmond, CA).

## 3. Results

### *3.1 In vitro petiole regeneration*

All petioles preserved their green colour for the first week, then turned light brown. Callus formation was the first response observed in petiole explants; indeed, a yellowish/greenish spongy callus started to develop at the cut ends of explants, from which increased rapidly, up to cover the entire surface of the explants (Fig. 1a). Statistical analysis, carried out at the end of the experiment, on the percentage of explants producing callus did not evidence a significant difference for the factor "Culture Medium Composition" (Table 1). After two weeks of culture, root regeneration was observed in 5 petioles, three of which were cultured on MS-BAP0 medium; the other two petioles with roots were observed in MS-BAP2 and MS-BAP4 culture media (Fig. 1b). Statistical analysis evidenced that the absence of BAP or its presence at low concentrations has a statistically significant influence on root regeneration; indeed, the statistically highest percentage of explants producing roots was observed in media MS-BAP0 and in MS-BAP2 (Table 1). The number of roots produced per single explant was very variable, depending, mainly, on the BAP concentration; up to 10 roots per explants were recorded from one explant cultured on MS-BAP0 medium. Statistical analysis evidenced that the petiole rooting response was influenced by BAP concentration in the culture media, but there was not a direct correlation between the increasing concentration of BAP and the increasing number of roots or *viceversa*; indeed, the statistically highest number of roots was observed in explants cultured in the medium MS-BAP0 (3.2), while the lowest in the medium MS-BAP4 (1.4). The longest roots (26 mm) were observed in medium

MS-BAP2; actually, statistical analysis evidenced that medium MS-BAP2 induced roots to develop, mainly, more than MS-BAP0 and MS-BAP6.

Together with the emergence of the first roots, calli formed organogenic centers in explants cultured in MS-BAP0 and MS-BAP4 media. After 3 weeks in culture, some of the organogenic centers turned in small shoots. Mostly indirect organogenesis was observed (Fig. 1c); but, even though at very low rate (4.48%), also direct shoot regeneration was observed, only in MS-BAP0 medium (Fig 1d). This result is in accordance with those reported by Zayova et al. (2012); indeed, their studies showed that, in eggplant, BAP promotes the organogenesis from callus. However, it is necessary to keep in mind that the pathway leading to shoot regeneration is strongly affected by plant species and genotype (Gercheva et al., 2000; Korban et al., 1992)

Shoot regeneration continued for all the period in which the explants were kept in culture and it was observed from the explants cultured in all media; shoots carried on growing in length and forming well developed leaves (Fig. 1e).

Statistical analysis carried out on the percentage of explants producing shoots did not evidenced a significant difference for the factor “Culture Medium Composition” (Table 1). Equally, regarding the parameter “n° of shoots”, no significant differences have been detected (2.4 shoots per explant, on the average), but it was possible to register a total of 67 adventitious shoots, independently on culture medium considered. Shoot length varied from 1 to 20 mm and statistical analysis showed that explants cultured on the medium MS-BAP8 produce shoots statistically longer than MS-BAP2 and MS-BAP4 (Table 1).

The analysis of the MTR, calculated considering the explant response during the 14 weeks, evidenced that explants cultured on the medium MS-BAP0 needed statistically less time than the ones cultured on media with BAP to produce new shoots; within explants in culture on BAP-containing media (MS-BAP8), the highest concentration of BAP statistically slowed down the regeneration process (Table 1). Same trend was observed, considering RE: actually, MS-BAP0 induced explants to regenerate much more synchronously and faster than the other media considered; culturing petioles on a medium containing a high amount of BAP (MS-BAP8) determined the statistically lower RE (3.32%) (Table 1).

*3.2 Flow cytometry analysis of petiole regenerants*

Cytofluorimetric analysis, carried out on 67 petiole regenerants revealed that 66 regenerants were diploid, as the mother plant (Fig. 2a) and only one of them was tetraploid (Fig. 2b); whereas,

other kind of ploidy variation, such as mixoploid or octoploid, were not recorded. The only mutated regenerant was obtained from one petiole cultured on MS-BAP8. Because of the limited number of obtained tetraploid, it was not possible to carry out a statistical analysis.

*3.2 Microcutting encapsulation*

In the thirty days of the experiment, all microcuttings, both the naked and the encapsulated, maintained their viability, showing a bright green color over the entire period in which they were kept in culture (Fig. 3a). Regrowth (Fig. 3b) varied from 74% to 100% and it was statistically lower for EMC-0 encapsulated microcuttings and for microcuttings encapsulated in artificial endosperm with the highest concentrations of BAP (EMC-2) (Table 2). Together with the naked microcuttings, also the encapsulated ones produced shoots (respectively 1.3 shoots for MC and 1.1 per EMC), demonstrating that hop microcuttings well respond to encapsulation process (Table 2). Statistical analysis revealed that the parameter "n°of shoots" was influenced by BAP concentration; indeed, by increasing the percentage of this growth regulator, the number of produced shoots decreased (Table 2). Medium containing 6.66 µM BAP (EMC-1.5) induced shoots to elongate statistically more than the ones obtained in the other media (Table 2). The analysis of MRET and REE, carried out considering the explant response during 30 days in culture, showed that the statistically shortest time to regrowth (10.8 dd) and the highest REE (9.6%) in microcuttings was observed in encapsulated microcuttings in 6.66 µM BAP (EMC-1.5), respect to microcuttings ancaspulated in EMC-0, meaning that this artificial endosperm composition induces microcutting to regrowth faster and in a more synchronous way (Table 2).

Overall the conversion was high for all explants (Fig. 3c), with the exception of EMC-0 explants that showed a conversion value significantly lower than the other ones (8.0%). Statistical analysis revealed that BAP concentration influenced the number of roots produced per single explant; indeed, the statistically highest number of roots was recorded in microcuttings encapsulated with AE containing 6.66 µM BAP (1.8%), while the absence of BAP or its highest concentration in the AE seems to inhibit the root formation. The longest root (35 mm) was observed in AE containing 8.88 µM BAP; moreover, it seems that BAP induces root elongation, indeed the significantly shorter roots were observed in naked microcuttings (MC) and in the ones encapsulated in artificial endosperm without BAP (EMC-0) (Table 2). Considering the parameters MCT and CE, AE containing 6.66 µM BAP seems to reduce significantly the time needed for the encapsulated microcuttings to produce roots, making the conversion a much more synchronous process.

Overall, EMC-1.5 had the best performance in terms of regrowth, conversion, root number and length, and shoot length, although by adding only 4.44 µM BAP it was possible to obtain results almost comparable with those achieved with a much higher concentration of BAP, reducing significantly the production costs.

**4. Discussion**

As a rule, plant regeneration is influenced by several factors including culture medium composition, the duration of culture, genotype and explant type (Ganeshan et al., 2002; Liu et al., 2010). In hop, numerous are the studies reporting somatic regeneration, starting from different types of explants, such as leaf portions, internodes, petioles (Krishna and Singh 2007). In literature, many authors reported outstanding results in hop regeneration using internodal explants (Motegi 1979; Batista et al. 1996; Gurriarán et al. 1999). However, it is well known that in hop, as in other species, one of the main factors influencing regeneration response is the genotype (Gurriarán et al. 1999).

Therefore, the purpose of this research was to study the regeneration phenomenon from petioles of hop, cv "Gianni", evaluating the effect of the type and the concentration of growth regulators. In this study, callus initiation represented the first manifestation of the organogenic process. Similar to previously obtained results (Koroch et al., 2001), callus, starting to develop at the end of explants, increased notably up to cover the entire petiole surface. The percentage of explants producing callus was very high, although there were no significant differences among considered media; this is in contrast with results obtained by Gurriarán et al. (1999), who reported that caulogenic response of Brewers Gold explants decreased when IAA was added in media containing BAP, pointing out that, in hop, different hop genotypes have different reaction to regeneration inductive treatment.

Together with callus, petiole explants produced roots, either directly or through callus formation. In hop, there is a lack in literature about root induction from petioles; however, even as petiole would seem to be less responsive than other explants sources (Škof et al., 2007), in this investigation, a high rooting potential has been observed; similar result was reported in a previous study (unpublished), on regeneration from leaves of the same hop genotype.

Type and concentration of cytokinin represent a key factor affecting the organogenic ability in hop (Šuštar-Vozlič et al. 1999); however, in this investigation shoot regeneration was detected in all media, regardless of growth regulator concentration, with an average percentage of 13.3%.

Since, indirect organogenesis is well known to induce genetic variability (Larkin and Scowcroft, 1981), petiole regenerated shoots were subjected to cytofluorimetric analysis to check their ploidy level. In this study only one regenerant, over 67 analyzed (1.5%), was tetraploid, a notably lower rate in comparison to that recorded in a previous study, in which 7.2% of regenerants obtained from hop leaves, cv. Gianni, was tetraploid (unpublished); moreover, in this study, the only tetraploid regenerant was obtained from a petiole cultured on the medium containing the highest concentration of BAP (MS-BAP8); also in the just cited study on hop leaf regeneration, the highest percentage of tetraploids (66.7%) was obtained from leaf portions cultured on the same medium. Nevertheless, It should be noted that the mutation rate obtained using petiole explants is considerably lower than that detected in regenerants obtained from leaf regeneration; therefore in hop, although the regeneration from leaves makes it possible to obtain a greater number of regenerants (112 adventitious shoots) in comparison to petioles (67 adventitious shoots), probably the induction of regeneration from petioles could represent a more appropriate method to produce plants true to original starting material; anyway, even if the percentage of tetraploids is very low, before using petiole regeneration as true-to-type propagation method, it will, always, be necessary to check the genetic correspondence of regenerants.

In order to avoid the occurrence of mutations in hop micropropagation process, resorting to encapsulation of unipolar propagules can be a valuable solution; indeed, skipping the de- and re-differentiation phase reduce significantly the mutation occurrence (Standardi and Piccioni 1998). As a matter of fact, several are the scientific studies, also in a specie strictly related to hop, such as *Cannabis sativa* L., reporting that one of the potential advantages of synthetic seed technology is that it allows true-to type plantlet obtainment (Nyende et al., 2003; Lata et al., 2011; Chandrasekhara Reddy et al., 2012). Other than, encapsulation technology has other numerous advantages potential applications, among which the most interesting is its use as a valuable method for mass propagation of plant species; indeed, to date, numerous are the studies concerning the application of encapsulation technology to several plant species, including fruit tree crops, ornamentals, cereals and vegetables (Rai et al., 2009), but until now, this technology was never applied to hop propagation. Several are the factors that markedly influence in vitro encapsulated propagule behaviour, among which the most important are the initial choice of plant materials, both in terms of genotype and of type of explant, artificial endosperm and culture medium composition and growth conditions (Rai et al., 2009). In this study, in which hop microcuttings were encapsulated, different artificial endosperm compositions were tested, in

order to evaluate, first of all, the suitability of hop, cv. Gianni, microcuttings to be encapsulated, then to study how their vegetative performances are influenced by the artificial endosperm composition, in terms of BAP concentration. All the encapsulated explants, independently on artificial endosperm composition, showed a high viability (100%), throughout the experiment, demonstrating that hop microcuttings could absorb the water and nutrients they need from the capsule; results reported in this study are in line with those obtained in other species, such as *Actinidia deliciosa, Malus domestica, Olea europaea, Carrizo citrange* (Gardi et al., 1999; Micheli and Standardi, 2005; Germanà et al., 2011; Micheli et al., 2019).

Researchers working on synthetic seed technology (Adriani et al., 2000; Micheli et al., 2019) agree on the importance of regrowth, but most of all, of conversion in making this technology really valuable. Our results showed that hop microcutting regrowth and conversion were strongly influenced by the presence of growth regulators. Being this the first study on hop synthetic seed technology, there is a complete lack of literature to compare our results with, the only study that could be useful is the one about the nodal segment encapsulation of Cannabis sativa, a plant species genetically similar to hop, indeed, both species belong to *Cannabaceae* family (Lata et al., 2009). In this study, in which thidiazuron, instead of BAP, was added to artificial endosperm, at a way higher concentration (10 fold), up to 77% of conversion was obtained; moreover, in Cannabis, around 21 days were needed to capsules to convert (Lata et al., 2009); both results are similar to those reported in this research; indeed, in hop, up to 82% of conversion was obtained in 20 dd, on the average, but, adding in the artificial endosperm a concentration 10 fold lower of cytokinin.

In this study, BAP was added at different concentrations to induce regrowth and conversion in encapsulated hop, cv. Gianni, microcuttings. After one month of culture, the highest BAP concentration used (8.88 µmol) appears to inhibit the regrowth process in hop, as reported by Badr-Elden (2013) in strawberry capsules; on the contrary, in *Mimosa pudica* L., Banu at al. (2014) observed the best results, in terms of regrowth, from microcuttings encapsulated with artificial endosperm containing the same BAP concentration (8.88 µmol). Conversion of encapsulated hop, cv. Gianni, was highly stimulated by the presence of BAP in the artificial endosperm, independently on its concentration; results in contrast with those reported in this study are those reported in *Celastrus paniculatus* (Fonseka et al., 2019) and in Prata-anã' banana's microshoots, clone Gorutuba, (Pereira et al., 2017) which the best performance, in terms of conversion, was obtained from explants encapsulated in 8.88µmol BAP enriched artificial endosperm.

As above reported in some examples, consulting the literature about the influence of artificial endosperm composition on vegetative parameters of several plant species encapsulated microcuttings, results are extremely different, demonstrating, once more, the strong influence of the genotype on their *in vitro* response.

**5. Conclusion**

A biotechnological approach to propagate *Humulus lupulus* L. represents a valid instrument to obtain an elevate number of plants in a relatively short time. In this work two methods of *in vitro* hop propagation have been described: regeneration from petioles and, for the first time in our knowledge, encapsulation technology. The first one confirmed the possibility of using petioles as a method of hop propagation; however, ploidy analysis detected the presence of a certain, even though low, percentage of tetraploids, and, therefore, the nedd of a continuous check of genetic correspondence, due to the possibility of the occurrence of genetic mutations. Encapsulation technology, resorting to nonembryogenic (unipolar) plant propagules that allow to by-pass the de-re-differentiation phase, represents an alternative to traditional methods exploiting the regeneration process from meristematic centers.

In less than one month, a very high percentage of explants producing shoots was obtained (up to 100%), proving that hop microcuttings are suitable for encapsultation. This result represents a first step towards the applications of the encapsulation technology for other purposes, such as hop germplasm conservation. However, further investigations and insights are required to assess the practical applicability of these techniques to different hop genotypes.

**References**

Adams A.N. (1975). Elimination of viruses from the hop (*Humulus lupulus*) by heat therapy and meristem culture. J. Hort. Sci. 50:151-160.

Adriani M.; Piccioni E.; Standard A. (2000) Effect of different treatments on the conversion of 'Hayward' kiwifruit synthetic seeds to whole plants following encapsulation of in vitro-derived buds. 28(1), 59-67. https://doi.org/10.1080/01140671.2000.9514123.

Bapat V.A., Mhatre M., Rao P.S (1987). Propagation of *Morus indica* L. (mulberry) by encapsulated shoot buds. Plant Cell Rep 6:393–395.

Banu L.A., Rashid M.H.O., Miah M.A.B. (2014). Development of artificial seed and preservation in Mimosa pudica L., an important medicinal plant in Bangladesh. J. Bio-Sci. 22:89-99. https://doi.org/10.3329/jbs.v22i0.30013

Barlass M., Skene K.G.M. (1982). *In vitro* plantlet formation from *Citrus* species and hybrids. Sci Hortic 17:333–341.

Barth-Hass G. (2016). The Barth Report, Nuremberg, Germany.

Batista D., Sousa M.J., Pais M.S. (1996). Plant regeneration from stem and petiole-derived callus of *Humulus lupulus* (hop) clone Bragança and var. Brewers Gold. In Vitro Cell Dev Biol Plant 32: 37–41.

Benson E.E. (2008). Cryopreservation of phytodiversity: a critical appraisal of theory & practice. Crit Rev Plant Sci 27:141–219.

Bouafia S., Lairy G., Blanc A., Bonnel E., Dereuddre J. (1995). Effets d'une pre´culture sur la tole´rance a` la conge´lation dans l'azote liquide des apex axillaires de vitroplants de Solanace´es tube´rife`res (*Solanum phureia* et *S. tuberosum*): e´tude apre`s enrobage et de´shydratation. Acta Bot Gall 142:393–402.

Carbone K., Cherubini D. (2016). Il luppolo fresco "made in Italy": qualità e competitività per l'industria brassicola. In *Conference, Criticità e opportunità per lo sviluppo sostenibile della filiera brassicola,* Roma, 26 Ottobre 2016.

Chandrasekhara Reddy M., Sri Rama Murthy K., Pullaiah T. (2012). Synthetic seeds: A review in agriculture and forestry. Afr. J. Biotechnol. 11, 14254-14275. https://doi.org/10.5897/AJB12.770

Duncan, R.R. (1997). Tissue culture-induced variation in crop improvement. Adv. Agron. 58, 201-240. https://doi.org/10.1016/S0065-2113(08)60256-4.

Fabre J., Dereuddre J. (1990). Encapsulation-dehydration: a new approach to cryopreservation of Solanum shoot tips. Cryo-letters 11: 413–426.

Fonseka D.L.C.K, Wickramaarachchi W.W.U.I., Madushani R.P.S. (2019). Synthetic seed production as a tool for the conservation and domestication of Celastrus paniculatus: A rare medicinal plant. Annu Res Rev Biol 32(4), 1-8. https://doi.org/10.9734/arrb/2019/v32i430092

Gantait S., Kundu S. (2017). Artificial Seed Technology for Storage and Exchange of Plant Genetic Resources in Advanced Technologies for Crop Improvement and Agricultural Productivity, C.P. Mali, Eds, Jodhpur: Agrobios (International).

Ganeshan S., Caswell K.L., Kartha K.K., Chibbar R.N. (2002). Shoot regeneration and proliferation. In: Khachatourians GG, McHughen A, Scorza R, Nip WK, Hui YH (eds) Transgenic plants and crops. Marcel Dekker, Inc, New York, pp 69–84.

Gardi T., Piccioni E., Standardi A. (1999). Effect of bead nutrient composition on regrowth ability of stored vitro-derived encapsulated microcuttings of different woody species. J Microencapsulation 16(1):13–25.

Gercheva P., Andinova T., Ivanova K. (2000). Plant regeneration from leaf tissues of apple cultivars Granny Smith, Morspur Golden and Starkrimson. Bulg J Agric Sci 6:637–643.

Germanà M.A., Micheli M., Chiancone B., Macaluso L., Standardi A. (2011). Organogenesis and encapsulation of *in vitro*-derived propagules of *Carrizo citrange* (*Citrus sinesis*(L.) Osb.×*Poncirius trifoliate*(L.) Raf.). Plant Cell Tissue Organ Cult;106:299–307.

Gray D.J., M.E. Compton, R.C. Harrell, D.J. Cantliffe (1995). Somatic embryogenesis and the technology of synthetic seed. Pages 127–151 in YPS Bajaj, ed. Biotechnology in agriculture and forestry. Vol 30. Somatic embryogenesis and synthetic seed I. Springer, Berlin

Gurriarán M.J., Revilla M.A., Tamés R.S. (1999). Adventitious shoot regeneration in cultures of *Humulus lupulus* L. (hop) cvs. Brewers Gold and Nugget. Plant Cell Rep 18:1007–11. https://doi.org/10.1007/s002990050699

Harding K. (2004). Genetic integrity of cryopreserved plant cells: a review. Cryo Lett 25:3–22.

Krishna H., Alizadeh M., Singh D., Chauhan N., Eftekhari M., Sadh R.K. (2016). Somaclonal variations and their applications in horticultural crops improvement. 3 Biotech 6:54. https://doi.org/10.1007/s13205-016-0389-7

Korban S.S., O'Connor P.A., Elobeidy A. (1992). Effects of thidiazuron, naphthaleneacetic acid, dark incubation and genotype on shoot organogenesis from Malus leaves. J Hortic Sci 67:341–349.

Koroch A.R., Juliani H.R., Kapteyn J., Simon J.E. (2001) *In vitro* regeneration of Echinacea purpurea from leaf explants. Plant Cell Tissue Organ Cult 69:79–83.

Krishna H., Singh S.K. (2007). Biotechnological advances in mango (*Mangifera indica* L.) and their future implication in crop improvement—a review. Biotechnol Adv 25:223–243. https://10.1016/j.biotechadv.2007.01.001

Larkin P.J., Scowcroft W.R. (1981). Somaclonal variation- a novel source of variabilità from cell cultures for plant improvement. Theor Appl Genet 60:197–214.

Lata H., Chandra S., Natascha T., Khan I.A., ElSohly M.A. (2011). Molecular analysis of genetic fidelity in Cannabis sativa L. plants grown from synthetic (encapsulated) seeds following in vitro storage. Biotechnol. Lett. 33, 2503-2508. https://doi.org/10.1007/s10529-011-0712-7

Lata H., Chandra S., Khan I.A., ElSohly M.A. (2009). Propagation through alginate encapsulation of axillary buds of Cannabis sativa L. -an important medicinal plant. Physiol. Mol. Boil. Plant 15, 79-86. https://doi.org/10.1007/s12298-009-0008-8

Liberatore C.M., Mattion G., Rodolfi M., Ganino T., Fabbri A., Chiancone B. (2018). Chemical and physical pretreatments to improve *in vitro* seed germination of Humulus lupulus L., Cv. Columbus. Sci. Hortic. 235, 86–94. https://doi.org/10.1016/j.scienta.2018.02.077

Liu C., Callow P., Rowland L.J., Hancock J.F., Song G-q. (2010). Adventitious shoot regeneration from leaf explants of southern highbush blueberry cultivars. Plant Cell Tissue Organ Cult 103:137–144.

Micheli M., Standardi A., Fernandes da Silva D. (2019). Encapsulation and Synthetic Seeds of Olive (Olea europaea L.): Experiences and Overview, in: Faisal, M., Alatar, A., (Eds.) Synthetic Seeds. Springer, Cham. The Netherlands, pp. 347-361. https://doi.org/10.1007/978-3-030-24631-0_9

Micheli M., Hafiz I.A., Standardi A. (2007). Encapsulation of *in vitro* derived explants of olive (*Olea europaea* L. cv. Moraiolo) II. Effects of storage on capsule and derived shoots performance. Sci Hortic 113:286–292.

Micheli M., Standardi A. (2005). Encapsulation of *in vitro*-derived explants of olive (cv. Moraiolo). In: Effect of pretreatments, their size and the coating. Curr Top Biotecnol 2:81–8.

Micheli M., Gardi T., Standardi A. (2003). La tecnologia dell'incapsulamento per la diffusione e/o la conservazione di materiale vivaistico. Italus Hortus 10(4):259–262.

Micheli M., Mencuccini M., Standardi A. (1998). Encapsulation of "*in vitro*" proliferated buds of olive. Adv Hortic Sc.

Motegi T. (1979). Differentiation of shoots from hop stem callus culture. Kyoyubokenkyu Neupo Iwate Ika Daigaku 14:15–17.

Murashige T., Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15:473–479. http://dx.doi.org/10.1111/ j.1399-3054.1962.tb08052.x

Na H.Y., Kondo K. (1996) Cryopreservation of tissue cultured shoot primordia from shoot apices of cultured protocorms in Vanda pumila following ABA preculture and desiccation. Plant Sci 118:195–201. Neve, R.A., 1991. Hops. Chapman and Hall, New York.

Neve R.A. (1991). Hops. Chapman and Hall, New York.

Niino T, A Sakai, H Yakuwa 1992 Cryopreservation of dried shoot tips of mulberry winter buds and subsequent plant regeneration. Cryo-letters 13:51–54

Nyende A.B., Schittenhelm S., Mix-Wagner G., Greef J. (2003). Production, storability, and regeneration of shoot tips of potato (Solanum tuberosum L.) encapsulated in calcium alginate hollow beads. In vitro Cell. Dev. Biol. Plant 39, 540-544. https://doi.org/10.1079/IVP2003442

Pereira J.C.G., Rocha S.S., Cardoso Nogueira Londe L., Batista Da Mota M.C., Santos Alves P.F., Silva Viana W. (2017). Growth of 'prata-ana' banana's microshoots clone gorutuba from synthetic seeds:substrates and bap concentration. Rev. Bras. Frutic. 39(5), e-892. http://dx.doi.org/10.1590/0100-29452017892

Piccioni E. (1997) Plantlets from encapsulated micropropagated buds of M.26 apple rootstock. Plant Cell Tissue Organ Cult 47:255–260.

Piccioni E., Barcaccia A., Falcinelli M., Standardi A. (1997). Estimating alfalfa somaclonal variation in axillary branching propagation and indirect somatic embryogenesis by RAPD fingerprinting. Int J Plant Sci 158:556–562.

Piccioni E., Standardi A. (1995). Encapsulation of micropropagated buds of six woody species. Plant Cell Tissue Organ Cult 42:221–2261995b .

Rai M.K., Asthana P., Singh S.K., Jaiswal V.S. Jaiswal U. (2009). The encapsulation technology in fruit plants—a review. Biotechnology Advances 27: 671–679.

Redenbaugh K. (1993). Introduction. Pages 3–7 in K Redenbaugh, ed.Synseeds: applications ofsynthetic seeds to crop improvement. CRC, Boca Raton, Fla.

Samy G., Welvoert W. (1983). Producing a "nuclear stock" of virus free hop plants. Med. Fac. Landbouw. Rijksuniv. Gent 48:877-881.

Škof S., Bohanec B., Kastelec D., Luthar Z. (2007). Spontaneus induction of tetraploidy in hop using adventitious shoot regeneration method. Plant Breed 126:416–421.

Smith R.H. (2000). Plant Tissue Culture: Techniques and Experiments, Ed 2. Academic Press, San Diego.

Standardi A., Piccioni E., Micheli M. (1999). Recent strategies in plant biotechnology: Micropropagation and synthetici seed, International Conference Tempus(Phare) CME 03066-97 (Bucharest, march 15-22), pp. 38-53.

Standard A.; Piccioni E. (1998). Recent perspectives on synthetic seed technology using non embryogenic in vitro-derived explants. Int. J. Plant Sci. 159:968–978.

Šuštar-Vozlič J., Javornik B., Bohanec B. (1999). Studies of somaclonal variation in hop (*Humulus lupulus* L.). Phyton – Ann Rei Bot 39:283–287.

Vine S.J., Jones O.P. (1969). The culture of shoot tips of hop (*Humulus lupulus* L) to eliminate viruses. J Hort Sci 44:181–28.

Wang B., Wang R.R., Cui Z.H., Bi W.L., Li J.W., Li B.Q, Ozudogru E.A., Volk G.M., Wang Q.C (2014). Potential applications of cryogenic technologies to plant genetic improvement and pathogen eradication. Biotech Adv 32:583–595.

Zanoli P., Zavatti M. (2008). Pharmacognostic and pharmacological profile of *Humulus lupulus* L. Journal of Ethnopharmacology 116:383–396.

Zayova E., Vassilevska-Ivanova R., Kraptchev B., Stoeva D. (2012). Indirect shoot organogenesis of eggplant (Solanum melongena L.). J. Cent. Eur. Agr. 13(3): 446-457.

**Tables**

Table 1. Influence of "Culture medium composition" on several vegetative parameters of *in vitro* cultured hop, cv. Gianni, petioles, after 14 weeks of culture.

| Culture medium composition | Viability (%) | Explants with callus (%) | Explants with roots (%) | Explants with shoots (%) | N° of roots (n°)* | L. of roots (mm) | N° of shoots (n°)* | L. of shoots (mm) | MRT (dd) | RE (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| MS-BAP0 | 84.1 | 90.7 | 40.0 a | 3.0 | 3.2 a | 5.3 b | 2 | 5.0 ab | 9.5 c | 11.3 a |
| MS-BAP2 | 94.9 | 94.9 | 10.9 ab | 7.1 | 2.5 ab | 11.7 a | 2.8 | 3.9 b | 25.3 b | 7.1 ab |
| MS-BAP4 | 95.7 | 95.7 | 7.3 bc | 7.1 | 1.4 b | 10.8 ab | 2.6 | 4.3 b | 57.3 ab | 6.0 ab |
| MS-BAP6 | 87.3 | 96.4 | 5.8 bc | 8.1 | 3.0 ab | 7.3 b | 2.4 | 5.1 ab | 32.2 b | 5.8 b |
| MS-BAP8 | 94.5 | 91.5 | 2.7 c | 15.0 | 1.7 ab | 8.4 ab | 2.4 | 9.4 a | 61.6 a | 3.3 b |

One-way analysis of variance (ANOVA), $p \leq 0.05$, followed by Tukey's test. *Non parametric test (Kruskal-Wallis' test, $p \leq 0.05$) followed by Dwass-Steel-Critchlow-Fligner'test. Per each column, values followed by different letters are statistically different.

**MS-BAP0:** MS-HF culture medium supplemented with 0.1 µM of NAA; **MS-BAP2:** MS-BAP0 culture medium supplemented with 8.88 µM of BAP; **MS-BAP4:** MS-BAP0 culture medium supplemented with 17.77 µM of BAP; **MS-BAP6:** MS-BAP0 culture medium supplemented with 26.66 µM of BAP**; MS-BAP8:** MS-BAP0 culture medium supplemented with 35.55 µM of BAP **MRT**: Mean Regeneration Time. **RE**: Regeneration Energy.

Table 2. influence of encapsulation and artificial endosperm composition on several vegetative parameters of hop, cv. Gianni, microcuttings, after 4 weeks of culture

| Thesis | Viability (%) | Regrowth (%) | Conversion (%) | N° of shoots n° | L. of shoots (mm) | N° of roots n° | L of roots (mm) | MRET (dd) | REE (%) | MCT (dd) | CE (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MC-HF | 100 | 94.0 a | 72.0 a | 1.3 a | 5.5 c | 1.00 d | 5.8 b | 15.0 ab | 6.4 ab | 17.1 b | 5.9 ab |
| EMC-0 | 100 | 74.0 b | 8.0 b | 1.2 a | 2.5 d | 1.25 c | 2.3 b | 18.1 a | 4.5 b | 28.0 a | 3.6 b |
| EMC-0.5 | 100 | 98.0 a | 78.0 a | 1.1 ab | 13.4 b | 1.32 b | 9.5 a | 6.4 b | 7.6 ab | 24.0 a | 4.8 b |
| EMC-1.0 | 100 | 100.0 a | 68.0 a | 1.1 ab | 11.2 b | 1.29 b | 9.9 a | 13.4 ab | 8.9 ab | 18.3 b | 6.6 ab |
| EMC-1.5 | 100 | 96.0 a | 82.0 a | 1.0 b | 19.3 a | 1.76 a | 13.2 a | 10.8 b | 9.6 a | 15.8 b | 7.2 a |
| EMC-2.0 | 100 | 90.0 b | 67.5 a | 1.0 b | 14.9 b | 1.04 c | 11.1 a | 12.1 ab | 8.2 ab | 16.0 b | 6.7 ab |

One-way analysis of variance (ANOVA), followed by Tukey's test; $p \leq 0.05$. Per each column, values followed by different letters are statistically different.

**MC-HF**: naked microcuttings, cultured on Hormon Free (HF) culture medium; **EMC-0**: microcuttings encapsulated with Artificial Endosperm (AE); **EMC-0.5**: microcuttings encapsulated in Artificial Endosperm (AE) with 2.22 µM BAP; **EMC-1.0**: microcuttings encapsulated AE in 4.44 µM BAP; **EMC-1.5**: microcuttings encapsulated in AE with 6.66 µM with BAP; **EMC-2.0:** microcuttings encapsulated in AE with 8.88 µM BAP. **MRET**: Mean Regrowth Time. **REE**: Regrowth Energy; **MCT**: Mean Regrowth Time. **CE**: Conversion Energy.

**Figures**

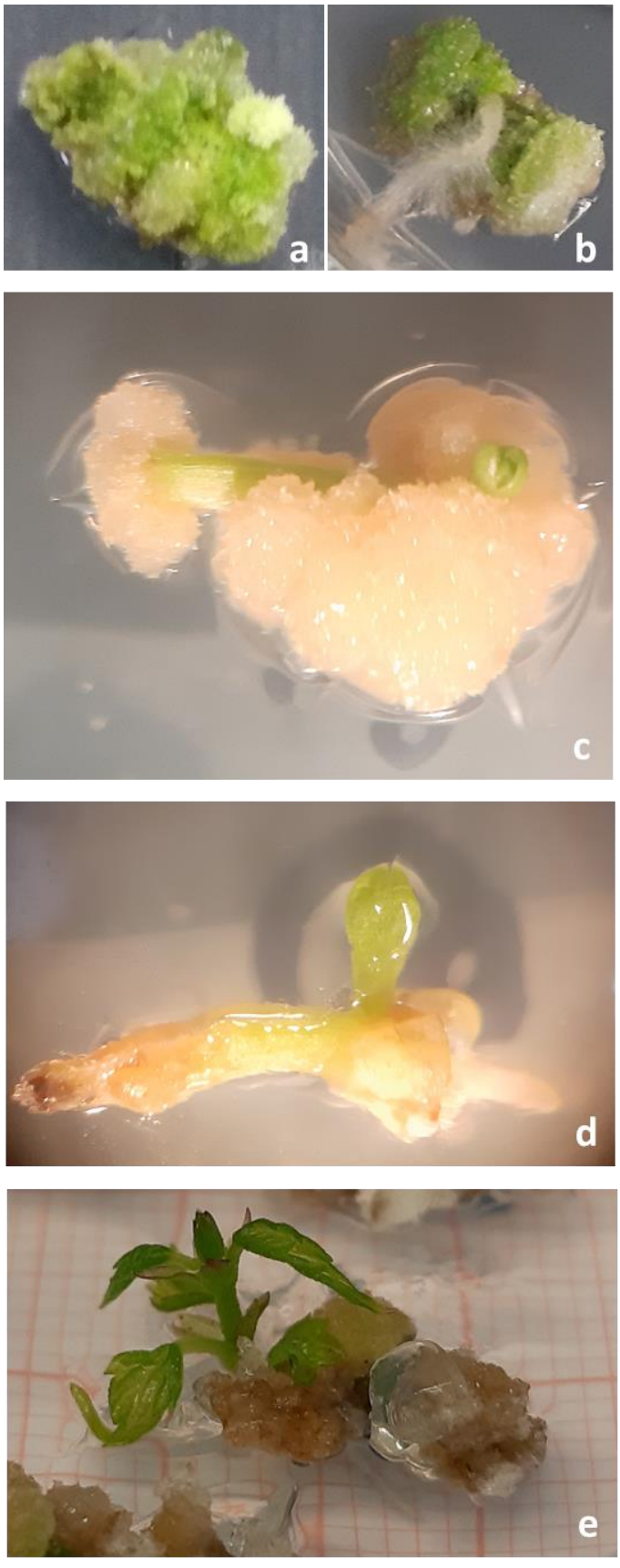


Figure 1. Organogenesis from hop, cv. Gianni, petioles: **a)** callus covering the petiole surface; **b)** first root regeneration; **c)** indirect organogenesis; **d)** direct organogenesis; **e)** shoot development.

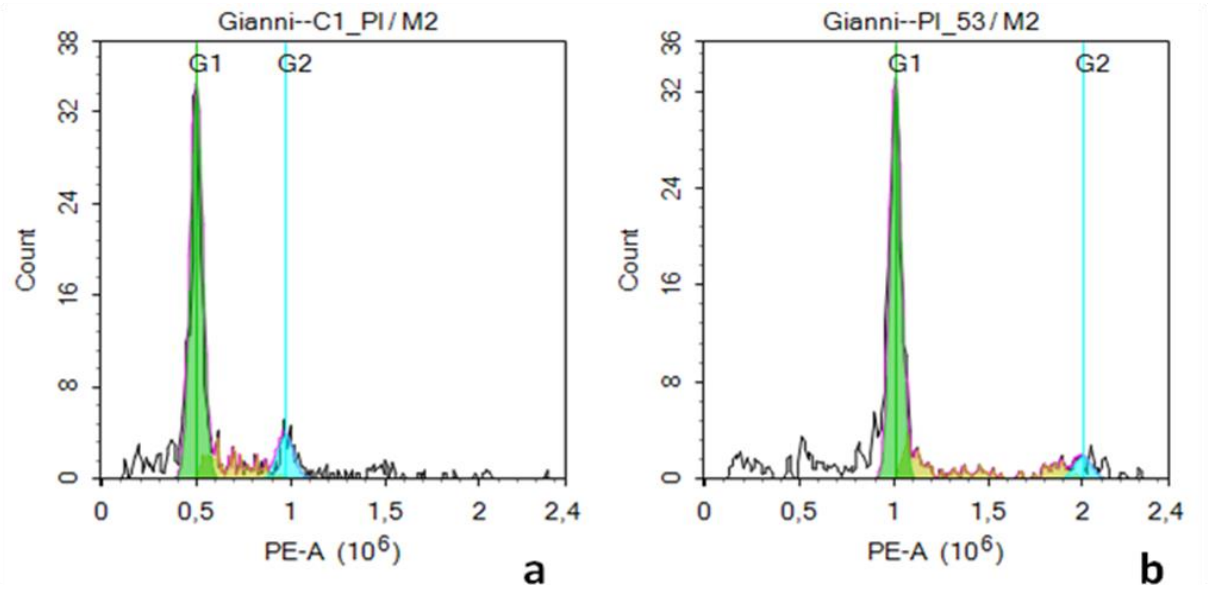


Figure 2. Cytofluorimetric analysis of hop, genotype Gianni: **a)** mother plant; **b)** tetraploid regenerant.

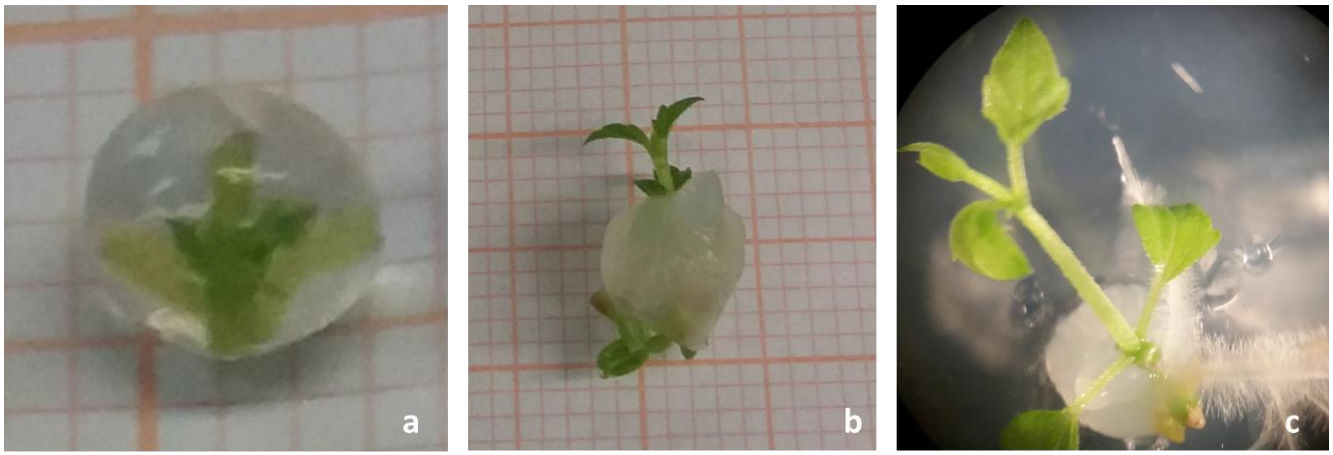


Figure 3. Hop, cv. Gianni, encapsulated microcutting development: **a)** viable encapsulated microcutting; **b)** microcutting regrowth; **c)** microcutting conversion.

***Hop agrobiodiversity enrichment***

## Hop agrobiodiversity enrichment

**Study 1**: Chemical and physical pre-treatments to improve *in vitro* seed germination of *Humulus lupulus* L., cv. Columbus

**Study 2**: Phenological phases of flowering in hop (*Humulus lupulus* L.) and their correspondence with microsporogenesis and microgametogenesis

**Study 3**: Organogenesis and somaclonal variation from leaf *in vitro* culture of *Humulus lupulus* (L.), cv. “Gianni”

# Study 1 - Chemical and physical pre-treatments to improve *in vitro* seed germination of *Humulus lupulus* L., cv. Columbus

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**Abstract**

The most widespread breeding method for hop (*Humulus lupulus* L.), based on the selection of interesting genotypes within segregating progenies, is a time-consuming process, requiring no less than 8-10 years. The process is further slowed down by the very low germination percentage of hop seeds. This study provides an efficient protocol to obtain, in a relatively short time and out of the natural season, a high number of" hop seedlings, cv. "Columbus, evaluating the influence of several pre-treatments (timing of chemical scarification, cold stratification, imbibition in water and in gibberellic acid solutions) on *in vitro* hop seed germinative power; moreover, the application of a sex-linked molecular marker allowed the precocious individuation of seedling gender. At the end of the study, an efficient protocol to obtain a high *in vitro* hop seed germination percentage was set up, resorting to a chemical scarification with sulfuric acid and the use of gibberellic acid; moreover, in a relatively short time, it was possible to determine the sex ratio of cv. "Columbus" obtained seedlings, that was around 70% of females and 30% of males. Further studies will be carried out to increase the final germination percentage and to individuate a sex-linked marker, based on morphological or physiological characters.

**Keywords**: chemical scarification, cold stratification, gibberellic acid, hop, imbibition, sex ratio determination

## 1. Introduction

Hop (*Humulus lupulus* L.), belongs to the order of *Urticales* and family of *Cannabaceae*; it is a perennial, dioecious, climbing plant that grows in the temperate climate zone. Hop is widely cultivated for resins and oils, secreted by lupulin glands, distributed all over the plant, but most of all in its infructescenses (cones), that carry small fruits called achenes, a single-seeded fruit that does not open. Achenes and, consequently, seeds are a precious resource for breeding studies; indeed, they represent a high source of variability within which it is possible to make selection programs and to pursue hop breeding goals, such as disease resistance (*Verticillium* wilt, downy mildew and powdery mildew), increasing resin content and high and stable yield (Stajner et al., 2008). Unfortunately, the use of seeds as a breeding method is a long-term process; indeed, at least 8-10 years are necessary from seed collection to a variety release.

Moreover, hop seeds have a very low germination percentage (3-5%) (Raum 1929; Suciu et al., 1977), due to dormancy that could be caused by seed tegument impermeability and by the presence of resins that prevent the embryo from absorbing water and oxygen (Suciu et al., 1977). Dormancy can be removed resorting to several treatments, such as cold stratification (Fang et al., 2003; Frisby and Seeley 1993; Garcìa-Gusano et al., 2004; Haut 1934; Mehanna et al., 1985; Seeley et al., 1998; Zhou et al., 2003), application of growth regulators and physical and chemical treatments (Duan et al., 2004; Fang et al., 2003; Macchia et al., 2001; Rascio et al., 1998; Rehman et al., 2000; Wada and Reed, 2011a,b). To better control breaking dormancy factors, to obtain a higher number of viable seedlings and to further accelerate the breeding process, hop seeds can be *in vitro* cultured, regardless of the natural season. Unfortunately, research reporting *in vivo* hop seed germination is scarce (Haunold and Zimmermann, 1974; Keller 1953; Suciu et al., 1977; William and Weston, 1957), and there is a complete lack of information about *in vitro* hop seed germination.

On the contrary, several are the studies investigating the *in vitro* seed germination in other crops such as papaya (Bhattacharya and Khuspe, 2001), olive (Germanà et al., 2009; Germanà et al., 2014) and apricot (Burgos and Ledbetter, 1993). As already reported *in vivo*, pre-treatments, such as different temperature of culture, light, presoaking in water or gibberellic acid ($GA_3$) solutions, removal of seed coat, have been used to stimulate *in vitro* seed germination of various crops (Bhattacharya and Khuspe, 2001; Ghimeray et al., 2014; Khanna et al., 2014).

Breeding programs for hop are slowed down by the uncertain gender of F1 progeny; two seasons of growth are, indeed, necessary for the definitive sex determination, based on physiological and morphological characters (McAdam et al., 2013). The individuation in hop of sex-linked molecular markers helped to overcome these problems, drastically reducing breeding program costs and duration (McAdam et al., 2013). For this reason, many researches have been carried out investigating sex determination in hop (McAdam et al., 201; 3Polley et al., 1997; Shephard et al., 2000), setting up the use of numerous molecular markers associated with the Y chromosome, such as RAPD markers (Buck et al., 2009; Polley et al., 1997), inter simple sequence repeat (ISSR) markers (Danilova and Karlov, 2006), microsatellites (Jakse et al., 2008; Rodolfi et al., 2015) and cytogenetic markers (Divashuk et al., 2011). Other than resorting to molecular marker analysis, several studies were carried out, in many species, but not in hop, aiming at establishing a relationship between seedling gender and some characters easy to be observed, such as germination rate and dormancy breaking (Korpelainen, 2002; Purrington and Schmitt, 1995).

In this research, to our knowledge for the first time, seeds of the hop cultivar "Columbus" were *in vitro* cultured. This study provides an efficient protocol to obtain, in a relatively short time and out of the natural season, a high number of hop seedlings, through *in vitro* seed culture; specifically, the influence of several pre-treatments (timing of chemical scarification, cold stratification, imbibition in water and in gibberellic acid solutions) on *in vitro* hop seed germinative power was evaluated and sex ratio of obtained seedlings was early individuated resorting to sex-linked markers.

## 2. Material and methods

### *2.1 Plant material*

Seeds were isolated from cones of hop, cultivar "Columbus", hand harvested in September, from plants, grown in the collection field of Marano sul Panaro (MO, Italy). Columbus, a cross obtained in 1982 is characterized by a superior yield of cones, by a high resins content (Krofta, 2002).

### *2.2 In vitro germination of hop seeds*

Setting up of the protocol for hop seed sterilization

In order to efficiently *in vitro* culture Columbus seeds, a sterilization protocol had to be set up. With this aim, seeds were subjected to several sterilizing treatments before *in vitro* culture. Several trial sets were carried out. In the first set, under flow cabinet, seeds were treated with 96% ethanol

for 5 minutes, then with commercial bleach (3.5% sodium hypochlorite), at different concentrations and for different times; seeds were then washed with sterile distilled water for three times. Specifically, the treatments were the following: 1) 25% bleach for 20 min; 2) 25% bleach for 40 min; 3) 50% bleach for 20 min; 4) 50% bleach for 40 min. In the second set of trials, seeds were pre-treated, before the proper sterilization: 1) no pre-treatment; 2) 95-97% $H_2SO_4$ (Sigma-Aldrich, Germany) for 5 min; 3) 100% methanol (VWR Chemicals, France) for 5 min; 4) 96% ethanol (VWR Chemicals, France) for 5 min. After the pre-treatments, seeds were sterilized under flow cabinet with 100% ethanol for 5 minutes, 25% bleach for 20 min and washed with sterile distilled water for three times. In the third set of trials, some pre-treatments were combined: 1) no pre-treatment; 2) 95-97% $H_2SO_4$ for 5 min; 3) 100% methanol for 5 min; 4) 95-97% $H_2SO_4$ for 5 min and 100% methanol for 5 min; after the pre-treatments, seeds were sterilized as above reported.

Sterilized seeds were placed in sterile Petri dishes (60x15 mm) (ten seeds for each Petri dish, ten Petri dishes per treatment) containing 10 ml of culture medium. Culture medium used (HF) contained Murashige and Skoog (1962) salt mixture at half concentration, Murashige and Skoog ()1962 vitamin mixture (1x) and 30 g/l of sucrose (pH 5.8). Petri dishes were then sealed and placed in growth-room in the dark at 25±1°C. Culture media were sterilized in autoclave for 20 minutes at 121 °C. In each trial cultured seeds were monitored, every day, for 7 days, with a stereomicroscope (Leica Microsystems, Germany) to record seed contamination.

Experiment 1: effect of pre-treatments (timing of chemical scarification, duration of cold stratification and imbibition) on in vitro hop seed germination

In order to stimulate hop seed germination, seeds were subjected to a combination of inductive treatments: cold stratification, chemical scarification and imbibition. For cold stratification, seeds were stored for 0, 15, 30 and 90 days at 4°C; for chemical scarification, seeds were treated with 95-97% $H_2SO_4$ for 5 min, before or after cold stratification; for imbibition, before or after cold stratification, scarified seeds were imbibed in sterile distilled water, for 24 hours at 25°C, in the dark. The different treatments, corresponding to the combinations among pre-treatments, are reported in table 1.

After pre-treatments, seeds were sterilized, under flow cabinet, with 96% ethanol for 5 minutes, with 25% commercial bleach for 20 min and washed with sterile distilled water for three times. In

each pre-treatment combination, seeds were *in vitro* cultured on Petri dishes (60x15 mm), containing 10 ml of HF culture medium (10 seeds per Petri dish, 10 Petri dishes per treatment).

Experiment 2: effect of exogenous gibberellic acid on in vitro hop seeds germination

In this experiment, seed germination response to imbibition and to gibberellic acid, added to the imbibition solution, in the culture medium or in both, was evaluated. After being pre-treated with 95-97% $H_2SO_4$ for 5 minutes, seeds were imbibed in sterilized water or in two different $GA_3$ aqueous solutions. Prior to *in vitro* culture, seeds were sterilized under flow cabinet for 5 minutes in 96% ethanol, 20 minutes in 25% commercial bleach and rinsed three times with sterile distilled water. After the sterilization, seeds were cultured directly (C) or imbibed for 24 hours in sterile distilled water (IC) or in sterile $GA_3$ enriched solutions, 0.5mM (I0.5mM) and 1mM (I1mM) (Table 2). Both not-imbibed (C) and imbibed seeds were cultured on the three culture media: HF, HF added with 0.5mg/l of $GA_3$ and HF added with 1.0mgl/l of $GA_3$ (Table 2). In each treatment 100 seeds were cultured, 10 seeds per each Petri dish (60x15 mm), each containing 10 ml of medium (10 Petri dishes per treatment).

*2.3 In vivo seedling acclimatization*

Seedlings forming the first photosynthetically active leaves were moved to jars, containing 100 ml of HF culture medium. When seedlings showed an efficient and well developed root and foliar system, after around 60 days, they were moved from *vitro* to *vivo* conditions. Specifically, plantlets were extracted from the culture medium, the root apparatus was washed with distilled water and seedlings were planted in 2x2 cm plateaux, containing 10 ml of a sand and peat mixture (2:1 w/w). Plateaux were stored in a growth chamber, at 25±1°C at 16 h photoperiod. Seedlings were watered with 2 ml of distilled water once per week. After 30 days, seedlings were planted in 5x5 cm plateaux containing sand and peat mixture (2:1 w/w) and stored at greenhouse conditions.

*2.4 DNA analysis for sex identification*

Simultaneously with the seedling acclimation, a portion of the epigeal system was isolated and used for molecular analysis. Genomic DNA was extracted following the CTAB (cetyl trimethylammonium bromide) procedure (Doyle and Doyle 1987). For DNA amplification one couple of primers, named F0R0, was used, according to Rodolfi and coworkers (2015).

PCR amplification was optimized in thermal cycler MJ PCT 100 Research (Watertown, Mass.), programming a first passage at 95°C for 5 min. followed by 30 cycles of 45" at 94°C, 45" at 60°C, and 90" at 72°C, for denaturation, annealing and primer extension, respectively; at the end of the cycles were allowed 8 minutes of incubation at 72°C. PCR products were analyzed by electrophoresis on horizontal 2% agarose gel, stained by ethidium bromide and visualized under UV light.

*2.5 Data analysis*

From the first day after culture, seeds germination was monitored every day, for 60 days. At the end of both experiments, final germination percentage (FGP), mean germination time (MGT), germination energy (GE), peak value (PV) and germination value (GV) were calculated; the formulae used were the following: FGP=(n° of germinated seeds*100)/total n° of cultured seeds; MGT=∑ƒ*x/∑ƒ (ƒ=seeds germinated at day x) (Kader, 2005); GE=(n° of germinated seeds before the peak*100)/total n° of cultured seeds (Czabator 1962); PV= cum. of ∑ germ/days (Czabator 1962) and GV= PV * Mean Daily Germination (MDG) (Czabator 1962).

Three way ANOVA (influence of duration of cold stratification, timing of chemical scarification and imbibition in water) and two way ANOVA (influence of different concentrations of $GA_3$ in the imbibition solution and in the culture medium) were used to evaluate differences among treatments; mean separation was performed with Tukey's test ($p \leq 0.05$) (SYSTAT 13.1, Systat Software, Inc; Pint Richmond, CA). One-way ANOVA was carried out to evaluate the influence of $GA_3$, whether in the imbibition solution and in the culture medium, but also it was used to evidence the effect of each factor, when previous analysis evidenced a statistical significance.

In order to individuate a sex-linked marker based on seed response, MGT was calculated for the seedlings analysed resorting to a sex-linked molecular marker.

**3. Results**

*3.1 In vitro germination of hop seeds*

Setting up of the protocol for hop seed sterilization

In the first trial, seeds showed a beginning of fungal contamination, already as early as after 24 hours of culture. After one week, the treatment that allowed the best sterilization was that with 25% commercial bleach for 20 min (10% of contaminated seeds). The stereomicroscope observations evidenced that contaminated seeds still presented lupulin grains on their surface (data not shown).

It was hypothesized that fungal spores kept stuck on lupulin grains, reducing the sterilization treatment efficiency. For this reason, the second trial was performed, using chemical products that could dissolve lupulin. The best results were obtained using 95-97% $H_2SO_4$ for 5 min or methanol for 5 min, with a dramatic reduction of contaminated seeds (0% and 2.5% respectively). In the third trial, where the best pre-treatments were combined, no better results were obtained (data not shown). For this reason, the sterilization protocol chosen for hop seeds was the following: pre-treatment with 95-97% $H_2SO_4$ for 5 min, followed by sterilization, under flow cabinet, with 96% ethanol for 5 min, 25% commercial bleach for 20 min and three washes with sterile distilled water.

*In vitro* seedling development

Observations performed for the 60 days of culture allowed the complete description of the germinative process of hop, cv. Columbus, seeds (Fig. 1). In particular, it was quite clear that the chemical scarification pre-treatment caused, besides the elimination of lupulin, also a tegument thinning, that favored radicle protrusion, speeding up the germinative process.

Seeds have started germinating after 24 hours in culture and carried on germinating for the following 60 days; each seed followed normal development, forming, after the radicle, the hypocotyl and the cotyledons (Fig. 1). When seedlings were moved to the jars, they continued their development forming epicotyl and the first photosynthetically active leaflets.

Experiment 1: effect of pre-treatments (timing of chemical scarification, duration of cold stratification and imbibition) on *in vitro* hop seed germination

After 60 days of culture, the influence of the combinations of pre-treatments on seed germination is evidenced, mainly, in FGP and MGT parameters. Statistical analysis evidenced that, among the analysed factors, “Duration of cold stratification” was the decisive factor for the germinative process (Table 3). Mean separation showed that the 15 days cold stratification treatment induced a percentage of seeds to germinate (23.0%) statistically higher than in non-treated seeds (14.6%) (Fig. 2 a). Regarding the MGT, statistical analysis showed that stratified seeds reduced the time needed for germination from 27.9 days for the untreated (C) to 17.5 for 15dd, 13.3 for 30dd and 19.3 for 90dd (Fig. 2 b). The pre-treatments did not affect at all GE, PV and GV parameters (Table 3).

In figure 3 is reported the germination trend, monitored every day, for 60 days, for all the tested treatments. The number of germinated seeds increased progressively until the 60$^{th}$ day in culture,

after which the germination process stopped. Observing the curves (Fig. 3 a, b), it is evident that the germination process of seeds treated with sulfuric acid before the cold stratification treatment (T1) shows a similar trend: seeds stratified for 15 days, indeed, germinated markedly faster and in higher percentage than non stratified seeds or seeds stratified for longer time, independently if they were imbibed or not. The germination curves of seeds treated with sulfuric acid, after the cold stratification treatment (T2) are somehow different (Fig. 3 c, d); the cold pre-treatment appears to have a more intense influence, particularly, seeds stratified for 30 and 90 days began to germinate faster and reached a higher final germination percentage (Fig. 3 d).

Experiment 2: effect of exogenous gibberellic acid on *in vitro* hop seeds germination

The analysis of data, considering the presence or the absence of $GA_3$, demonstrated that the presence of $GA_3$, both in the imbibition solution and in the culture medium, has a statistically significant influence on "Columbus" FGP, from 35.3% in the control to 47.2% in the presence of $GA_3$. The influence of gibberellic acid, whether in the imbibition solution, in the culture medium or in both, is shown in table 4. Specifically, after 60 days of culture, a significant interaction was recorded between the two factors, "Imbibition" and "Culture medium" for the FGP, MGT, GE and PV parameters (Table 4). For all the parameters above cited, statistical analysis showed that, in comparison with the control (C/HF), $GA_3$ at the highest concentration, both in the culture medium or in the imbibition solution, has a positive significant influence on the seed germinative response (Table 4).

To better understand the role of each factor separately, looking at one-way ANOVA of the results for FGP parameter (Fig. 4), it is evident that $GA_3$ gives a lighter effect if it is added to the imbibition solution (Fig. 4 a) than to the culture medium (Fig. 4 b); indeed, whilst if $GA_3$ is added to the imbibition solution, the main statistically significant differences are observed between the control (C) and the 24 hours imbibition treatment in 1mM $GA_3$ solution; if $GA_3$ is added to the culture medium significant statistical differences are observed between media with $GA_3$ (0.5 mg/l and 1mg/l) and the culture medium without $GA_3$ (HF) (Fig. 4b). The stronger influence of the "Culture medium" factor is confirmed for the other considered parameters. The culture medium composition has a greater effect than the imbibition solution on the germinative process, and determines a higher number of early germinated seeds (Fig. 5, Table 4).

As reported for the trial on the influence of pre-treatments, also in this trial a progressive increase of germinated seeds number was observed for the first 60 days of seed culture (Fig. 6). By observing the curves built up to describe the influence of each treatment on the germination process, a similar trend can be noticed; particularly, between the curves representing the germination course of not imbibed and imbibed seeds with sterile distilled water, differences are highlighted for the two control treatments (C/HF and IC/HF) (Fig. 6 a, b); the curves representing the course of germination of imbibed seeds with gibberellic acid have indeed a comparable trend, with a slightest difference in the final germination response rate (Fig. 6 c, d).

3.2 In vivo *acclimatization*

After 60 days in culture, 840 seedlings developed from the 2,800 seeds put in culture, representing a germination percentage of 30%. Seedlings with photosynthetically active leaves, moved to jars, kept developing and formed good foliar and root apparatuses. Of these, only 300 young plantlets (36% of the obtained seedlings) were considered sufficiently developed and strong to be acclimatized *in vivo*. During the acclimatization phase, plantlets became more vigorous, hardened and got ready to be moved to greenhouse conditions. In this last phase, a 50% of survival was recorded, with a total of 154 seedlings acclimated in *in vivo* conditions.

*3.3 DNA analysis for sex identification*

Molecular analysis carried out on acclimatized seedlings allowed the identification of their gender. Specifically, in the experiment 1, 73.1%$\pm$8.3 female seedlings were obtained; while in the experiment 2 the female seedlings were the 67.4%$\pm$5.3. No big differences were observed between the results obtained in the two experiments; for this reason, it is reasonable to state that for the hop cultivar “Columbus”, the female/male ratio is 70%/30%.

The parameter “MGT”, used to evaluate if female seeds behave differently than males, did not highlight any peculiar difference: MGT varied from 11 to 23 days for female and from 13 to 25 for males. Data analysis did not evidence any relationship between seed gender and MGT.

**4. Discussion**

Many temperate species disperse their seeds when environmental conditions are not suitable for seedling survival; for this reason, these plants have developed mechanisms, such as dormancy, that

prevent germination just after seed dispersal (Schütz and Rave, 1999). Seed dormancy and low germination rates are considered a barrier to the *ex situ* cultivation of commercial plant species (Sharma and Sharma, 2010), such as *Humulus lupulus* L. In hop, the hard and waterproof seed coat and the presence of lupulin determine the very difficult spontaneous germination, that reaches 3-5% at most (Raum, 1929; Suciu, 1977).

In spite of the increasing commercial importance of this species and the continuous request of new genotypes carrying peculiar characteristics, very few are the studies, neither *in vivo* nor *in vitro*, dealing with methods to reduce hop seed dormancy and to increase, consequently, germination.

In this study, several methods to set up an efficient protocol to obtain, in a short time, a high number of hop seedlings were tested. First of all, sulfuric acid, used before the *in vitro* sterilization, contributed to reduce the contamination incidence, but most of all determined a thinning in the seed coat that allowed easier water uptake, oxygen diffusion (Smith, 1970; Montorio et al., 1997) and, thus, a cracking within 24 hours. Indeed, sulfuric acid has been used widely to improve germination of several hard-seeded species (Tigabu and Odén, 2001; Wada and Reed, 2011a, b); in hop, sulfuric acid pre-treatments did not seem to improve seed germinative response; for example, William and Weston (1957) registered a damage of root tips after a treatment of 9 minutes and Keller (1953) obtained a very scarce germinative response (4.4%), in comparison with other treatments tested.

Moreover, other than sulfuric acid, several are the pre-treatments that can affect seed dormancy; cold stratification is regarded as the most important way to break dormancy in seeds of summer annuals and most temperate perennials (Baskin and Baskin, 1988; Probert ,1992). Treating seeds with low temperature activates $GA_3$ synthesis that favours embryo growth, until its protrusion from the external structure (Finch-Savage and Leubner-Metzger, 2006; Sharma and Sharma, 2010). In hop, Keller (1953) and Haunold and Zimmerman (1974) obtained a high germination percentage (more than 80%) in cold stratified seeds; moreover, Smith (1939) and Suciu (1977) reported a correlation between hop seed germination percentage and time of cold stratification at 5°C. In this research, in which hop seeds were cold stratified for several periods of time (0, 15, 30 and 90 days), it seems that storing seeds at 4°C contributed to increasing the *in vitro* seed germination percentage (14.6% vs 20%). These results are in accord with Bressman (1931), who reported better *in vivo* germination percentage, resorting to 10 days of cold stratification; but they are in contrast with

Smith (1939) and Suciu (1977), who described that storing hop seeds for more than 15 days at 4°C does not further increase hop seed germination response.

Gibberellic acid has been shown to break dormancy and increase germination in seeds of several genera (Evans 2001; Machado de Mello et al., 2009). The effect of gibberellic acid could be ascribed to stimulus of various hydrolytic enzymes and, consequently, to increased availability of nutrients for embryo growth. $GA_3$ also regulates seed germination by loosening the mechanical restraints of the testa and endosperm to permit easy protrusion of the radicle (Sharma and Sharma, 2010). In hop, previous studies did not observe any positive influence of gibberellic acid treatment on *in vivo* seed germination (Suciu ,1977; Neve, 1991). In the present study, instead, the addition of gibberellic acid in the imbibition solutions and in the culture medium significantly increased *in vitro* seed germination (35.3% vs. 47.2%); moreover, the highest concentration of $GA_3$, added both to the imbibition solution (1mM) and to culture medium (1mg/l), induced the highest percentage of "Columbus" seeds to germinate, also with respect to 15 days cold stratification alone (56.3% vs. 23.0%). Results here reported are in agreement with those reported by Sharma and Sharma (2010) who compared different treatments to stimulate *Inula racemosa* (Hook. f.) seed germination and observed that the imbibition in a 1.0mM of $GA_3$ solution gave better results than cold stratification (30 days at 1°C), combined with imbibition in distilled water for 24 hours (100% vs. 92%).

In this research, in a relatively short time and out of the germination natural season, a satisfying number of perfectly healthy seedlings were obtained and analysed to identify their gender. The use of a sex-linked marker set up by Rodolfi et al. (2015) guaranteed a rapid and reliable result. Molecular markers assure reproducible results, but they need a very expensive technology. Individuating a sex-linked marker, based on physiological and morphological characters, detectable during the early stages of seedling development, could help to determine precociously the gender of seedlings, in a less expensive way. For example, Purrington and Schmitt (1995), suggested that, in *Silene latifolia*, a sex dimorphism in both survivorship and dormancy of buried seeds is detectable. Also in *Rumex acetosa*, Korpelainen (2002) observed that male seeds germinated at a lower rate. The speculation carried out in this study to evaluate a possible relationship between mean germination time and sex in hop, cv. "Columbus", did not highlight any considerable sex-related differences.

**5. Conclusions**

This study, that in our knowledge is the first on *in vitro* culture of hop seeds, gives new insights on the mechanisms of hop seed germination. First of all, a sterilization protocol has been developed for *in vitro* culture hop seeds, minimizing the contamination incidence. Furthermore, to speed up the germination process, hop seeds were treated with physical and chemical agents. Cold stratification for 15 days determined an improvement in seed germination response, in comparison with the non-treated seeds; a better result was obtained adding gibberellic acid in the imbibition solution and in the culture medium, with an increase in *in vitro* hop seed germination from 23% to 56%. Thanks to the molecular analysis carried out for sex determination of obtained seedlings, it was possible to conclude that in the hop cultivar "Columbus", the female/male ratio was 70%/30% and no relationship between mean germination time and seedling gender was detected. Results obtained are really encouraging and will be the base for new studies, aiming at a further increase of hop seed germination percentage. Moreover, thanks to the application of *in vitro* technologies, it was possible, in a relatively short time, to *in vivo* acclimate 154 seedlings that will be soon ready for further breeding programs. What is more, the relatively high number of seedlings was obtained in winter, out of the natural germination season. Further studies will be carried out, combining different pre-treatments, to increase the final germination percentage and further speed up the hop germinative process. Attention will be given to the quality of seedlings coming from the different treatments, in view of their survival rates.

**References**

Baskin, C.C., Baskin, J.M., 1988. Germination ecophysiology of herbaceous plant species in a temperate region. Am. J. Bot. 75,286-305. doi:10.2307/2443896.

Bhattacharya, J., Khuspe, S.S., 2001. *In vitro* and *in vivo* germination of papaya (*Carica papaya* L.) seeds. Sci. Hortic. 91,39-49. doi:10.1016/S0304-4238(01)00237-0.

Bressman, E.N., 1931. Developing new varieties of hops. Science 74,202-203. doi:10.1126/science.74.1912.202-a

Buck, E.J., Wiedow, C., Carlisle, C., Chagné, D., Beatson, R., 2009. Validation of new sex specific DNA markers for hops (*Humulus lupulus* L.). Acta Hortic. (ISHS) 848,323-328. doi:10.1126/science.74.1912.202-a

Burgos, L., Ledbetter, C., 1993. Improve efficiency in apricot breeding: effect of embryo development and nutrient media on *in vitro* germination and seedling establishment. Plant Cell Tiss. Organ Cult. 35:217-222. doi:10.1007/BF00037273.

Czabator, F.J., 1962. Germination value: an index combining speed and completeness of pine seed germination. Forest Science 8,386-396.

Danilova, T.V., Karlov, G.I., 2006. Application of inter simple sequence repeat (ISSR) polymorphism for detection of sex-specific molecular markers in hop (*Humulus lupulus* L.) Euphytica 151,15-21. doi:10.1007/s10681-005-9020-4.

Divashuk, M.G., Alexandrov, O.S., Kroupin, P.Y., Karlov, G.I., 2011. Molecular cytogenetic mapping of *Humulus lupulus* sex chromosomes. Cytogenet Genome Res. 134,213-219. doi:10.1159/000328831.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19,11-15.

Duan, C., Wang, B., Liu, W. et al.,2004. Effect of chemical and physical factors to improve the germination rate of *Echinacea angustifolia* seeds. Colloids Surf. B. Biointerf. 37,101-105. doi:10.1016/j.colsurfb.2004.07.003.

Evans, J., Luna, T., Wick, D. 2001. Propagation protocol for production of container *Pedicularis groenlandica* Retz. plants (172ml containers); Glacier National Park, West Glacier, Montana. In: Native Plant Network. URL: http://www.nativeplantnetwork.org

Fang, S., Yang, W., 2003. Prospects for silviculture and utilization of *Cyclocarya paliurus* resources. Forest. Sci. Technol. 17(1),49-51 (in Chinese with an English abstract)

Finch-Savage, W.E., Leubner-Metzger, G., 2006. Seed dormancy and the control of germination. New Phytol. 171,501-523. doi:10.1111/j.1469-8137.2006.01787.x.

Frisby, J.W., Seeley, S.D., 1993. Chilling of endodormant peach propagules: seed germination and emergence. J. Am. Soc. Hort. Sci. 118,248-252.

Garcia-Gusano, M., Martinez-Gomez, P., Dicenta, F., 2004. Breaking seed dormancy in almond (*Prunus dulcis* (Mill.) D.A. Webb). Sci. Hortic. 99,363-370. doi:10.1016/S0304-4238(03)00117-1.

Germanà, M.A., Chiancone, B., Hammami, S.B.M., Rapoport, H.F., 2014. Olive embryo *in vitro* germination potential: role of explant configuration and embryo structure among cultivars. Plant Cell Tiss. Organ Cult. 118(3),409-417. doi:10.1007/s11240-014-0493-5.

Germanà, M.A., Patricolo, G., Chiancone, B., 2009. *In vitro* germination of stoneless and isolated embryos of 14 Sicilian cultivars of *Olea europaea* L. Acta Hortic 839(1),173-179. doi:10.17660/ActaHortic.2009.839.20.

Ghimeray, A.K., Lee, H.W., Lee, B., Sharma, P., Shim, I.S., Park, C.H., 2014. Effect of storage conditions and scarification on *in vitro* seed germination in *Lorathus tanakae* Hosok. Korean J. Plant Res. 27(3),263-270. doi:10.7732/kjpr.2014.27.3.263.

Haunold, A., Zimmermann, C.E. 1974. Pollen collection, crossing, and seed germination of hop. Crop Sci. 14,774-776

Haut, I.C. 1934. The effect of various low temperatures upon the afteripening of fruit tree seeds. Amer. Soc. Hort. Sei. Proc. 30,365-367.

Jakse, J., Stajner, N., Kozjak, P., Cerenak, A., Javornik, B., 2008. Trinucleotide microsatellite repeat is tightly linked to male sex in hop (*Humulus lupulus* L.). Mol. Breed. 21,139-148. doi:10.1007/s11032-007-9114-x.

Kader, M., 2005. Varying temperature regimes affect osmotically primed sorghum seeds and seedlings. International Sorghum and Millets Newsletter 42,39.

Keller, K.R. 1953. Seed germination in hops, *Humulus lupulus* L. Agron. J. 45,146-150.

Khanna, S., Jenkins, H., Bucalo, K., Determann, R.O., Cruse-Sanders, J.M., Pullman, G.S., 2014. Effects of seed cryopreservation, stratification and scarification on germination for five rare species of pitcher plants. CryoLetters 35,29-39.

Korpelainen, H., 2002. A genetic method to resolve gender complements investigations on sex ratios in *Rumex acetosa*. Mol. Ecol. 11(10),2151-2156. doi:10.1046/j.1365-294X.2002.01593.x.

Krofta, K., 2002. Comparison of quality parameters of Czech and foreign hop varieties. Plant Soil Env. 49,261-268.

Macchia, M., Angelini, L.G., Ceccarini, L., 2001. Methods to overcome seed dormancy in *Echinacea angustifolia* DC. Sci. Hortic. 89,317-324. doi:10.1016/S0304-4238(00)00268-5.

Machado De Mello, A., Streck, N.A., Blankenship, E.E., Paparozzi, E.T., 2009. Gibberellic acid promotes seed germination in *Penstemon digitalis* cv. Husker Red. HortSci. 44,870-873.

McAdam, E.L., Freeman, J.S., Whittock, S.P. et al.,2013. Quantitative trait loci in hop (*Humulus lupulus* L.) reveal complex genetic architecture underlying variation in sex, yield and cone chemistry. BMC Genomics 14,360-386. doi:10.1186/1471-2164-14-360.

Mehanna, H.T., Martin, G.C., Nishijuma, C., 1985. Effects of temperature, chemical treatments and endogenous hormone content on peach seed germination and subsequent seedling growth. Sci. Hortic. 27,63-73. doi:10.1016/0304-4238(85)90056-1.

Montorio, G.A., Braccini, A.L., Scapim, V.R.O., Braccini, M.C.L., 1997. Avaliação de métodos para superação da dormência das sementes de capim braquiária (Brachiaria brizantha cv. Marandu). Revista Unimar, Marília 19,797-809.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15,473-479. doi:10.1111/j.1399-3054.1962.tb08052.x.

Neve, R.A., 1991. Hops. Chapman and Hall, London.

Polley, A., Ganal, M.W., Seigner, E., 1997. Identification of sex in hop (*Humulus lupulus*) using molecular markers. Genome 40(3),357-361.

Probert, R.J., 1992. The role of temperature in germination ecophysiology, in: Fenner, M., (Ed.), Seeds: the ecology of regeneration in plant communities. CAB International, Wallingford, pp. 285-325. doi:10.1079/9780851994321.0261.

Purrington, C., Schmitt, J., 1995. Sexual dimorphism of dormancy and survivorship in buried seeds of *Silene latifolia*. J. Ecol. 83(5),795-800. doi:10.2307/2261416.

Rascio, N., Mariani, P., Vecchia, F.D., et al.,1998. Effects of seed chilling or $GA_3$ supply on dormancy breaking and plantlet growth in *Cercis siliquastrum* L. Plant Growth Regul. 25,53-56. doi:10.1023/A:1005960708857.

Raum, H., 1929. Über sortenwesen im bayerischen hopfenbau und wege der hopfenzüchtung. Fortschr. Landw. 4,342-345.

Rehman. S., Park, I.H., 2000. Effect of scarification, GA and chilling on the germination of goldenrain-tree (*Koelreuteria paniculata Laxm*.) seeds. Sci. Hortic. 85,319-324. doi:10.1016/S0304-4238(00)00126-6.

Rodolfi, M., Mongelli, A., Marieschi, M., Ganino, T., Bruni, R., Torelli, A., 2015. Optimization of PCR marker for early sex identification in Hop, in: EBC-European Brewering Convention, Porto, 24-28 May 2015.

Schütz, W., Rave, G., 1999. The effect of cold stratification and light on the seed germination of temperate sedges (Carex) from various habitats and implications for regenerative strategies. Plant Ecol. 144,215-230.

Seeley, S.D., Ayanoglu, H., Frisby, J.W., 1998. Peach seedling emergence and growth in response to isothermal and cycled stratification treatments reveal two dormancy components. J. Am. Soc. Hort. Sci. 123,776-780.

Sharma, S., Sharma, R., 2010. Seed physiological aspects of pushkarmool (*Inula racemosa*), a threatened medicinal herb: response to storage, cold stratification, light and gibberellic acid. Current Sci. 99(12),1801-1806.

Shephard, H.L., Parker, J.S., Darby, P., Ainsworth, C.C., 2000. Sexual development and sex chromosomes in hop. New Phytol. 148,397-411. doi:10.1046/j.1469-8137.2000.00771.x.

Smith, C.J., 1970. Seed dormancy in *Sabi panicum*. Proceedings of the International Seed Testing Association 36(1),81-97.

Smith, D.C., 1939. Influence of moisture and low temperature on the germination of hop seeds. J. Agric. Res. 58(5),369-382.

Stajner, N., Satovic, Z., Cerenak, A., Javornik, B., 2008. Genetic structure and differentiation in hop (*Humulus lupulus* L.) as inferred from microsatellites. Euphytica 161,301-311. doi:10.1007/s10681-007-9429-z.

Suciu, T., Salontai, A., Muntean, L., Felecan, V., Vaida, L., 1977. Recherches concernant la germination des semences de houblon Notulae Botanicae Hortu Agrobotanici 9,79-84.

Tigabu, M., Odén, P.C. 2001. Effect of scarification, gibberellic acid and temperature on seed germination of two multipurpose *Albizia* species from Ethiopia. Seed Sci. Technol. 29,11-20.

Wada, S., Reed, B.M., 2011a. Optimized scarification protocols improve germination of diverse *Rubus* germplasm. Sci. Hortic. 130, 660-664. doi:10.1016/j.scienta.2011.08.023.

Wada, S., Reed, B.M., 2011b. Standardizing germination protocols for diverse raspberry and blackberry species. Sci. Hortic. 132,42-49. doi:10.1016/j.scienta.2011.10.002.

Williams, I.H., Weston, E.W., 1957. Hop propagation. I. The germination of hop seeds. In: Annual Report. Wye College, Univ. of London, England pp. 108-118

Zhou, L., Wu, J., Wang, S., 2003. Low-temperature stratification strategies and growth regulators for rapid induction *of Paris polyphylla* var. *yunnanensis* seed germination. Plant Growth Regul. 41,179-183. doi:10.1023/A:1027310507257.

**Tables**

Table 1. Combinations of the pre-treatments performed to stimulate hop seed germination

| Treatment | Denomination | Pre-treatments | | |
|---|---|---|---|---|
| | | Timing of chemical scarification | Imbibition | Duration of cold stratification (4°C) |
| 1 | T1/C/0dd | T1 | C | 0dd |
| 2 | T1/C/15dd | T1 | C | 15dd |
| 3 | T1/C/30dd | T1 | C | 30dd |
| 4 | T1/C/90dd | T1 | C | 90dd |
| 5 | T1/IC/0dd | T1 | IC | 0dd |
| 6 | T1/IC/15dd | T1 | IC | 15dd |
| 7 | T1/IC/30dd | T1 | IC | 30dd |
| 8 | T1/IC/90dd | T1 | IC | 90dd |
| 9 | T2/C/0dd | T2 | C | 0dd |
| 10 | T2/C/15dd | T2 | C | 15dd |
| 11 | T2/C/30dd | T2 | C | 30dd |
| 12 | T2/C/90dd | T2 | C | 90dd |
| 13 | T2/IC/0dd | T2 | IC | 0dd |
| 14 | T2/IC/15dd | T2 | IC | 15dd |
| 15 | T2/IC/30dd | T2 | IC | 30dd |
| 16 | T2/IC/90dd | T2 | IC | 90dd |

**T1:** pre-treatment with sulfuric acid for 5 minutes, before the cold stratification; **T2:** pre-treatment with sulfuric acid for 5 minutes, after the cold stratification; **C:** no imbibition; **IC:** sterile distilled water imbibition for 24 hours; **0dd/15dd/30dd/90dd:** cold stratification period at 4°C.

Table 2. Combinations of the imbibition solution composition and the concentration of gibberellic acid, performed to stimulate hop seed germination

| Treatment | Denomination | Imbibition | $GA_3$ concentration in culture medium |
|---|---|---|---|
| 1 | C/HF | C | 0mg/l |
| 2 | C/0.5mg/l | C | 0.5mg/l |
| 3 | C/1.0mg/l | C | 1.0mg/l |
| 4 | IC/HF | IC | 0mg/l |
| 5 | IC/0.5mg | IC | 0.5mg/l |
| 6 | IC/1.0mg | IC | 1.0mg/l. |
| 7 | I0.5mM/HF | I0.5mM | 0mg/l |
| 8 | I0.5mM/0.5mg | I0.5mM | 0.5mg/l |
| 9 | I0.5mM/1.0mg | I0.5mM | 1.0mg/l. |
| 10 | I1mM/HF | I1mM | 0mg/l |
| 11 | I1mM/0.5mg | I1mM | 0.5mg/l |
| 12 | I1mM/1.0mg | I1mM | 1.0mg/l |

**C**: no imbibition; **IC**: imbibition in sterile distilled water for 24 hours; **I0.5mM**: imbibition in 0.5mM $GA_3$ solution, for 24 hours; **I1.0mM**: imbibition in 1.0mM $GA_3$ solution. for 24 hours; **0mg/l**: no $GA_3$ added in the culture medium; **0.5mg/l**: 0.5mg/l $GA_3$ added to the culture medium; **1.0mg/l**: 1.0mg/l $GA_3$ added to the culture medium.

Table 3. Influence of several pre-treatments (timing of chemical scarification, duration of cold stratification and imbibition) on in vitro seed germination of hop, cultivar Columbus, after 60 days in culture

| **Treatment** | **FGP (%)**±SE | **MGT (dd)** ±SE | **GE (%)**±SE | **PV (%/dd)** ±SE | **GV (%/dd)** ±SE |
|---|---|---|---|---|---|
| T1/C/0dd | 10.8±3,6 | 29.4±10,1 | 15.0±2,9 | 0.3±0,1 | 0.1±0,1 |
| T1/C/15dd | 26.0±4,4 | 20.4±1,8 | 13.5±5,0 | 2.1±1,1 | 2.1±0,2 |
| T1/C/30dd | 14.4±5,8 | 12.1±1,3 | 16.0±0,3 | 0.3±0,1 | 0.2±0,1 |
| T1/C/90dd | 14.3±3,7 | 14.4±1,8 | 13.3±0,5 | 1.5±0,6 | 0.4±0,1 |
| T1/IC/0dd | 18.4±4,4 | 27.7±2,7 | 13.9±2,0 | 0.7±0,6 | 0.3±0,2 |
| T1/IC/15dd | 28.4±4,3 | 19.9±4,4 | 17.1±2,6 | 1.4±0,6 | 1.4±0,2 |
| T1/IC/30dd | 16.6±4,1 | 12.3±1,7 | 17.1±2,6 | 0.5±0,1 | 0.2±0,1 |
| T1/IC/90dd | 17.5±6,9 | 19.6±1,4 | 20.2±5,8 | 0.6±0,2 | 0.3±0,1 |
| T2/C/0dd | 10.8±3,6 | 29.4±1,9 | 15.0±2,9 | 0.3±0,1 | 0.1±0,1 |
| T2/C/15dd | 17.7±4,7 | 15.0±2,0 | 17.5±2,1 | 1.6±1,2 | 1.6±0,3 |
| T2/C/30dd | 26.9±6,5 | 14.5±3,3 | 21.0±1,5 | 0.6±0,2 | 0.4±0,1 |
| T2/C/90dd | 24.8±4,3 | 20.4±5,1 | 21.6±4,4 | 1.1±0,2 | 0.7±0,2 |
| T2/IC/0dd | 18.4±3,0 | 25.2±2,3 | 13.9±2,0 | 0.7±0,6 | 0.3±0,2 |
| T2/IC/15dd | 19.7±6,0 | 15.0±2,7 | 16.1±1,0 | 1.0±0,1 | 0.6±0,1 |
| T2/IC/30dd | 21.7±5,4 | 14.2±5,2 | 21.5±4,4 | 0.6±0,1 | 0.3±0,0 |
| T2/IC/90dd | 16.3±6,1 | 22.8±5,6 | 16.9±2,4 | 1.2±0,4 | 0.5±0,1 |
| Statistical analysis of factors[a] | | | | | |
| CHEMICAL SCARIFICATION (CS) | 0.806 | 0.862 | 0.469 | 0.537 | 0.597 |
| IMBIBITION (I) | 0.763 | 0.926 | 0.970 | 0.961 | 0.745 |
| DURATION OF COLD STRATIFICATION (S) | 0.048 | 0.001 | 0.132 | 0.423 | 0.934 |
| CS*S | 0.067 | 0.455 | 0.647 | 0.284 | 0.284 |
| CS*I | 0.209 | 0.869 | 0.949 | 0.588 | 0.680 |
| I*S | 0.685 | 0.649 | 0.709 | 0.688 | 0.777 |
| CS*I*S | 0.878 | 0.993 | 0.174 | 0.537 | 0.888 |

[a]Three-way analysis of variance (ANOVA), followed by Tukey's test; $p \leq 0.05$

**T1:** pre-treatment with sulfuric acid for 5 minutes, before the cold stratification; **T2:** pre-treatment with sulfuric acid for 5 minutes, after the cold stratification; **C:** no imbibition; **IC:** sterile distilled water imbibition for 24 hours; **0dd/15dd/30dd/90dd:** cold stratification period at 4°C.

**FGP**: Final Germination Percentage; **MGT**: Mean Germination Time; **GE**: Germination Energy; **PV**: Peak Value; **GV**: Germination Value.

Table 4. Influence of the gibberellic acid addition to the imbibition solutions and to the culture media on the in vitro seed germination of hop cultivar Columbus, after 60 days in culture.

| **Treatment** | **FGP (%)**±SE | **MGT (dd)** ±SE | **GE (%)**±SE | **PV (%/dd)** ±SE | **GV (%/dd)** ±SE |
|---|---|---|---|---|---|
| C/HF | 10.8±3.6 | 29.6±9.9 | 14.3±3.5 | 0.3±0.1 | 0.1±0.0 |
| C/0.5mg/l | 30.7±6.9 | 19.1±3.0 | 23.0±2.3 | 1.3±0.2 | 1.3±0.5 |
| C/1.0mg/l | 54.6±11.3 | 19.0±2.6 | 44.3±10.8 | 2.2±0.6 | 2.6±0.8 |
| IC/HF | 18.4±4.4 | 24.2±5.4 | 21.0±3.8 | 0.9±0.2 | 0.6±0.2 |
| IC/0.5mg/l | 48.7±7.9 | 20.6±2.6 | 41.8±6.3 | 1.6±0.3 | 1.8±0.7 |
| IC/1.0mg/l | 48.6±11.5 | 17.8±2.1 | 46.0±10.7 | 2.0±0.4 | 2.3±0.7 |
| I0.5mM/HF | 40.3±8.8 | 15.0±3.2 | 43.0±3.4 | 1.6±0.3 | 2.0±0.4 |
| I0.5mM/0.5mg/l | 48.5±7.2 | 22.0±1.1 | 34.0±3.2 | 1.1±0.3 | 1.2±0.3 |
| I0.5mM/1.0mg/l | 46.2±4.7 | 21.0±1.0 | 34.4±3.6 | 1.7±0.1 | 2.1±0.7 |
| I1.0mM/HF | 48.8±9.0 | 17.0±2.5 | 45.6±5.0 | 1.8±0.3 | 1.8±0.3 |
| I1.0mM/0.5mg/l | 42.9±4.9 | 19.6±3.6 | 31.9±2.7 | 1.3±0.1 | 1.4±0.1 |
| I1.0mM/1.0mg/l | 56.3±5,0 | 22.5±1.7 | 42.9±5.9 | 1.8±0.6 | 3.0±1.7 |
| Statistical analysis of factors[a] | | | | | |
| IMBIBITION (I) | 0.021 | 0.351 | 0.067 | 0.161 | 0.354 |
| CULTURE MEDIUM (M) | 0.000 | 0.014 | 0.030 | 0.000 | 0.005 |
| I*M | 0.036 | 0.003 | 0.013 | 0.005 | 0.055 |

[a]Two-way analysis of variance (ANOVA), followed by Tukey's test; $p \leq 0.05$

**C**: no imbibition; **IC**: imbibition in sterile distilled water for 24 hours; **I0.5mM**: imbibition in 0.5mM $GA_3$ solution for 24 hours; **I1.0mM**: imbibition in 1.0mM $GA_3$ solution. for 24 hours; **0mg/l**: no $GA_3$ added in the culture medium; **0.5mg/l**: 0.5mg/l $GA_3$ added in the culture medium; **1.0mg/l**: 1.0mg/l $GA_3$ added in the culture medium.

**FGP**: Final Germination Percentage; **MGT**: Mean Germination Time; **GE**: Germination Energy; **PV**: Peak Value; **GV**: Germination Value.

**Figures**

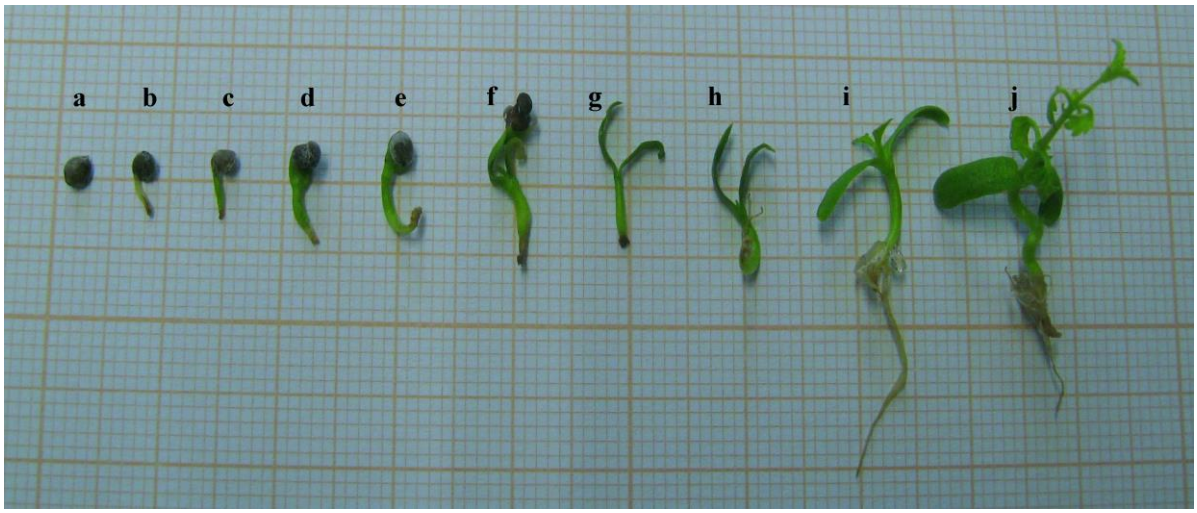


**Figure 1.** Description of the germinative process of hop, cv. Columbus, seeds: stage **a)**: seed with broken coat; stages **b-d)**: rootlet protrusion; stages **e-f)**: hypocotyl development; stages **g-h)**: cotyledon appearance; stages **i-j)**: epicotyl growth.

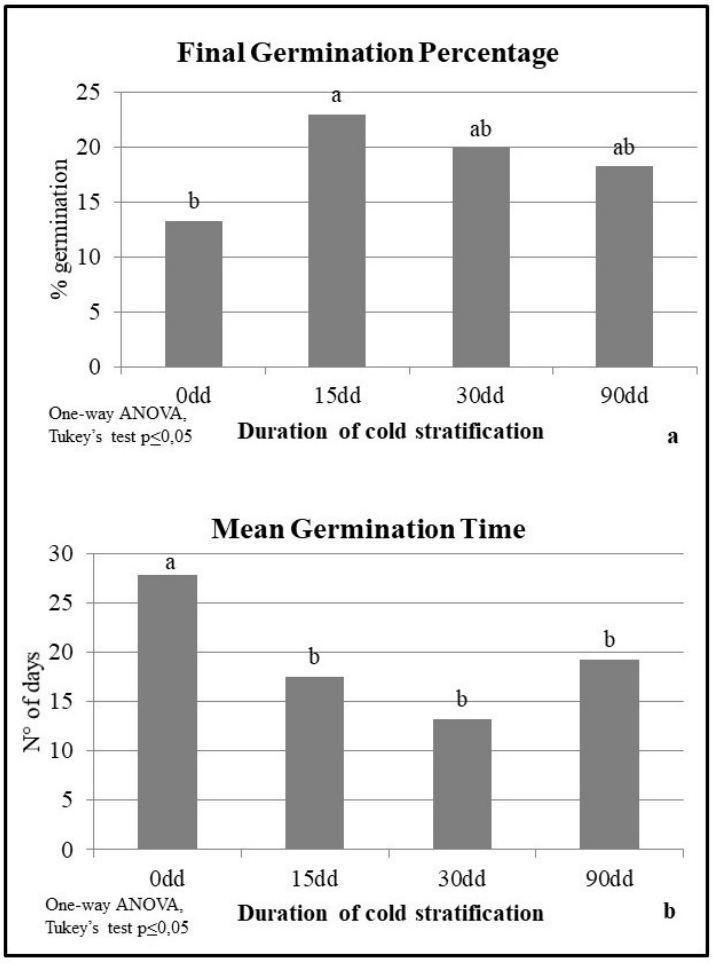


**Figure 2. a)** One-way ANOVA of the parameter Final Germination Percentage for the factor "Duration of cold stratification"; **b)** One-way ANOVA of the parameter Mean Germination Time for the factor "Duration of cold stratification".

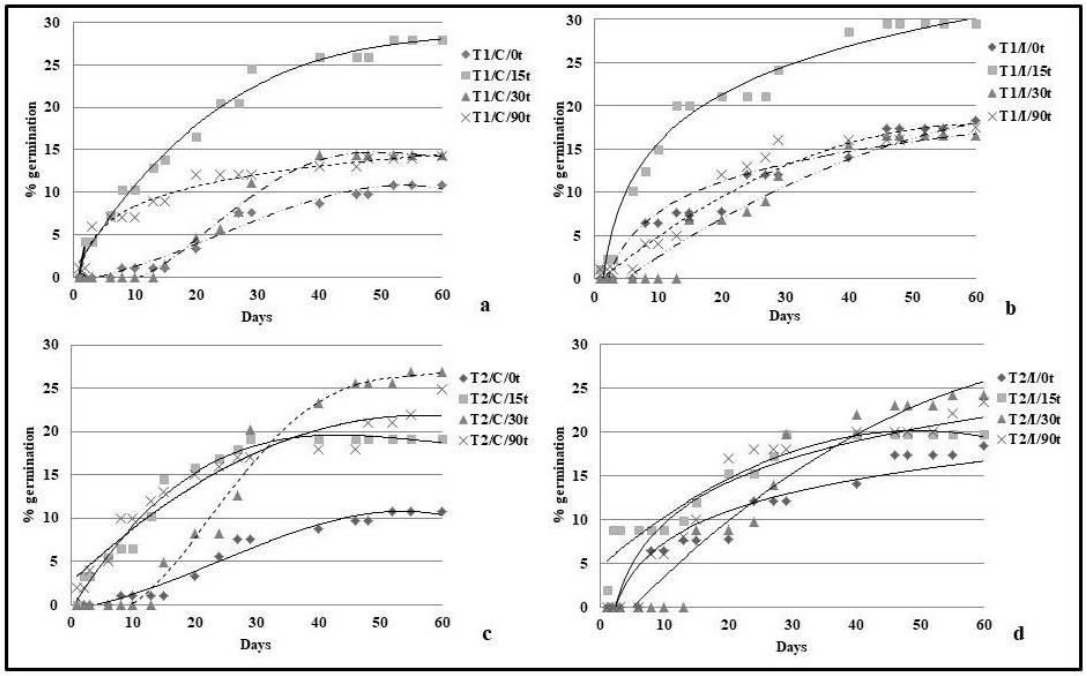


**Figure 3.** Seed germination curves of hop seeds, cv. "Columbus", subjected to chemical scarification, with sulfuric acid: **a**) before the cold stratification treatment (T1) without imbibition (C); **b**) before the cold stratification treatment (T1) with imbibition in water (IC); **c**) after the cold stratification treatment (T2) without imbibition (C); **d**) after the cold stratification treatment (T2) with imbibition (IC) in water.

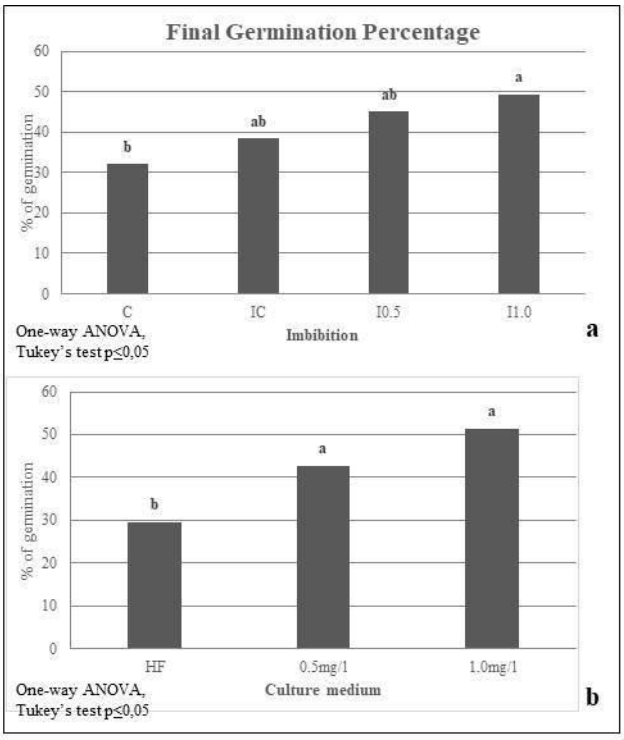


**Figure 4. a)** One-way ANOVA of the parameter Final Germination Percentage for the factor "Imbibition"; b) One-way ANOVA of the parameter Final Germination Percentage for the factor "Culture medium".

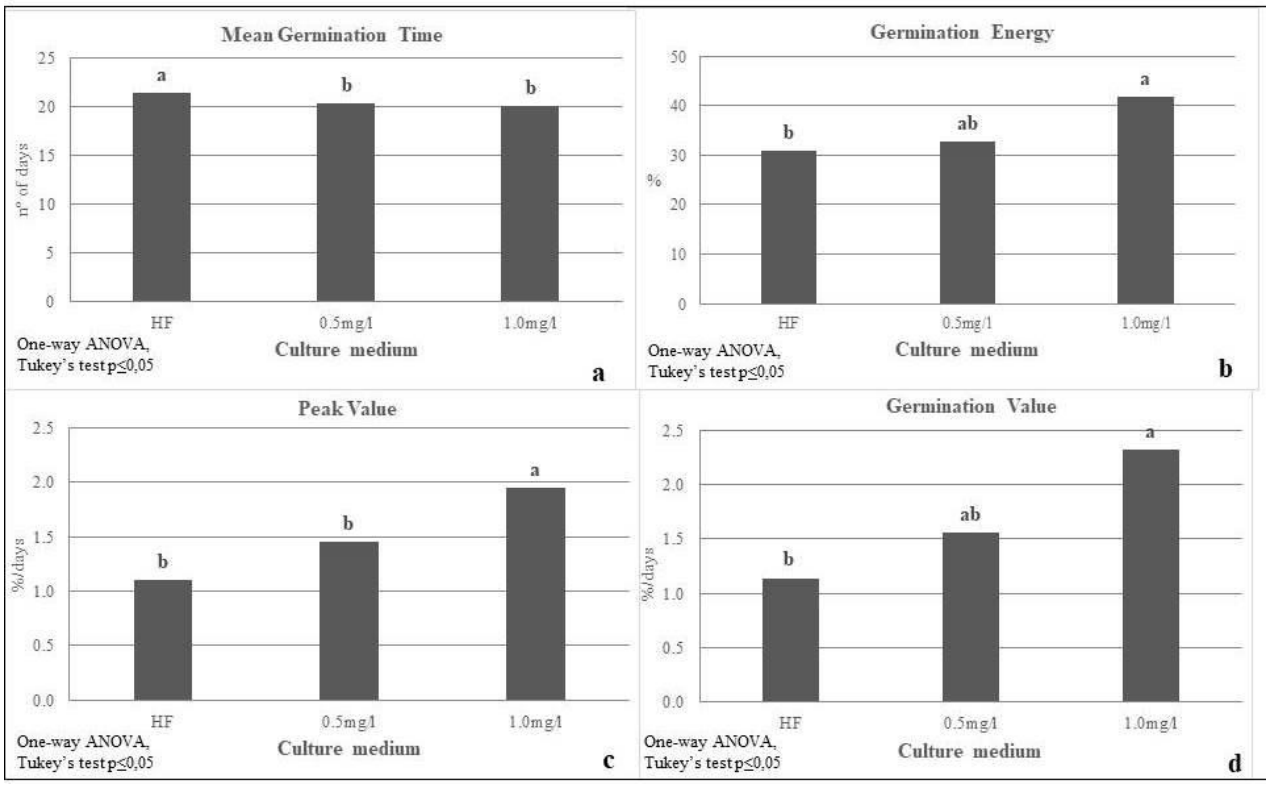


**Figure 5.** One-way ANOVA for the factor "Culture medium" of the parameters **a**) Mean Germination Time; **b**) Germination Energy; **c**) Peak value; **d**) Germination Value.

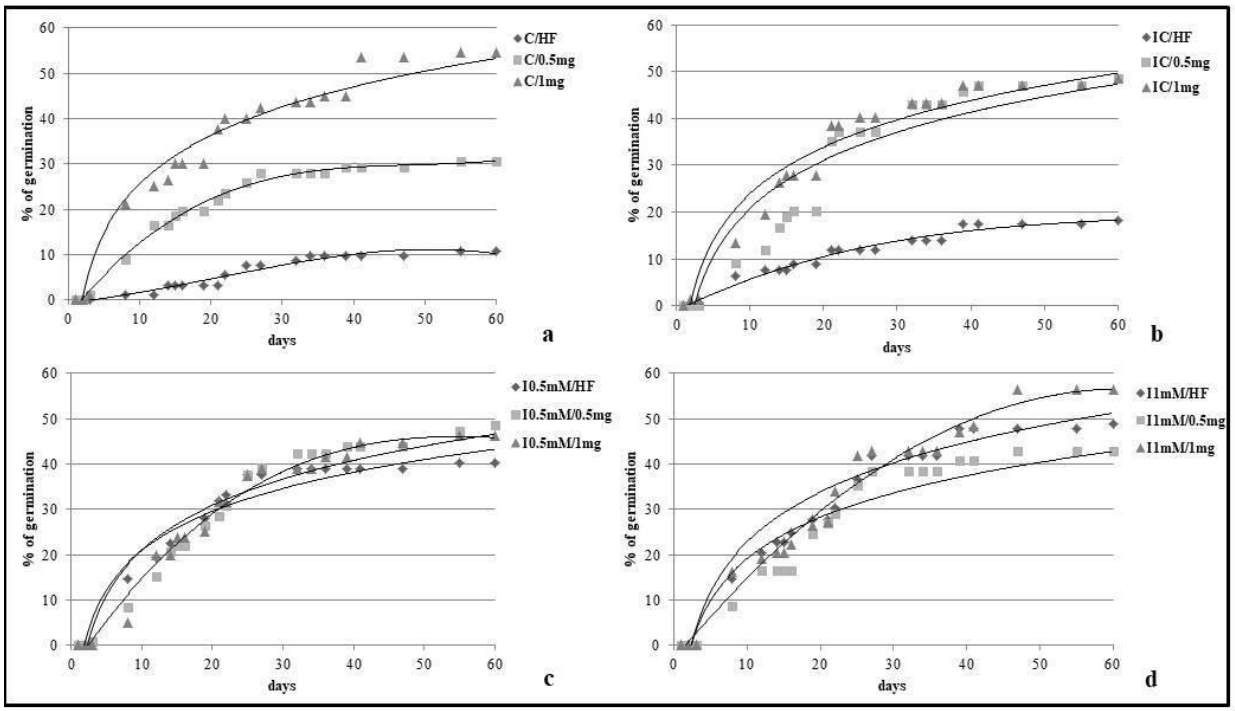


**Figure 6.** Seed germination curves of hop seeds of cv. "Columbus", *in vitro* cultured on culture media with different concentrations of $GA_3$ (0mg/l, 0.5mg/l, 1.0mg/l) and **a**) not imbibed; **b**) imbibed for 24 hours in sterile distilled water; **c**) imbibed for 24 hours in sterile 0.5mM $GA_3$ aqueous solution; **d**) imbibed for 24 hours in sterile 1.0mM $GA_3$ aqueous solution.

# Study 2 - Phenological phases of flowering in hop (*Humulus lupulus* L.) and their correspondence with microsporogenesis and microgametogenesis

**Running head:** Microsporogenesis and microgametogenesis in hop

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**Abstract**

Hop (*Humulus lupulus* L.) suffered, as many other crops, a shrinkage of its intraspecific agrobiodiversity. Biotechnological methods of breeding would offer new opportunities to produce improved varieties with interesting phytochemical profiles and adaptable to the challenging conditions of climate change. Doubled haploid (DH) technology could be a useful tool to increase hop agrobiodiversity but, unfortunately, there is a complete lack of information about hop flower biology. For this reason, the main aim of this work is the study of the different phenological phases of flowering in hop and the corresponding developmental stages of microspores/pollen grains contained therein. The results obtained allowed the identification of morphological markers (anther and flower bud length), easy and fast to measure, that would speed up the selection of flower buds containing the highest percentage of vacuolated microspores and young pollen, the stages considered in most species as the most responsive to androgenesis. A further result, derived from the flower bud and anther microscopical observation, evidenced the increase of lupulin glands on bud and anther surface as the bud proceeds in development from microsporogenesis to microgametogenesis.

**Keywords:** anther length; bud length; lupulin glands; microspore; pollen; flower development.

**Abbreviations**

DIC: Differential Interference Contrast

DH: Doubled haploid

**1. Introduction**

Hop (*Humulus lupulus* L.) is a dioecious, anemophylus species belonging to the Cannabaceae family. In recent times, increasing demand of craft beers with innovative flavors and the widening of beer consumption (Barth-Hass, 2016) has resulted in a greater general attention for hop culture, and in particular, in the research of hop characterized by peculiar phytochemical profiles. This adds to the need for adaptation of this crop to the challenging conditions of climate change, as it happens for other beer ingredients such as barley (Xie *et al.,*2018). Unfortunately, during its domestication, hop suffered from a shrinkage of intraspecific agrobiodiversity (Patzak *et al.,* 2010a; Patzak *et al.,* 2010b), which makes these goals even more challenging. In this context, recent studies revealed the occurrence of different types of aneuploidy and chromosome rearrangements that contribute to segregation distortion, a phenomenon that seriously makes hop breeding difficult (Easterling *et al.,* 2018). To overcome these limitations, we are still far from having the biotechnological toolbox already available for other crops. Although the interest of brewing industry for hop resides in the female flower cones, rich in lupulin glands with bitter compounds, males are essential for breeding new hop varieties, as well as for developing advanced biotechnology-based breeding tools.

One of these tools is DH technology. In general, high yields in intensive horticulture are based on the use of hybrids between homozygous (pure) lines. Pure lines are traditionally obtained through classical techniques of self-fertilization and selection, which involves many years of work and important economic investments. As a much faster and cheaper alternative, pure lines can also be generated by DH technology. DHs are derived from haploid microspores reprogrammed towards embryogenesis. This reduces the process to a single generation, with huge savings of time and economic resources. These advantages make DHs a very interesting tool for breeding companies, which already use them in those crops where the technology is available. Therefore, the development of a reliable protocol for DH production in hop would be a powerful tool to speed up applied hop breeding. However, it is really surprising that, to the best of our knowledge, there is nothing published about DH production in hop. This means that in order to develop such a DH method, we must start from the very beginning, which is the understanding of hop male flower, microspore and pollen development, and its particularities. Unfortunately, there is not much information about microsporogenesis and microgametogenesis. Neither the classical studies of Ehara (1955, 1956) on hop morphology and development nor the extensive study of Shephard *et al.* (2000) on hop flower development covered microspore and pollen formation. Only some

palynological data are available, which define hop pollen grains as ~20 µm in size, circular to ovate with very thin and scabrate exine, and triporate, with slightly protrusive 2 µm pori that form a subtle annulus (Berger, 2018). Intine is also very thin, although it thickens beneath pores, forming an oncus.

The most efficient way to produce DHs is the experimental induction of androgenesis *in vitro*, either by anther culture or by isolation and culture of microspores in liquid medium. This can only be achieved in a narrow developmental window during microsporogenesis and microgametogenesis. In particular, this window is restricted, in all known species, to the transition from vacuolated microspores to young, bicellular pollen grains (Seguí-Simarro, 2010). Therefore, the first step to develop a DH protocol in hop would be to characterize male flower development, and to find morphological and easily measurable parameters to identify flower buds and anthers with microspores/pollen at the right developmental window. In this work, we present a study of male hop flower development, characterizing buds, anthers and microspores/pollen at all developmental stages from meiocytes to mature pollen, which would correspond to stages 8-10 of hop male flower development according to Shephard et al. (2000). Based on this study, we propose a criterion to identify the buds containing microspores at the inducible stages, in order to maximize the efficiency of further *in vitro* anther or microspore culture. In order to check the influence of genotype in the measured parameters, this study has been carried out in parallel in three different hop Italian genotypes. To the best of our knowledge, this is first study that reports, firstly, the characterization of the microspore/pollen grain developmental stages in hop, but also that exists a clear correlation between hop microspore/pollen grain developmental stage and male flower bud/anther size. Then, the results presented herein would represent the first step towards the establishment of the optimal parameters to initiate androgenesis induction in hop.

## 2. Materials and methods

### *2.1 Plant material*

Male flowers were isolated from three hop accessions (MA1, Prismi 2, Santa Clara). Donor plants were grown in the collection field of Manaro sul Panaro (Modena, Italy) during 2018. Plants bloomed and samples were taken in July 2018.

*2.2 Characterization of flower bud, anther, microspore and pollen development*

In order to study the parallel development of microspore/pollen grains and anthers, 20 flower buds per genotype were selected. 50 anthers were randomly isolated, measured from the filament insertion to the apical end with a stereomicroscope equipped with a calibrated eyepiece, separated in dimensional classes, squashed in a glass slide with 10 µl of 7.5 µg/ml 4', 6-diamidino-2-phenylindole (DAPI), and observed with a Zeiss Axiovert 40 CFL inverted microscope. For each anther, the developmental stage of 200 randomly chosen microspores/pollen grains was determined. Data collected were used to calculate percentages of each stage with respect to the total. For each genotype the average anther and flower bud lengths were calculated, correlated and a correlation function was built. Finally, in order to confirm the validity of the correlation function, 50 flower buds were measured with the stereomicroscope from the pedicel insertion to the top of the corolla and divided into dimensional classes. For each dimensional class, 20 anthers were isolated and the developmental stage of 200 randomly chosen microspores/pollen grains was determined as described above. Data collected were used to calculate percentages, and images were taken for characterization of microspore and pollen developmental stages. In addition, 50 male flower buds per genotype were observed with a stereomicroscope to evaluate the presence of trichome hairs and lupulin glands on their bud and anther surface.

**3. Results and Discussion**

*3.1 Characterization of microspore and pollen development*

First, we studied the parallel development of microspores/pollen grains (Figures 1A-G, A'-G'), anthers (Figures 1A''-G'') and flower buds (Figures 1A'''-G'''), covering from tetrads to mature pollen grains. Tetrads contained four independent microspores still enclosed within the post-meiotic cell walls (Figures 1A-A'). At this stage, anthers were small (around 0.5 mm length) and green-yellowish (Figure 1A''), buds were also small (around 1 mm), green and fusiform (Figure 1A'''). Young microspores, upon release from the tetrad, were slightly oval and presented a still thin pollen coat and a centrally located nucleus (Figures 1B, B'). Anthers enlarged dramatically, almost doubling its length with respect to the previous stage (Figure 1B''). The increase in size of anthers, however, was not reflected in a similar size increase in buds, which presented a similar, or slightly larger size (Figure 1B'''). Mid microspores (Figures 1C, C') were similar to young microspores in terms of shape and size, the only remarkable difference being the displacement of the nucleus to the cell periphery,

as a consequence of the formation of the central vacuole, typical of this stage. Consequently, anthers (Figure 1C'') and buds (Figure 1C''') underwent minimal changes in size, shape and color. At the vacuolated (mature) microspore stage, remarkable changes were observed. Microspores increased in size and adopted a round morphology (Figure 1D). In addition, a thicker microspore coat was evidenced in DIC images. In fluorescence images (Figure 1D'), an increase in autofluorescence of this coat suggested a compositional change as well. DAPI images also evidenced a nucleus closely apposed to the plasma membrane, indicating the imminence of the first pollen mitosis. In parallel, anthers became larger. Buds enlarged too, adopting an oval shape.

The transition of vacuolated microspores to young bicellular pollen grains (Figures 1E, E') involved the formation of a vegetative and a generative cell, defined by different levels of chromatin condensation in their nuclei, as revealed by the different intensity of DAPI staining. Typically, the vegetative nucleus, transcriptionally active during pollen development, presents lower DNA condensation, whereas the generative nucleus, precursor of the sperm cells and transcriptionally inactive, presents highly condensed DNA. This transition implied no apparent changes in pollen size or shape, but the formation of a thicker and denser pollen coat, thickened at the apertures. It also implied a notable enlargement of anthers, which adopted a pale yellow color (Figure 1E''), and of flower buds (Figure 1E'''). The mid bicellular pollen stage was defined by a thickening of the coat at the level of apertures, now visible at DIC (Figure 1F) and fluorescence images (Figure 1F'), a movement of the generative nucleus from the cell periphery to a central position, a transition of anther color from pale yellow to yellow as pollen matures (Figure 1F''), and a progressive growth of both anthers and flower buds (Figure 1F'''). At the mature pollen stage, enlarged and densely filled grains were found (Figure 1G). DAPI staining revealed the presence of three nuclei (Figure 1G'), indicative of the occurrence of the second pollen mitosis. Anthers at this stage (Figure 1G'') were nearly 2 mm long and yellow, indicative of pollen maturity. In turn, flower buds were wider, and the separation between sepals became more evident (Figure 1G'''), which was indicative of imminent anthesis. Further stages consisted on anthesis and anther dehiscence, with no changes in pollen grains other than desiccation (data not shown).

*3.2 Development of lupulin glands in anthers and male flower buds*

During all the studied stages of flower bud development, buds remained closed, changing from a fusiform (at early stages) to an oval shape, typical of late stages, in agreement with previous studies

that described that hop male flowers open only after pollen maturity has been achieved (Shephard *et al.,* 2000). During all these stages, trichome hairs were abundantly observed on their surface, but no changes in abundance or bulk number were noticed. We also observed the presence of lupulin glands on the bud surface at all stages. However, their presence was scarce at the first stages and increased notably at the transition from vacuolated microspores to pollen grains (arrows in Figures 1A'''-G'''), coinciding with the increase in bud size and the change in shape. We also found lupulin glands on the surface of anthers at all stages. Again, their number and size increased remarkably in the transition from microspores to pollen and onwards. These newly formed lupulin glands appeared only at the interthecal groove region (arrows in Figures 1A''-G''), as previously suggested (Shephard et al., 2000). Lupulin glands produce lupulin, composed by essential oils, bitter acids and polyphenols, that contribute to beer bitterness and flavor. Our observations on lupulin glands confirm the results of Nagel *et al.* (2008), who measured a significant presence of polyphenols, and specifically, of xanthohumol in hop male flowers. According to our observations, the presence of xanthohumol would be due to the increase in lupulin glands observed on the bud surface, but principally, on the interthecal grooves of anthers.

*3.3 Morphological markers to identify anthers and buds enriched in vacuolated microspores and young pollen grains*

As explained above, male flowers do not open until the end of microgametogenesis. In addition, we did not observe any macroscopic change during the entire process studied, other than size and a slight widening of the flower bud. Therefore, we focused on these changes in order to establish criteria to easily identify buds at the right developmental window to induce androgenesis. In the three accessions studied, young anthers (1.5-1.8 mm) were characterized by a very homogeneous population of meiocytes/tetrads, comprising 100% of the population in two out of three genotypes. However, as soon as microspores were released from tetrads, anther locules presented a coexisting heterogeneous population of microspores at two or three different stages. Heterogeneity, as is usual for all plant species studied, increased upon gametogenesis, with up to four different stages in the same anther. To identify the intervals containing the microspore/pollen stages most responsive to androgenesis induction (around the first pollen mitosis), we selected those containing mostly vacuolated microspores and young bicellular pollen (bolded rows in Tables 1, 2). In cases where two intervals contained a clear majority of these stages, we selected the range with a majority of

vacuolated microspores with respect to young pollen, since they are at a stage immediately prior to mitosis, and would enter it soon, while young pollen would be exiting this stage. MA 1 anthers contained these stages at the 1.6-1.8 mm interval, whereas Prismi 2 contained them at the 1.7-1.9 mm interval and Santa Clara at the 1.8-2.0 mm interval. The slight genotype-dependent discrepancy in bud and anther size stresses the need for this type of studies in each particular genotype, as for other species (Parra-Vega *et al.,* 2013; Salas *et al.,* 2012; Seguí-Simarro and Nuez, 2005).

As seen, anther length increased in parallel to bud length for the three genotypes. Indeed, we plotted the paired bud and anther lengths (Figure 2) and they matched in all cases to a linear model with remarkably high $R^2$ coefficients (0,9358 for MA 1, 0,9319 for Prismi 2, 0,9009 for Santa Clara). Dioecy implies that male buds contain only anthers. This, together with the fact that buds remain closed up to the mature pollen stage, likely contributes to this high correlation. Since all three genotypes showed the same linear model, it was possible to develop the following correlation function, $y = 0.9643x - 0.4722$, that will be very useful to infer the anther length (y), knowing the flower bud length (x). Finally, by correlating the percentage of microspores at the different developmental stages with flower bud length, the validity of this function was confirmed. Specifically, the flower bud dimensional class containing the highest percentage of vacuolate microspores (2.1-2.3 mm for MA1 and Prismi2; 2.5-2-7 for Santa Clara; Table 2) corresponded, using the formula mentioned above, to the most suitable anther length (1.6-1.8 mm interval for MA1, 1.7-1.9 mm interval for Prismi2 and 1.8-2 mm interval for Santa Clara). This is positive in order to be able to use both anther and bud length indistinctly as a reliable criterion, since in other species, buds open or stop grow at intermediate stages of microspore/pollen development (Parra-Vega et al.,2013; Salas et al., 2012; Seguí-Simarro and Nuez, 2005), which precludes such use. However, the strong correlation between buds and anther length observed for the three hop genotypes, makes us propose to use exclusively bud length as the criterion to identify the appropriate stages. This would constitute a fast and easily measurable marker for suitable stages. In case of doubt, or as a second confirmation, anther length or the abundant presence of lupulin glands on the anther groove could also be used.

**4. Conclusion**

This work is, to the best of our knowledge, the first detailed characterization of the different microspore/pollen stages during hop male flower development. It represents a first step towards

the development of DH technology in hop. First of all, a detailed description of male flowers has been provided, with a particular focus on development of microspores and pollen grains and their features. Furthermore, a correlation between microspore/pollen developmental stages and flower bud/anther size was established. We also showed that in the three genotypes studied, vacuolated microspores and young bicellular pollen are contained in anthers with slightly different lengths. By means of microscopical analysis, it was possible to evidence that lupulin glands on buds and anthers surface, increase in parallel to bud and anther development. Thanks to these results, it will be easier and faster to select flower buds containing microspores at the most suitable stage of development to induce androgenesis. This, in turn, may allow for the development of innovative techniques such as DH technology for hop breeding.

**Author contribution**

CML and ACS performed all the experimental work required for this study. MR contributed to sample harvesting and preparation, BC helped in sample preparation, analysis of results and writing of the manuscript. JMSS designed the experiments, analyzed the results and wrote the manuscript.

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**Declarations of interest:** none

**References**

Barth-Hass, G., 2016. The Barth Report, Nuremberg, Germany.

Berger, U.E., 2018. Pollen Atlas. www.pollenwarndienst.at - Medical University of Vienna.

Easterling, K., Pitra, N., J. Jones, R., G. Lopes, L., R. Aquino, J., Zhang, D., D. Matthews, P., Bass, H., 2018. 3D Molecular Cytology of Hop (Humulus lupulus) Meiotic Chromosomes Reveals Non-disomic Pairing and Segregation, Aneuploidy, and Genomic Structural Variation. Front Plant Sci 9, 1501.

Ehara, K., 1955. Comparative Morphological Studies On The Hop (*Humulus Lupulus* L.) And The Japanese Hop (*H. Japonicus* Sieb. ET ZUCC.). I. Journal of the Faculty of Agriculture, Kyūshū University 10, 209-232.

Ehara, K., 1956. Comparative Morphological Studies On The Hop (*Humulus Lupulus* L.) And The Japanese Hop (*H. Japonicus* Sieb. ET ZUCC.). II. Journal of the Faculty of Agriculture, Kyūshū University 10, 307-324.

Nagel, J., Culley, L.K., Lu, Y., Liu, E., Matthews, P.D., Stevens, J.F., Page, J.E., 2008. EST Analysis of Hop Glandular Trichomes Identifies an O-Methyltransferase That Catalyzes the Biosynthesis of Xanthohumol. Plant Cell 20, 186-200.

Parra-Vega, V., González-García, B., Seguí-Simarro, J.M., 2013. Morphological markers to correlate bud and anther development with microsporogenesis and microgametogenesis in pepper (*Capsicum annuum L.*). Acta Physiol. Plant. 35, 627-633.

Patzak, J., Nesvadba, V., Henychová, A., Krofta, K., 2010a. Assessment of the genetic diversity of wild hops (*Humulus lupulus* L.) in Europe using chemical and molecular analyses. Biochemical Systematics and Ecology 38, 136-145.

Patzak, J., Nesvadba, V., Krofta, K., Henychova, A., Marzoev, A.I., Richards, K., 2010b. Evaluation of genetic variability of wild hops (*Humulus lupulus* L.) in Canada and the Caucasus region by chemical and molecular methods. Genome 53, 545-557.

Salas, P., Rivas-Sendra, A., Prohens, J., Seguí-Simarro, J.M., 2012. Influence of the stage for anther excision and heterostyly in embryogenesis induction from eggplant anther cultures. Euphytica 184, 235-250.

Seguí-Simarro, J.M., 2010. Androgenesis revisited. Bot. Rev. 76, 377-404.

Seguí-Simarro, J.M., Nuez, F., 2005. Meiotic metaphase I to telophase II is the most responsive stage of microspore development for induction of androgenesis in tomato (*Solanum lycopersicum*). Acta Physiol. Plant. 27, 675-685.

Shephard, H.L., Parker, J.S., Darby, P., Ainsworth, C.C., 2000. Sexual development and sex chromosomes in hop. New Phytol. 148, 397-411.

Xie, W., Xiong, W., Pan, J., Ali, T., Cui, Q., Guan, D., Meng, J., Mueller, N.D., Lin, E., Davis, S.J., 2018. Decreases in global beer supply due to extreme drought and heat. Nature Plants 4, 964-973.

**Tables**

Table 1. Distribution of the stages of microspore/pollen development at different lengths, during anther development in MA 1, Prismi 2 and Santa Clara.

| **MA 1** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Anther length (mm)** | **M+T** | **YM** | **MM** | **VM** | **YP** | **MP** | **LP** |
| 1.0-1.2 | 90.5±7.4 | 9.5±7.4 | | | | | |
| 1.3-1.5 | | 49.5±14.47 | 33.2±8.3 | 17.4±7.2 | | | |
| **1.6-1.8** | | | **6.4±3.7** | **73.2±8.4** | **20.4±12.0** | | |
| 1.9-2.1 | | | 3.0±0.5 | 12.8±0.5 | 84.2±0.0 | | |
| ≥2.2 | | | | 12.4±1.1 | 64.6±0.7 | 19.0±0.8 | 4.0±1.3 |
| **Prismi 2** | | | | | | | |
| **Anther length (mm)** | **M+T** | **YM** | **MM** | **VM** | **YP** | **MP** | **LP** |
| 1.1-1.3 | 100±0 | | | | | | |
| 1.4-1.6 | 61.9±15.6 | 32.4±12.3 | 5.7±3.3 | | | | |
| **1.7-1.9** | | | **18.3±7.5** | **60.8±13.3** | **20.9±12.3** | | |
| ≥2 | | | 0.8±0.5 | 7.0±3.1 | 70.9±4.5 | 19.9±5.2 | 1.5±0.9 |
| **Santa Clara** | | | | | | | |
| **Anther length (mm)** | **M+T** | **YM** | **MM** | **VM** | **YP** | **MP** | **LP** |
| 1.1-1.3 | 100±0 | | | | | | |
| 1.5-1.7 | | 39.1±16.9 | 32.1±10.2 | 28.8±13.2 | | | |
| **1.8-2.0** | | | **1.3±0.3** | **85.0±11.3** | **13.7±11.6** | | |
| 2.1-2.3 | | | 0.8±0.0 | 34.1±1.1 | 65.1±1.0 | | |
| >2.4 | | | | 12.3±0.1 | 58.0±0.5 | 27.7±0.4 | 2±0.2 |

The number of microspores/pollen grain units counted at each stage is expressed as the mean ± s.d. of the percentages of total counted microspores/pollen grains. **M+T**: meiocytes + tetrads; **YM**: young microspores; **MM**: mid microspores; **VM**: vacuolated microspores; **YP**: young pollen grains; **MP**: mid pollen grains; **LP**: late (mature) pollen grains. The bolded row represents the anther length where a majority of vacuolated microspores can be found

Table 2. Distribution of the stages of microspore/pollen development at different lengths, during bud development in MA 1, Prismi2 and Santa Clara.

| **MA 1** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bud length (mm)** | **M+T** | **YM** | **MM** | **VM** | **YP** | **MP** | **LP** |
| 1.5-1.7 | 82.5±20.3 | 17.5±10.1 | | | | | |
| 1.8-2.0 | | 52.1±19.2 | 20.9±9.0 | 27±14.3 | | | |
| **2.1-2.3** | | | | **80.0±1.2** | **20.0±1.2** | | |
| 2.4-2.6 | | | | 34.5±18.7 | 65.5±18.7 | | |
| ≥2.6 | | | | 12.4±0.7 | 65.1±0.7 | 18.6±0.8 | 430±0.8 |
| **Prismi 2** | | | | | | | |
| **Bud length (mm)** | **M+T** | **YM** | **MM** | **VM** | **YP** | **MP** | **LP** |
| 1.5-1.7 | 100±0 | | | | | | |
| 1.8-2.0 | 34.1.±23.6 | 51.9±18.3 | 11.2±3.9 | 2.8±1.6 | | | |
| **2.1-2.3** | | | **8.4±1.5** | **78.3±5.6** | **13.3±7.1** | | |
| ≥2.4 | | | 9.1±6.6 | 16.3±7.6 | 59.5±10.1 | 13.9±6 | 1.3±0.8 |
| **Santa Clara** | | | | | | | |
| **Bud length (mm)** | **M+T** | **YM** | **MM** | **VM** | **YP** | **MP** | **LP** |
| 1.6-1.8 | 100±0 | | | | | | |
| 1.9-2.1 | 47.8±27.7 | 48.0±25.3 | 4.2±2.8 | | | | |
| 2.2-2.4 | | 9.8±5 | 37.9±12.8 | 52.3±17.0 | | | |
| **2.5-2.7** | | | **1.3±0.3** | **85.0±11.3** | **13.7±11.6** | | |
| >2.8 | | | 0.4±0.2 | 23.2±6.3 | 61.6±2.1 | 13.9±8 | 1±0.6 |

The number of microspores/pollen grain units counted at each stage is expressed as the mean ± s.d. of the percentages of total counted microspores/pollen grains. **M+T**: meiocytes + tetrads; **YM**: young microspores; **MM**: mid microspores; **VM**: vacuolated microspores; **YP**: young pollen grains; **MP**: mid pollen grains; **LP**: late (mature) pollen grains. The bolded row represents the bud length where a majority of vacuolated microspores can be found.

## Figures

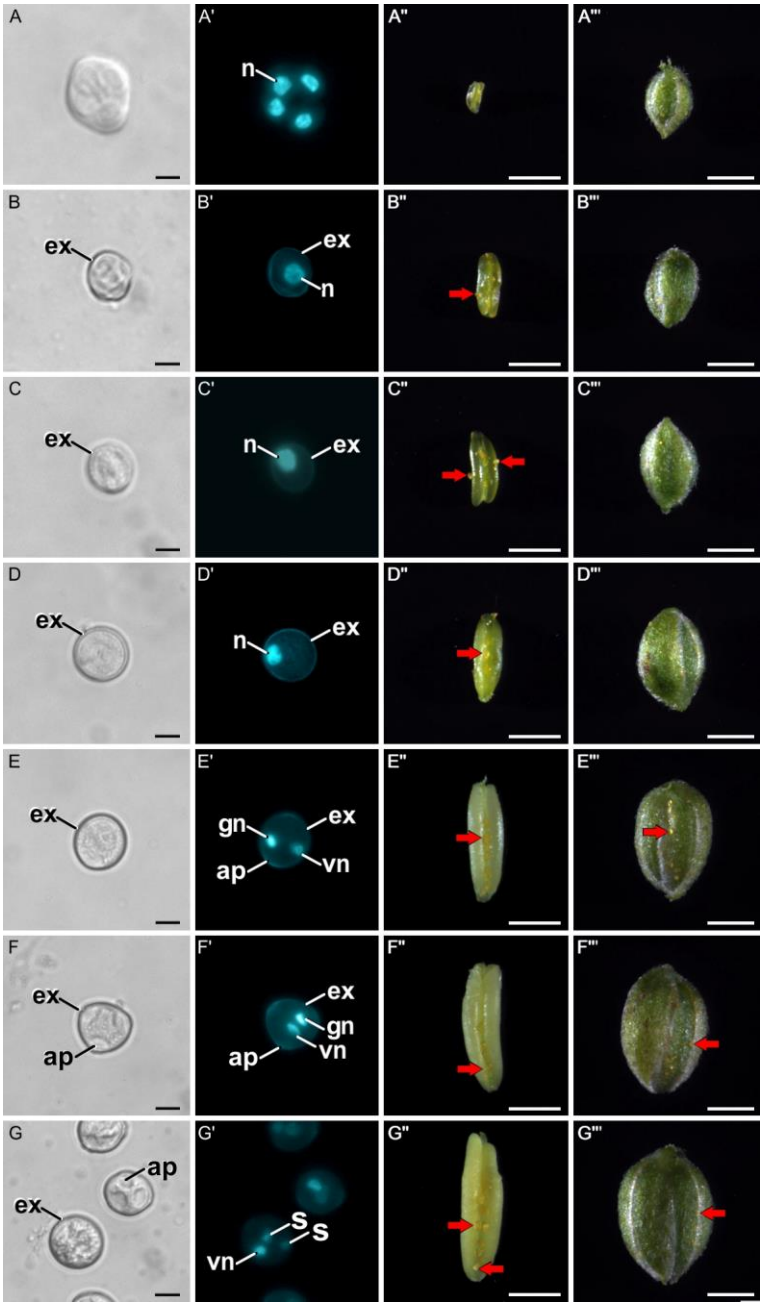


**Figure 1**. Changes during microspore/pollen (A-G: DIC images; A'-G': DAPI staining), anther (A''-G''), and bud development (A'''-G''') in hop accession MA 1. A-A''': meiocytes and tetrads. B-B''': Young microspores. C-C''': Mid microspores. D-D''': Vacuolated microspores. E-E''': Young bicellular pollen. F-F''': Mid pollen. G-G''': Mature pollen. ap: aperture; ex: exine microspore/pollen coat; gn: generative nucleus; n: nucleus; s: sperm cell; vn: vegetative nucleus. Arrows point to lupulin glands on the surface of anthers and flower buds. Bars in A-G and A'-G': 10 µm; A''-G'' and A'''-G''': 1 mm.

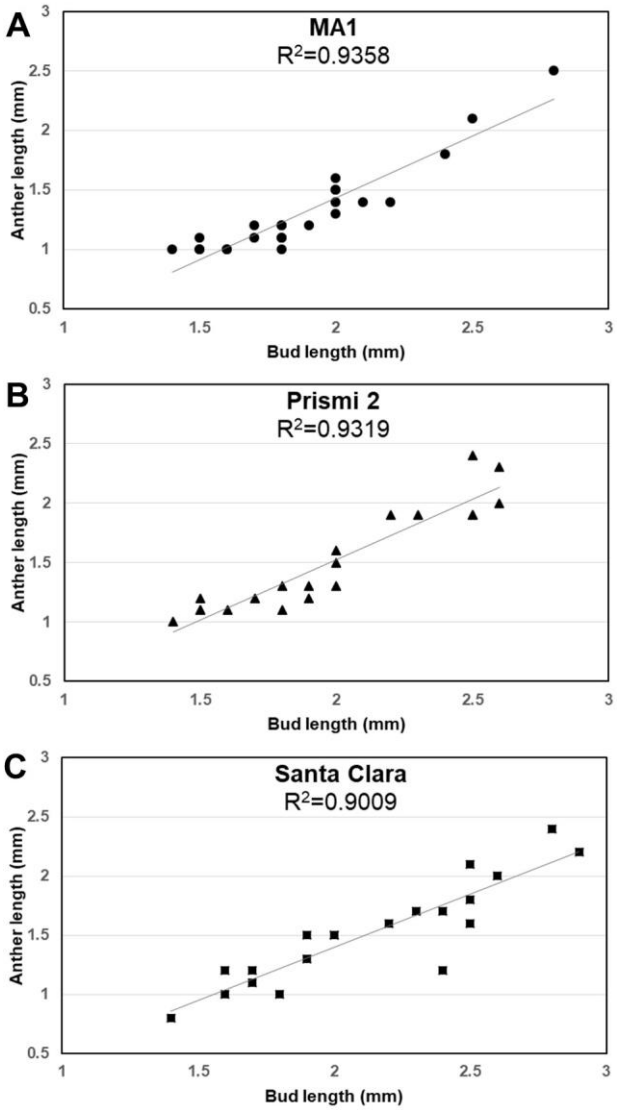


**Figure 2**. Correlation between anther and flower bud length in hop male buds of MA 1 (A), Prismi 2 (B) and Santa Clara (C). A regression line and the corresponding $R^2$coefficient are shown.

## Study 3 - Induction of indirect *in vitro* organogenesis from hop, cv. "Gianni", leaf portions and detection of somaclonal variants

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**Abstract**

The exploitation of somaclonal variation potentialities could be a valid strategy to overcome the depletion of hop intraspecific agrobiodiversity. To increase the possibilities of somaclonal variation induction, it is possible to resort to several strategies: a differentiated starting material, such as leaves, roots and stems, an extended time in which starting material is kept in culture, a well-balanced citokinin/auxin ratio. In this research, firstly indirect organogenesis was induced from hop, cv. "Gianni", leaves, testing i) the influence of type and concentration of growth regulators in the culture medium and ii) the effect of the period of culture (6, 12 and 18 weeks) in which leaves were kept in culture; secondly, to verify the occurrence of somaclonal variation, regenerants were characterized by cytofluorimetric analysis to check their ploidy, and by RAPD analysis to verify any possible polymorphism.

Adventitious shoots were obtained in all media containing BAP (except the one with BAP at lowest concentration), with no influence detected by period of culture. 16.8% of regenerants, analysed by flow cytometer and RAPD markers, were mutated, some of which were tetraploids. More than half of tetraploids recovered were obtained on culture containing the highest BAP concentration; while mutants, detected by RAPD analysis, originated independently on the medium composition and period of culture considered. A strong influence of the explant was observed instead: almost half of mutants were obtained by the same leaf portion. Further studies are needed to characterize the field performance of mutants.

**Keywords:** Adventitious shoots, flow cytometry, Humulus lupulus L., leaf portion culture, organogenesis, RAPD, somaclonal variation

## 1. Introduction

Hop (*Humulus lupulus* L.) is a dioecious plant, cultivated mainly for its female inflorescences, used to add bitterness, aroma and flavour to beer. In the last years, the increasing interest on craft brewing and the widening of beer consumption in new markets has led to the research of hops characterized by peculiar phytochemical profiles. Unfortunately, during its domestication and breeding process, hop suffered from a shrinking of intraspecific agrobiodiversity that brought to an impoverishment of the genetic basis. Breeding is the solution to individuate hop genotypes endowed with interesting phytochemical profiles, but also suitable for sustainable cultivation and

adaptable to climate changes. Fundamental for starting breeding programs is the increasing of variability, that can be reached through crossing or by manipulating the plant ploidy level; biotechnologies offer tools for speeding up the breeding programs, exploiting the variation that can arise through the in vitro culture of plant cells, tissues and organs, known as 'somaclonal variation' (SV) (Larkin and Scowcroft, 1981). Several are the factors that influence the occurrence of somaclonal variation; firstly, it is possible to obtain more variability using strongly differentiated tissues, such as roots, leaves and stems, rather than explants with meristematic cells, like axillary buds and shoot tips (Duncan, 1997; Pijut, et al. 2012); moreover, the longer is the time in which a culture is maintained *in vitro*, the greater is the chance of success (Kuznetsova et al., 2006; Gao et al., 2010; Farahani et al., 2011; Jevremović et al., 2012; Sun et al., 2013); for example, it has been reported that with the increasing of callus age, the occurrence of variant karyotypes increases and therefore the possibilities of variation in plants (Zayova et al., 2010). Finally, D'Amato (1985), in his studies seriously considered that, depending on their concentration, plant growth regulators can have a mutagenic effect; indeed, several are the studies indicating that plant growth regulators, added in culture media, have a strong influence on somaclonal variation (Gao et al., 2010).

In hop, somaclonal variation can be a valid tool to recovery polyploids, characterized by altered plant morphology, phenology and physiology (Levin, 2002; Roy et al., 2001). Specifically, in traditional breeding programs, tetraploids play an important role in hop breeding, as they can be crossed with diploid plants to obtain triploids (Roy et al., 2001). Actually, hop producers and, most of all, brewers are particularly interested in producing triploid cultivars which, producing non-functional reproductive cells, are infertile and therefore seedless (Dhooghe et al., 2011), since, seeds contain fats and proteins that compromise beer fermentation (Hildebrand et al., 1975).

Unfortunately, traditional breeding methods are time consuming; on the contrary, through tissue culture, tetraploids can be recovered in relatively short time. The mechanism that leads to tetraploid induction, during indirect organogenesis, is not well understood; it could be induced using plant growth regulators, such as cytokinins and auxins, that generate endomitosis phenomena or mutagenic activity as a by-product of culture process (Trojak-Goluch et al., 2015). Chromosomal breakage could be due to the disruption of normal cell cycle control that, during tissue culture, is not able to prevent cell division before the completion of DNA replication (Phillips et al., 1994). It has been reported that aberration in vitro could be from chromosomal damages and its consequences such as deletions, duplications, inversions, and translocations (Duncan, 1997).

Adelberg and Rhodes (1994) found that the type of explant is one of the most crucial factors affecting the occurrence of polyploidization. A large percentage of tetraploid regenerants was obtained from immature cotyledons of *Cucumis melon* L. compared with that obtained from apical meristem explants, arising from the same seeds. In the past years, to detect polyploids among all tissue culture regenerants, several were the approaches that researchers considered. Traditional cytological approaches to screen potential tetraploids, based on chromosomes count, are time consuming (Hamill et al., 1992). Another way to estimate ploidy level is the observation of stomatal characteristics; however, due to environmental effects, this method is not always reliable (Van Duren et al., 1996). Flow cytometric analysis of nuclear DNA content is being increasingly used for ploidy assessment.

As previously cited, somaclonal variation cannot induce only polyploidization, it can cause a wide range of mutations that need to be detected. Different tools based on the differences in morphological traits (Pérez et al., 2009; 2011; Nhut et al., 2013), biochemical (Vujović et al., 2010; Kar et al., 2014), molecular DNA markers (Hossain et al., 2003; Krishna and Singh, 2007; Pathak and Dhawan, 2012; Bello-Bello et al., 2014) or their combinations (Horáček et al., 2013; Dey et al., 2015; Stanišić et al., 2015) have been used. Specifically, since the probability of mutations is randomly distributed along the genome, an efficient way to analyze somaclonal variation could be based on PCR based markers, like random amplified DNA polymorphism (RAPD); RAPD has proved to be effective in several cases, allowing, also, a fast screening of the genome: in *Lolium* (Wang et al., 1993), *Triticum* (Brown et al., 1993), *Picea* (Isabel et al., 1993) and *Beta* (Munthali et al., 1996) changes in RAPD bands have been observed in somaclonal variants.

In this research, indirect in vitro organogenesis from hop, cv. Gianni, leaves and occurrence of somaclonal mutants were studied, considering i) the influence of type and concentration of growth regulators in the culture medium and ii) the effect of the period in which leaves were kept in culture. Regenerants were characterized by cytofluorimetric analysis to check their ploidy, and by RAPD analysis to verify any possible polymorphism.

## 2. Material and methods

### *2.1 Plant material*

Young and well expanded leaves of hop, cv. “Gianni”, were isolated from two-month-old plantlets, maintained, *in vitro*, in a growth chamber, at 25±1°C and light intensity of 20 µmol m−2 s−1, under 16 h photoperiod.

*2.2* In vitro *leaf regeneration*

Isolated leaves were cut in sections (0.5 x 0.5 mm) and put in culture, in sterile Petri dishes (ten explants for each Petri dish, ten Petri dishes per treatment), with the abaxial surface toward the culture medium. To facilitate medium uptake by tissue, several transverse cuts were made before the culture. In order to evaluate the influence of type and concentration of growth regulators in the culture medium on hop leaf regeneration, six culture media, with the following composition, were tested: (i) **MS-HF**: Murashige and Skoog (MS) salt mixture (Murashige and Skoog, 1962), MS vitamin mixture (1x) (Murashige and Skoog, 1962), 30 g $L^{-1}$ of sucrose, 8 g $L^{-1}$ of agar; (ii) **MS-BAP0**: MS-HF culture medium supplemented with 0.1 µM of 1-Naphthaleneacetic acid (NAA); **MS-BAP2**: MS-BAP0 culture medium supplemented with 8.88 µM of 6-Benzylaminopurine (BAP); **MS-BAP4**: MS-BAP0 culture medium supplemented with 17.77 µM of BAP; **MS-BAP6**: MS-BAP0 culture medium supplemented with 26.66 µM of BAP; **MS-BAP8**: MS-BAP0 culture medium supplemented with 35.55 µM of BAP. Culture media, after adjusting the pH to 5.8, were sterilized in autoclave for 20 min at 121 °C. Cultures were placed in a growth chamber, at 25±1°C and light intensity of 20 µmol $m^{-2}$ $s^{-1}$, under 16 h photoperiod.

*2.3 Experimental design and analysis of data*

The experiment was carried out in a completely randomized design with ten replications, and a total of 100 explants was used per each culture medium tested.

Cultures were monitored every week for 18 weeks and, to evaluate the influence on the regeneration potential and of the time in which the explants were kept in culture, three period of culture (PC) were individuated: first PC (**I PC**) after 6 weeks of culture, second PC (**II PC**) after 12 weeks of culture and third PC (**III PC**) after 18 weeks of culture. In correspondence of each PC, adventitious shoots were excised, and the following parameters were recorded: the number of explants producing callus, the number of explants producing roots, the number of explants producing shoots, the number of roots per each explant, the number of shoots per each explant, the

length of roots and the length of shoots. Data of explants with callus, roots and shoots were used to calculate percentage.

At the end of the experiment, Mean Regeneration Time (MRT) and Regeneration Energy (RE) were calculated; the formulae used were the following: MRT= ∑f*x/∑f (f=number of adventitious shoots at day x); RE = (n° of leaf portions with adventitious shoots before the peak*100)/total n° cultured leaf portions.

Two-way ANOVA (influence of Culture Medium Composition, CMC, and influence of the PC) was used to calculate the differences among treatments per each parameter considered; Tukey's test ($p \leq 0.05$) was used for mean separation (SYSTAT 13.1, Systat Software, Inc; Pint Richmond, CA). One-way ANOVA, followed by Tukey's test ($p \leq 0.05$), was used when only one factor showed statistically significant differences.

*2.4 Rooting and acclimatization*

After 6, 12 and 18 weeks of culture, leaf adventitious shoots, with the first leaflets fully expanded, were excised and cultured on MS-HF culture medium in 25 mL glass jars. When plantlets showed an efficient and well-developed root and foliar system, after around 60 days, they were moved from vitro to vivo conditions. Specifically, plantlets were extracted from the culture medium, the root apparatus was washed with distilled water and plantlets were transferred in 2x2 cm plateaux, containing sand and peat mixture (2:1 w/w).

*2.5 Flow cytometry analysis of regenerants*

Flow cytometry analysis was performed to evaluate the ploidy of regenerants, using a NovoCyte (Acea Biociences). Each regenerant was analysed three times. About 0.5 $cm^2$ of sample (leaves excised from regenerants) and 0.5 $cm^2$ of mother plant, cv. Gianni, were chopped with a razor blade for 30-90 s, in a plastic Petri dish containing 0.5 mL of extraction buffer (Partec CyStain PI Absolute P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany). The resulting extract was passed through a 30-µm filter into a 3.5-mL plastic tube, to which was then added 2.0 mL of Partec CyStain PI Absolute P Staining Buffer, containing 12 µl of Propidium Iodide Solution and 6 µl RNase A. Samples were kept in darkness for 30 min before analysis by flow cytometry. At least 5000 nuclei were analysed in each sample, Acea NovoExpress v.1.25 software was used.

*2.5 DNA extraction and RAPD analysis*

To verify any possible polymorphism, regenerants, obtained from hop, cv. Gianni, leaf *in vitro* cultured, were analysed using RAPD molecular markers. Per each regenerant and from mother plant, cv. Gianni, a portion of leaf was excised and used for molecular analysis. Collected leaves were stored at -80°C. Genomic DNA was extracted following the CTAB (Cetyl TrimethylAmmonium Bromide) procedure (Rodolfi et al., 2018). DNA was quantified by spectrophotometric method (Spectrophotometer Uvikon 930, Kontron Instruments Inc., Boston, MA, USA).

Genomic DNA was amplified using 16 decamer primers (Table 1). The amplification reaction was obtained in a volume of 25 µl containing: 1X Reaction Buffer (KAPA Taq Buffer w/loading dye, KAPA Biosystems, Wilmington, Massachusetts, US), 1.5 mM $MgCl_2$, 2.5 µM dNTPs (Amersham Biosciences, Little Chalfont, UK), 0.4 µM of primer (Sigma-Genosys Ltd, Dorset, UK), 1 unit of taq DNA polymerase (KAPA Taq DNA Polymerase, KAPA Biosystems, Wilmington, Massachusetts, US), and 40 ng DNA.

The amplification reaction was optimised in a thermal cycler MJ PCT 100 (MJ Research, Watertown, Mass.), programming a first step at 95°C for 5 min, followed by 40 cycles of 40 s at 94°C, 40 s at 36°C, 2 min at 72°C, for denaturation, annealing, and primer extension; the last step included 10 min of incubation at 72°C. Per each oligonucleotide utilized, the reaction was performed three times.

The products of amplification were separated on agarose gel at 2% in TAE buffer and stained with ethidium bromide. The gels were photographed with a Canon PowerShot A720IS digital camera. DNA molecular weight marker VII (0.081-8.57kbp) (Roche Diagnostics GmbH, Mannheim, DE) was used to estimate the approximate molecular weight of the amplified products, with the aid of the Kodak digital science 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY). The products of amplification were separated on agarose gel at 2% in TAE buffer and stained with ethidium bromide.

**3. Results**

*3.1 In vitro leaf regeneration*

Leaf portions in culture in all culture media tested maintained their green color for the first two weeks, then turned light brown and started producing a yellowish spongy callus that increased rapidly, covering the entire surface of the explants. Explants kept producing callus for the first 12 weeks, then stopped and the callus turned dark brown. Statistical analysis, carried out, after 18

weeks, on the percentage of explants producing callus, evidenced a significant interaction between the main factors ("PC" and "CMC") and demonstrated that the factor that mostly influenced leaf in vitro cultured response was the factor "CMC"; indeed, callus regeneration was recorded only in leaves cultured on media containing BAP, independently on its concentration (Table 2). After two weeks of culture, root regeneration was observed in one leaf portion cultured on MS-BAP0 medium; while the first roots produced by leaf portions cultured on media containing BAP (except for MS-BAP8) were registered after three weeks of culture (Fig. 1a). The percentage of explants producing roots increased from the first "PC" to the second, and then no more explants regenerating roots were observed. Statistical analysis evidenced that both factors influenced significantly the leaf portion response; specifically, regarding the "PC", the percentage of explants with roots in the first "PC" (11.8%) was statistically lower than those registered in the second and third "PC" (both 23.3%). Speaking about "CMC", differences statistically significant were observed between media containing BAP and media without (MS-HF and MS-BAP0); moreover, among media containing BAP, the medium with the highest BAP concentration (MS-BAP8) induced the statistically lowest percentage of explants to produce roots (Table 2). The number of roots produced per single explant was very variable, depending, mainly, on the culture medium composition; up to 10 roots per explant were recorded from one explant cultured on MS-BAP2 medium. Statistical analysis evidenced a significative interaction between factors "PC" and "CMC", with a major influence of the factor "CMC". In particular, in the first PC the statistically highest number of roots was recorded in explants cultured on medium MS-BAP6, while the lowest in MS-BAP8; in the other two PCs, increasing the concentration of BAP, a statistically significant decreasing in root number was observed (Table 2).

Root length measured at the end of the experiment showed a significant interaction between factors, with a strong influence of the factor "CMC"; the only differences statistically significant were observed in the first PC, in which the statistically longest roots, were observed in explants culture in medium MS-BAP8 (12.4 mm) (Table 2).

Together with the emergence of the first roots, calli formed organogenic centers in explants cultured in MS-BAP4 culture medium (Fig. 1b). After 4 weeks in culture, some of the organogenic centers turned in small shoots with well developed leaves (indirect organogenesis) (Fig. 1c). Direct organogenesis was not observed. Adventitious shoot regeneration continued for all the PCs without moving leaf portions in a fresh medium and it was observed from explants cultured in all media containing BAP, except for MS-BAP2.

The analysis of the MTR, calculated considering the explant response during the 18 weeks, evidenced a statistically significant difference only for the factor “PC”; indeed, from the first to the third PC, a significant decreasing of the time needed, on the average, to obtain shoot regeneration was observed; whilst, an opposite trend was observed for the parameter RE, which increased significantly from the first to the third PC (Fig. 2).

Statistical analysis carried on the percentage of explants with adventitious shoots evidenced a significant interaction between factors; the factor with the highest influence is the “CMC”; effectively, significant differences were observed only between the first and the other two PCs, but only for explants cultured on medium MS-BAP8, in which, in the first PC, the significantly lowest percentage of explants with shoots was observed.

Statistical analysis did not evidence any significant difference for any of the considered factors for the parameter “n° of shoots”, but it was possible to register a total of 138 adventitious shoots, independently on culture medium and PC considered, and up to 14 regenerants from one leaf portion, cultured on medium MS-BAP4 (data not shown).

The shoot length varied from 3 to 26 mm; statistical analysis evidenced a significant interaction between factors: specifically, in the third PC, the statistically longest shoots were observed in culture media MS-BAP6 and MS-BAP8 (respectively 16.5 and 9.1 mm); while, considering explants cultured on medium MS-BAP4, in the second PC the statistically longest shoots were observed (11.0 mm) (Table 2).

*3.2 Rooting and acclimatization*

After around 14 days of culture, excised shoots, cultured on MS-HF, started producing roots. Root formation occurred directly from the shoot cut end, without the callus phase. A very well-developed rooting system was observed within 30 days of culture. Around 60% of cultured shoots developed a well differentiated root apparatus.

*3.3 Flow cytometry analysis of regenerants*

All well-developed plantlets, derived from adventitious shoots, were subjected to flow cytometry to determine their ploidy. Cytometric analysis showed the presence of both diploid and tetraploid lines (Fig. 3a and b); other variations of ploidy, such as mixoploid or octoploid were not detected. In present study, 6 over 83 regenerants analysed (7.2%) were tetraploids. Even if it was not possible to

carry on a statistical analysis, because of the low number of tetraploids obtained, the efficiency of tetraploid recovery varied, mainly depending on the medium composition. Indeed, it is possible to state that culture medium MS-BAP8 induced the regeneration of the highest percentage of tetraploids (66.7%). The time in which leaf portions were kept in culture did not influence the ploidy variation of explants; 83.3% of regenerated tetraploid plants were obtained during the first six weeks of culture (I PC).

*3.4 RAPD analysis*

A total of 183 bands were generated using 16 RAPD decamer primers. For all regenerants, the number of bands generated by each primer varied from 5 (primer 544) to 17 (primer AI12); moreover, primer AI055 generated 5 fragments only with the regenerant 52. Each primer generated a set of fragments, ranging from 8570 bp to 149 bp. Results indicated that three of the sixteen primers tested revealed polymorphic DNA profiles and their list is reported in table 3: OPK16 for the regenerant 134 (Fig. 1), AI12 for regenerant 19A, AI05 for regenerants 7, 10, 48, 52, 78 and 112. In detail, OPK16 primer generated one RAPD amplification product (1625 bp) that was unique only for 134; in AI12 primer, two amplification products (1650 bp and 1501 bp) were absent for 19A regenerant; AI05 primer generated amplification products present in 7, 10, 48 regenerants and absent in 52, 78 and 112 regenerants (Table 3).

RAPD analysis showed that 8 over 83 regenerants considered (9.6%) revealed scoreable polymorphism. Due to the low number of mutants obtained, it was not possible to carry on a statistical analysis, but it can be observed that mutants were recovered in equal number from MS-BAP4 and MS-BAP8 medium, but none from MS-BAP6; moreover, the time in which explants were kept in culture does not seem to have a strong mutagenic effect in cv. Gianni hop leaves *in vitro* cultured: 5 out of 8 mutants were recovered during the first 6 weeks of culture (I RT) independently on the culture medium considered. To be highlighted is the fact that 3 mutants were regenerated by the same leaf portion, during the I RT, suggesting that, maybe, the explant origin is as important as the other considered factors.

**4. Discussion**

Culture medium composition and duration of culture are key factors influencing, not only the regeneration competence, but also the incidence of somaclonal variation. In hop, several are the

studies reporting somatic regeneration from different types of explants, such as internodes, petioles and leaf discs (Krishna and Singh, 2007). Many authors reported that the best regeneration, in hop, has been obtained from internodal cuttings (Motegi, 1979; Connell and Heale, 1992; Heale et al., 1989; Rakouský and Matoušek, 1994; Batista et al., 1996; Gurriarán et al., 1999; Šuštar-Vozlič et al., 1999; Horlemann et al., 2003), but it is also well known that the regeneration ability of hop is highly genotype dependent (Gurriarán et al., 1999); for this reason first aim of this study was the organogenesis induction from leaf portions of hop, cv. "Gianni", considering the influence of type and concentration of growth regulators in the culture medium and of the period of time in which explants remained in the culture.

In this study, the first sign of organogenesis was the callus formation, starting from the edges and, afterwards, covering all the leaf portion surface; callus formation was observed in a high percentage of leaf portions cultured on media containing BAP (91.5%), results in accordance with Rakouský and Matoušek (1994) who obtained similar values in in vitro cultured leaves of two Czech clones; on the contrary, Skof et al. (2007) reported a very scarce callus formation from leaf discs. Moreover, in this study, callogenesis was observed only in leaves cultured on media containing both BAP and NAA. The importance of auxin/cytokinin ratio in callogenesis is evidenced by Gurriarán et al. (1999) who obtained an increase in the number of callus forming explants in hop (cvs. Brewers Gold and Nugget) internodal segments, cultured on media containing both BAP and Indole-3-butyric acid (IBA).

Other than callus, leaf portions regenerated roots, if cultured on media containing NAA. No reports are available, to our knowledge, about root induction from hop leaves, but by comparing results reported in this study with those reported by Gurriarán et al. (1999) in internodal segments, it seems that a much higher percentage of explants with roots was obtained using leaf portions.

Together with roots, on the leaf portions, firstly, organic centers, and then adventitious shoots, appeared. In hop, the choice and concentration of cytokinin was often found to be essential for organogenic capacity (Šuštar-Vozlič et al., 1999). In this study, adventitious shoot regeneration was observed in all media containing BAP, except of MS-BAP2, with an average percentage of 8.3%. Very few are the reports in which leaf portions are used as starting material for organogenesis induction; in Skof et al. (2007) percentages of leaf discs producing shoots are highly genotype dependent, going from 5.1% in Savinjski to 20.0% Tettnanger. In cv. "Gianni", adventitious shoots appeared after around 4 weeks of culture, needing a two-fold longer time than the one reported by Skof et al. (2007), for the cvs. Aurora, Savinjski golding and Tettnanger.

Cassells (1979) and Gurriarán et al. (1999) observed that, due to apical dominance, the first shoot produces auxins that may inhibit regeneration of new shoots, and, for this reason, organogenic centers keep developing new buds only following shoot excision. In order to avoid this phenomenon, in this study, every 6 weeks, shoots were excised and cultured on MS-HF medium. The percentage of explants producing adventitious shoots increased significantly between the first and the other two period of culture, specifically for explants cultured on MS-BAP6 and MS-BAP8, demonstrating that the adventitious shoot regeneration capacity of cv. "Gianni" leaf portions was affected by the time in culture; similar results were obtained in cultivars Brewers Gold and Nugget, that showed an increase in regeneration rate from the first to the third subculture (Gurriarán et al., 1999).

Considering that plants regenerated from in vitro cultures can exhibit an array of genetic and epigenetic changes, known as 'somaclonal variation', leaf regenerated shoots were analysed, using flow cytometry and RAPD molecular markers, to evaluate their variability. Three are the main factors giving rise to SV: i) explant source, ii) callus formation and iii) organogenic process (Benzion and Phillips, 1988). Among these factors, it has been demonstrated that most abnormalities are accumulated if the organogenic process is indirect (Phillips et al., 1994; Roy et al., 2001). In hop varieties, Skof et al. (2007) demonstrated that indirect organogenesis could be considered a successful method to obtain polyploids. In this study, tetraploid hop, cv. Gianni, plantlets were obtained through regeneration from in vitro cultured leaf portions. Trojak-Goluch and Skomra (2013) compared diploid and tetraploid hop plants, assessing the effect of polyploidization on the morphological and chemical characteristics: they observed an increase in the size of the flowers, a delayed time of flowering (Trojak-Goluch and Skomra, 2013), a greater vigour and a higher yield. Moreover, tetraploids play an important role in hop breeding as they can be crossed with diploid plants in order to obtain triploid progeny, considered to be superior to both diploids and tetraploids (Roy et al., 2001), mostly because of their seedlessness.

In this study, some of the regenerants, analysed by RAPD molecular markers, although maintaining their diploid chromosomic set, showed a different DNA pattern, respect to the mother plant, with loss or gain of amplified products. Indeed, other than polyploidization, in vitro culture can cause. several genomic alternations, such as deletion/insertion between priming sites or in restriction sites (De Verno et al., 1999). Results reported in this research, with a total of 16.8% of mutated regenerants, tetraploids and RAPD detected variants, confirm what reported by several authors (Jain, 2001; Patzak, 2003; Hashmi et al., 1997; Al-Zahim et al., 1999; Rout, 2002): *in vitro* culture is a

mutagenic system with a mechanism comparable to what happens in nature (Linacero et al., 2000). It has been demonstrated that occurrence of mutations is at non-random in hop genome, but there are some hypervariable DNA regions where they can happen more frequently (Linacero et al., 2000; Polanco and Ruiz, 2002; Patzak, 2003).

**5. Conclusions**

In this study, indirect organogenesis from hop leaf portions, cv. Gianni, was induced adding, in the culture medium, both 6-Benzylaminopurine and 1-Naphtalenacetic acid. The percentage of leaf portions producing adventitious shoots increased with the period in which explants were kept in culture; moreover, longer was the period of culture, shorter was the time needed for regeneration. In vitro culture conditions could have mutagenic effect, most of all, if differentiated tissue are used as starting material. For this reason, it was necessary to evaluate the genetic correspondence of leaf portion regenerants. Flow cytometric and RAPD marker analysis evidenced that the 16.8% of the plantlets obtained from the adventitious shoots were mutated, some of them showed a tetraploid genome. The mutation rate registered was independent of the BAP concentration and not influenced by the period of culture. Mutants obtained will be, further, characterized to evaluate their field performance.

**References**

Adelberg JW, Rhodes BB (1994) Explant origin affects the frequency of tetraploid plants from tissue culture of melon. Hort Science 29:689–692. https://doi.org/10.21273/HORTSCI.29.6.689

Al-Zahim MA, Ford-Lloyd BV, Newbury HJ (1999) Detection of somaclonal variation in garlic (Allium sativum L.) using RAPD and cytological analysis. Plant Cell Rep18:473-47. https://doi.org/10.1007/s002990050606

Batista D, Sousa MJ, Pais MS (1996) Plant regeneration from stem and petiole-derived callus of *Humulus lupulus* L. (hop) clone Bragança and var. Brewers's Gold. In Vitro Cell Dev Pl 32:37–41. https://doi.org/10.1007/BF02823011

Bello-Bello JJ, Iglesias-Andreu LG, Avilés-Vinãs SA, Gómez-Uc E, Canto-Flick A, Santana-Buzzy N (2014) Somaclonal variation in habanero pepper (*Capsicum chinense* Jacq.) as assessed ISSR molecular markers. Hort Science 49(4):481–485. https://doi.org/10.21273/HORTSCI.49.4.481

Benzion G, Phillips RL (1988) Cytogenetic stability of maize tissue cultures: a cell line pedigree analysis. Genome 30:318–325. https://doi.org/10.1139/g88-056

Brown PTH, Lange FD, Krang E, Lorz H (1993) Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Gen Genet 237:311–317. https://doi.org/10.1007/BF00279433

Cassells AC (1979) The effect of 2,3,5-triiodobenzoic acid on caulogenesis in callus cultures of tomato and *Pelargonium*. Physiol Plant 46:159–64. https://doi.org/10.1111/j.1399-3054.1979.tb06550.x

Connell SA, Heale JB (1992) Sensitivity of cells and protoplasts of hop cultivars to cytotoxic components of culture filtrates of *Verticillium albo-atrum* isolates from hop. Plant Pathol 39(1):92–101. https://doi.org/10.1111/j.1365-3059.1990.tb02479.x

D'Amato F (1985) Cytogenetics of plant cell and tissue cultures and their regenerants. CRC Crit Rev Plant Sci 3:73–112. https://doi.org/10.1080/07352688509382204

De Verno LL, Park YS, Bonga JM, Barret JD (1999) Somaclonal variation in cryopreserved embryogenic clones of white spruce [*Picea glauca* (Moench) Voss.]. Plant Cell Rep 18:948–95. https://doi.org/10.1007/s002990050689

Dey T, Saha S, Ghosh PD (2015) Somaclonal variation among somatic embryo derived plants—evaluation of agronomically important somaclones and detection of genetic changes by RAPD in *Cymbopogon winterianus*. S Afr J Bot 96:112–121. https://doi.org/10.1016/j.sajb.2014.10.010

Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J (2011) Mitotic chromosome doubling of plant tissue *in vitro*. Plant Cell Tiss Organ Cult 104:359–373. https://doi.org/10.1007/s11240-010-9786-5

Duncan RR (1997) Tissue culture-induced variation in crop improvement. Adv Agron 58:201–240. https://doi.org/10.1016/S0065-2113(08)60256-4

Farahani F, Yari R, Masoud S (2011) Somaclonal variation in Dezful cultivar of olive (*Olea europaea* subsp. *europaea*). Gene Conserve 10:216–221

Gao X, Yang D, Cao D, Ao M, Sui X, Wang Q, Kimatu JN, Wang L (2010) *In vitro* micropropagation of *Freesia hybrid* and the assessment of genetic and epigenetic stability in regenerated plantlets. 3 Biotech (2016) 6:54 Page 13 of 18 54 123. J Plant Growth Regul 29:257–267. https://doi.org/10.1007/s00344-009-9133-4

Gurriarán MJ, Revilla MA, Tamés RS (1999) Adventitious shoot regeneration in cultures of *Humulus lupulus* L. (hop) cvs. Brewers Gold and Nugget. Plant Cell Rep 18:1007–11. https://doi.org/10.1007/s002990050699

Hamill SD, Smith MK, Dodd WA (1992) *In vitro* induction of banana autotetraploidy by colchicine treatment of micropropagated diploids. Aust J Bot 40:887–896. http://dx.doi.org/10.1071/BT9920887

Hashmi G, Huettel R, Meyer R, Krusberg L, Hammerschlag F (1997) RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. Plant Cell Rep 16:624–627. https://doi.org/10.1007/BF01275503

Heale JB, Legg T, Connell S (1989) *Humulus lupulus* L. (Hop): *In vitro* culture; attempted production of bittering components and novel disease resistance. In: Bajaj Y.P.S. (eds) Medicinal and Aromatic Plants II. Biotechnology in Agriculture and Forestry, vol 7. Springer, Berlin, Heidelberg, pp 264-285. https://doi.org/10.1007/978-3-642-73617-9_15

Hildebrand RP, Kavanagh TE, Clarke BJ (1975) Hop lipids and beer quality. Brewers Dig 50: 58–60.

Horáček J, Švábová L, ŠarhanováP, Lebeda A (2013) Variability for resistance to *Fusarium solani* culture filtrate and fusaric acid among somaclones in pea. Biol Plant 57(1):133–138. https://doi.org/10.1007/s10535-012-0131-1

Horlemann C, SchwekendiekA, Höhnle M, Weber G (2003) Regeneration and Agrobacterium-mediated transformation of hop (*Humulus lupulus* L.). Plant Cell Rep 22:210–217. https://doi.org/10.1007/s00299-003-0676-8

Hossain MA, Konisho K, Minami M, Nemoto K (2003) Somaclonal variation of regenerated plants in chili pepper (*Capsicum annuum* L.). Euphytica 130:233–239. https://doi.org/10.1023/A:1022856725794

Isabel N, Tremblay L, Michand M, Tremblay FM, Bousquet J (1993) RAPD as aids to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill.). Theor Appl Genet 86:81–87. https://doi.org/10.1007/BF00223811

Jain SM (2001) Tissue culture-derived variation in crop improvement. Euphytica 118:153–166. https://doi.org/10.1023/A:1004124519479

Jevremović S, SubotićA, MiljkovićD, TrifunovićM, PetrićM, Cingel A (2012) Clonal fidelity of *Chrysanthemum* cultivars after long term micropropagation by stem segment culture. Acta Hortic 961:211–216. https://10.17660/ActaHortic.2012.961.25

Kar B, Kuanar A, Singh S, Mohanty S, Joshi RK, Subudhi E, Nayak S (2014) *In vitro* induction, screening and detection of high essential oil yielding somaclones in turmeric (*Curcuma longa* L.). Plant Growth Regul 72(1):59–66. https://doi.org/10.1007/s10725-013-9836-1

Krishna H, Singh SK (2007) Biotechnological advances in mango (*Mangifera indica* L.) and their future implication in crop improvement—a review. Biotechnol Adv 25:223–243. https://10.1016/j.biotechadv.2007.01.001

Kuznetsova OI, Ash OA, Gostimsky SA (2006) The effect of the duration of callus culture on the accumulation of genetic alternations in pea *Pisum sativum* L. Russ J Genet 42:555–562. https://doi.org/10.1134/S1022795406050139

Larkin PJ, Scowcroft WR (1981) Somaclonal variation- a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214. https://doi.org/10.1007/BF02342540

Levin DA (2002) The role of chromosomal change in plant evolution. Oxford: Oxford University Press. https://doi.org/10.1600/036364404774195656

Linacero R, Freitas Alves E, Vázquez AM (2000) Hot spots of DNA instability revealed through the study of somaclonal variation in rye. Theor Appl Genet 100:506–511. https://doi.org/10.1007/s001220050066

Motegi T (1979) Differentiation of shoots from hop stem callus culture. Kyoyubokenkyu Neupo Iwate Ika Daigaku 14:15–17.

Munthali MT, Newbury HJ, Ford-Lloyd BV (1996) The detection of somaclonal variants of beet using RAPD. Plant Cell Rep 15:474–478. https://doi.org/10.1007/BF00232977

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15:473–479. http://dx.doi.org/10.1111/ j.1399-3054.1962.tb08052.x

Nhut DT, Hai NT, Thu PTM, Thi NN, Hien TTD, Tuan TT, Nam NB, Huy NP, Chien HX, Jain SM (2013) Protocol for inducing flower color somaclonal variation in torenia (*Torenia fournieri* Lind.). Methods Mol Biol 11013:455–462. doi: 10.1007/978-1-62703-074-8_34.

Pathak H, Dhawan V (2012) ISSR assay for ascertaining genetic fidelity of micropropagated plants of apple rootstock Merton 793. In Vitro Cell Dev Biol Plant 48:137–143. https://doi.org/10.1007/s11627-011-9385-0

Patzak J (2003). Assessment of somaclonal variability in hop (*Humulus lupulus* L.) *in vitro* meristem cultures and clones by molecular methods. Euphytica 131:343-350. https://doi.org/10.1023/A:1024096401424

Pérez G, Yanez E, Isidròn M, Lorenzo JC (2009) Phenotypic and AFLP characterization of two new pineapple somaclones derived from *in vitro* culture. Plant Cell Tissue Organ Cult 96:113–116. https://doi.org/10.1007/s11240-008-9463-0

Pérez G, Mbogholi A, Sagarra F, Aragòn C, Gonzàlez J, Isidròn M, Lorenzo JC (2011) Morphological and physiological characterization of two new pineapple somaclones derived from *in vitro* culture. In Vitro Cell Dev Biol Plant 47:428–433. https://doi.org/10.1007/s11627-011-9342-y

Pérez G, Yanez E, Mbogholi A, Valle B, Sagarra F, Yabor L, Aragòn C, Gonzàlez J, Isidròn M, Lorenzo JC (2012) New pineapple somaclonal variants: P3R5 and dwarf. Am J Plant Sci 3:1–11. https://doi.org/10.4236/ajps.2012.31001

Phillips RL, Kappler SM, Olhof R (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. Proc Natl Acad Sci USA 91:5222–5226. https://doi.org/10.1073/pnas.91.12.5222

Pijut PM, Beasley RR, Lawson SS, Palla KJ, Stevens ME, Wang Y (2012) *In vitro* propagation of tropical hardwood tree species—a review (2001–2011). Propag Ornam Plants 12(1):25–51

Polanco C, Ruiz ML (2002) AFLP analysis of somaclonal variation in Arabidopsis thaliana regenerated plants. Plant Sci 162:817–824. doi: 10.1016/S0168-9452(02)00029-8

Rakouský S, MatoušekJ (1994) Direct organogenesis in hop - a prerequisite for the application of *A. tumefaciens*-mediated transformation. Biol Plantarum 36:191–200. https://doi.org/10.1007/BF02921085

Rodolfi M, Silvanini A, Chiancone B, Marieschi M, Fabbri A, Bruni B, Ganino T (2018). Identification and genetic structure of wild Italian *Humulus lupulus* L. and comparison with European and

American hop cultivars using nuclear microsatellite markers. Genet Resour Crop Evol 65:1405–1422. doi: https://doi.org/10.1007/s10722-018-0622-9

Rout G.R. (2002). Direct plant regeneration from leaf explant of Plumbago species and its genetic fidelity through RAPD markers. Ann Appl Biol 140:305-313. DOI: https://doi.org/10.1111/j.1744-7348.2002.tb00186.x

Roy AT, Leggett G, Koutoulis A (2001) *In vitro* tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). Plant Cell Rep 20:489–495. https://doi.org/10.1007/s002990100364

Skof S, Bohanec B, Kastelec D, Luthar Z (2007) Spontaneous induction of tetraploidy in hop using adventitious shoot regeneration method. Plant Breed 126:416–421. https://doi.org/10.1111/j.1439-0523.2007.01378.x

Stanišić M, Raspor M, Ninković S, Milošević S, Ćalić D, Bohanec B, Trifunovića M, Petrića M, Subotić A, Jevremović S (2015) Clonal fidelity of *Iris sibirica* plants regenerated by somatic embryogenesis and organogenesis in leaf-base culture—RAPD and flow cytometer analyses. S Afr J Bot 96:42–52. https://doi.org/10.1016/j.sajb.2014.10.014

Sun S, Zhong J, Li S, Wang X (2013) Tissue culture-induced somaclonal variation of decreased pollen viability in torenia (*Torenia fournieri* Lind.). Bot Stud 54(1):36. https://doi.org/10.1186/1999-3110-54-36

Šuštar-Vozlič J, Javornik B, Bohanec B (1999) Studies of somaclonal variation in hop (*Humulus lupulus* L.). Phyton – Ann Rei Bot 39:283–287

Trojak-Goluch A, Kawka M, Czarnecka D (2015) The effects of explant source and hormone content on plant regeneration and induction of tetraploids in *Humulus lupulus* L. In Vitro Cell Dev Biol-Plant 51:152-https://doi.org/10.1007/s11627-014-9661-x

Trojak-Goluch A, Skomra U (2013) Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits. Breed Sci 63:393–399. https://doi:10.1270/jsbbs.63.393

Van Duren M, Mopurgo R, Dolezel J, Afza R (1996) Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. Euphytica 88:25–34. https://doi.org/10.1007/BF00029262

Vujović T, Ružić Đ, Cerović R, Momirović GŠ (2010) Adventitious regeneration in blackberry (*Rubus fruticosus* L.) and assessment of genetic stability in regenerants. Plant Growth Regul 61:265–275. https://doi.org/10.1007/s10725-010-9474-9

Wang ZY, Nagel J, Potrykus I, Spangenberg G (1993) Plants from cell suspension-derived protoplasts of *Lolium* species. Plant Sci 94:179–193. https://doi.org/10.1016/0168-9452(93)90019-V

Zayova E, Vassilevska IR, Kraptchev B, Stoeva D (2010) Somaclonal variations through indirect organogenesis in eggplant (*Solanum melongena* L.). Biol Divers Conserv 3:1–5

**Authors Contribution Statement**

C.L. carried out the *in vitro* leaf culture. C.L. and M.R. carried out the molecular marker analysis. B.C. did the statistical analysis with the support of C.L. T.G. and B.C. conceived the original idea. B.C. wrote the manuscript with support of C.L., T.G. and A.F.. B.C. supervised the project.

**Conflict of Interest**: The authors declare that they have no conflict of interest.

**Tables**

Table 1. Sequences of 20 decamer RAPD primers used for identifying unique bands from *Humulus lupulus* L. *in vitro* leaf regenerants

| Name | Sequence (5'a 3') | Name | Sequence (5'a 3') |
|---|---|---|---|
| OPA10 | GTGATCGCAG | AI05 | GTCGTAGCGG |
| OPK16 | GAGCGTCGAA | AI08 | AAGCCCCCCA |
| OPC16 | CACACTCCAG | AI11 | ACGGCGATGA |
| 544 | TAGAGACTCC | AI12 | GACGCGAACC |
| AH01 | TCGGCAACCA | AI14 | TGGTGCACTC |
| AH09 | AGAACCGAGG | AI16 | AAGGCACGAG |
| AH12 | TCCAACGGCT | OPB10 | CTGCTGGGAC |
| AH18 | GGGCTAGTCA | OPB20 | GGACCCTTAC |

Table 2: Effect of period of culture and of culture medium composition on callus, root and shoot formation from hop, cv. Gianni, leaf portions, after 18 weeks of culture

| PC | Culture Medium Composition | Explants with callus | Explants with roots | Explants with shoots | Mean n° of roots per explant | Mean n° of shoots per explant | Root length | Shoot length |
|---|---|---|---|---|---|---|---|---|
| | | %±SE | %±SE | %±SE | n°±SE | n°±SE | mm±SE | mm±SE |
| I | HF | 0.00±0.0 | 0.00±0.0 | 0.00±0.0 | - | - | - | - |
| I | BAP0 | 0.00±0.0 | 2.00±2.0 | 0.00±0.0 | - | - | - | - |
| I | BAP2 | 94.00±6.0 | 31.00±8.9 | 0.00±0.0 | 2.09±0.2 | - | 6.60±0.6 | - |
| I | BAP4 | 93.00±7.0 | 17.00±4.7 | 5.00±1.7 | 1.88±0.4 | 3.80±2.1 | 8.52±1.2 | 5.41±1.2 |
| I | BAP6 | 99.00±1.0 | 11.00±3.5 | 4.00±1.6 | 2.31±0.3 | 1.50±0.3 | 6.00±0.6 | 7.13±1.5 |
| I | BAP8 | 80.00±11.8 | 10.00±4.5 | 2.00±2.0 | 1.10±0.1 | 2.50±1.5 | 12.40±2.2 | 6.00±1.2 |
| II | HF | 0.00±0.0 | 0.00±0.0 | 0.00±0.0 | - | - | - | - |
| II | BAP0 | 0.00±0.0 | 2.00±2.0 | 0.00±0.0 | - | - | - | - |
| II | BAP2 | 94.00±6.0 | 50.00±9.7 | 0.00±0.0 | 2.10±0.2 | - | 6.58±0.5 | - |
| II | BAP4 | 93.00±7.0 | 39.00±8.9 | 11.00±4.1 | 1.69±0.2 | 5.33±1.8 | 7.82±0.9 | 11.00±2.8 |
| II | BAP6 | 99.00±1.0 | 42.00±10.3 | 13.00±9.5 | 1.70±0.2 | 2.73±0.5 | 6.39±0.5 | 13.08±2.5 |
| II | BAP8 | 80.00±11.8 | 15.00±6.4 | 14.00±3.7 | 1.27±0.3 | 3.22±0.6 | 9.58±1.5 | 11.60±2.2 |
| III | HF | 0.00±0.0 | 0.00±0.0 | 0.00±0.0 | - | - | - | - |
| III | BAP0 | 0.00±0.0 | 2.00±2.0 | 0.00±0.0 | - | - | - | - |
| III | BAP2 | 94.00±6.0 | 50.00±9.7 | 0.00±0.0 | 2.10±0.2 | - | 6.58±0.5 | - |
| III | BAP4 | 93.00±7.0 | 41.00±9.6 | 6.00±2.2 | 1.66±0.2 | 2.50±0.7 | 7.82±0.9 | 6.60±1.1 |
| III | BAP6 | 99.00±1.0 | 47.00±10.6 | 9.00±6.5 | 1.94±0.2 | 2.79±0.5 | 6.12±0.4 | 16.50±3.0 |
| III | BAP8 | 80.00±11.8 | 19.00±7.2 | 10.00±3.0 | 1.63±0.4 | 3.13±0.6 | 8.39±1.0 | 9.14±2.5 |
| [a]Statistical analysis of factors | | | | | | | | |
| RT | | 0.633 | 0.042 | 0.030 | 0.535 | 0.182 | 0.900 | 0.000 |
| CMC | | 0.000 | 0.000 | 0.000 | 0.000 | 0.439 | 0.004 | 0.650 |
| RT*CMC | | 0.000 | 0.000 | 0.000 | 0.000 | 0.521 | 0.000 | 0.000 |

[a]Two-way analysis of variance (ANOVA), followed by Tukey's test ($p \leq 0.05$). **PC:** Period of Culture; **CMC:** Culture Medium Composition. **I**: 6 weeks of culture; **II**: 12 weeks of culture; **III**: 18 weeks of culture. **HF**: 0μM NAA and 0μM BAP; **BAP0**: 0.1μM NAA and 0μM BAP; **BAP2**: 0.1μM NAA and 8.88μM BAP; **BAP4**: 0.1μM NAA and 17.77μM BAP; **BAP6**: 0.1μM NAA and 26.66μM BAP; **BAP8:** 0.1μM NAA and 35.55μM BAP.

Table 3. Presence of polymorphic RAPD fragments in regenerants obtained from leaf portion of hop, cv. “Gianni”, and in the mother plant (MP). The symbols + and – indicate, respectively, the presence or the absence of a RAPD marker.

| **RAPD bands** | **Regenerant/cultivar** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 7 | 10 | 19A | 48 | 52 | 78 | 112 | 134 | MP |
| OPK16-1625 | - | - | - | - | - | - | - | + | - |
| AI12-1650 | + | + | - | + | + | + | + | + | + |
| AI12-1501 | + | + | - | + | + | + | + | + | + |
| AI05-1110 | + | + | + | - | + | + | - | + | + |
| AI05-910 | - | + | - | - | - | - | - | - | - |
| AI05-866 | + | + | + | + | - | + | + | + | + |
| AI05-800 | + | + | - | - | - | - | - | - | - |
| AI05-760 | + | - | - | - | - | - | - | - | - |
| AI05-710 | + | - | + | + | - | - | - | + | + |
| AI05-630 | + | - | + | + | - | - | + | + | + |
| AI05-570 | + | - | - | - | - | - | - | - | - |
| AI05-515 | + | + | - | + | + | + | + | - | - |
| AI05-470 | + | + | + | + | - | + | - | + | + |

**Figures**

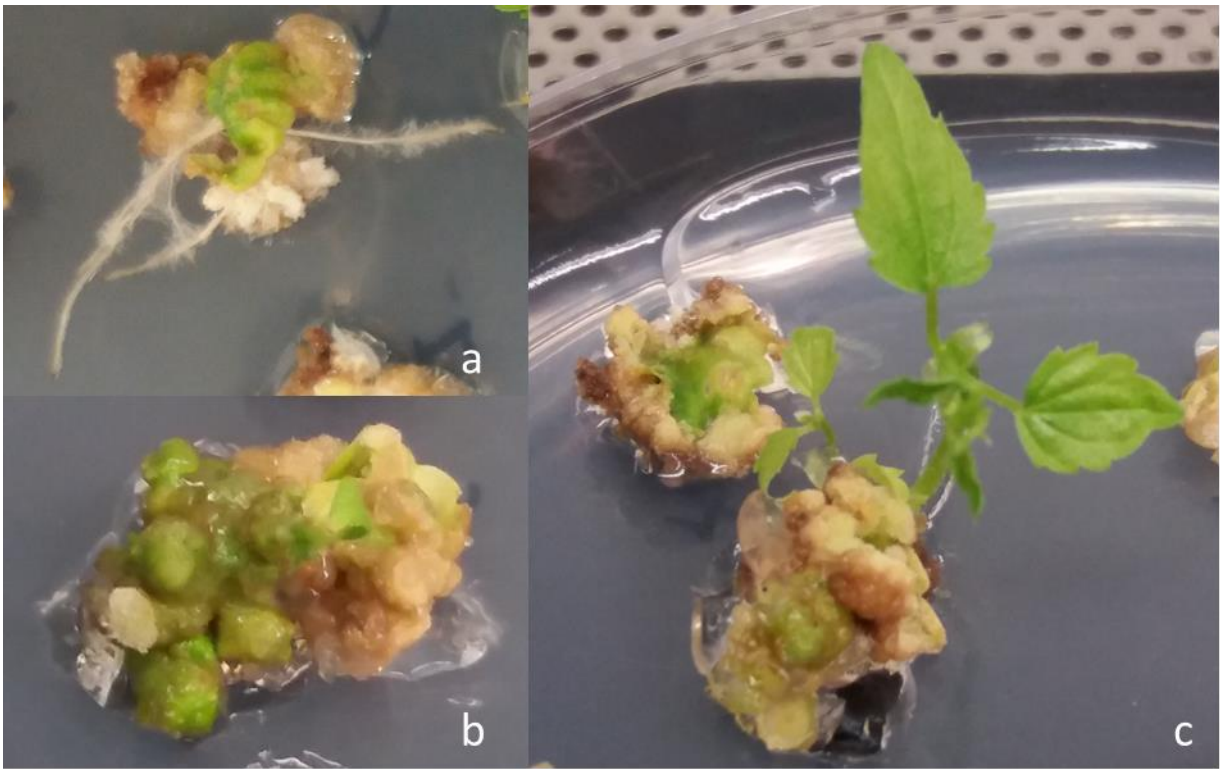
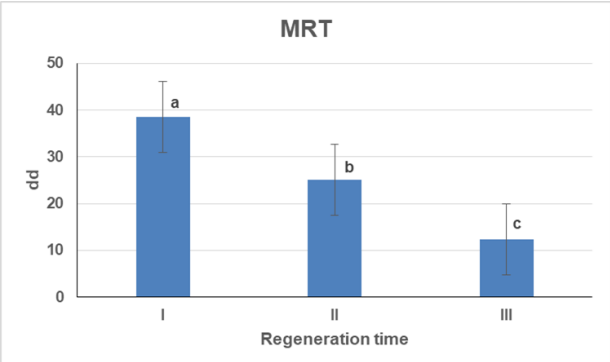



**Figure 1**. *In vitro* organogenesis from hop, genotype Gianni, leaves: **a)** indirect root regeneration; **b)** organogenic centers coming from undifferentiated callus; **c)** adventitious shoot regenerated from undifferentiated callus. **.**



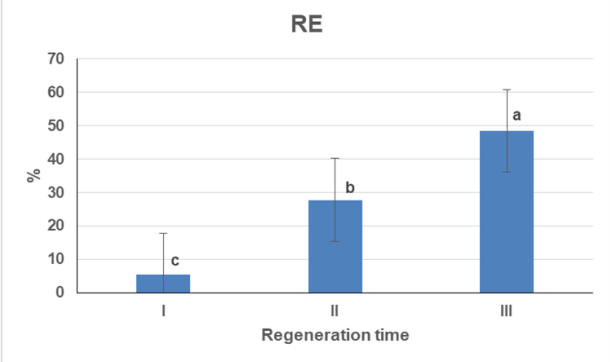




**Figure 2**. Mean Regeneration Time (MRT) (a) and Regeneration Energy (RE) calculated after 18 weeks of culture. One-way ANOVA, Tukey's test ($p \leq 0.05$).

**e**

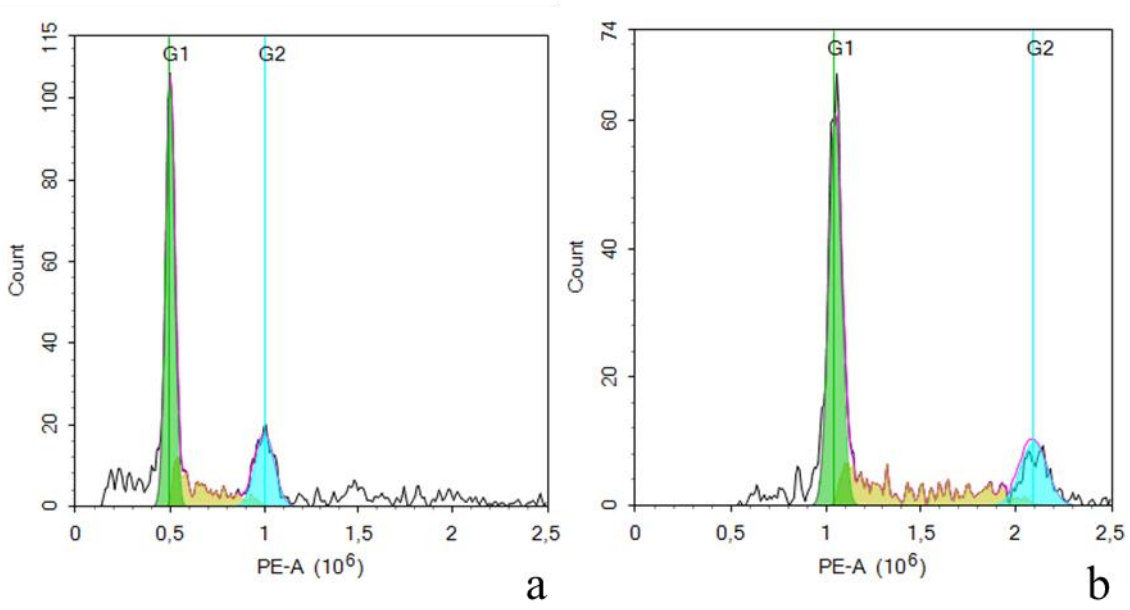



**Figure 3**. Cytofluorimetric analysis of hop, genotype Gianni, mother plant (a) and a tetraploid regenerant (b).

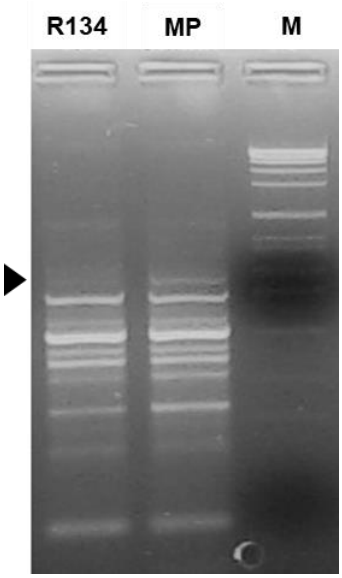



**Figure 4**. OPK16 RAPD marker showing difference in DNA profile amplification in mother plant, cv. Gianni, and one regenerant from hop, cv. Gianni, leaf. Lane R134 = leaf regenerant; Lane MP = hop, cv. Gianni, mother plant; Lane M = DNA molecular weight marker VII (M) (Roche Diagnostics GmbH, Mannheim, DE). Arrow indicates the differences in DNA profiles.

## Final Conclusions of the Ph.D. Thesis

The new trend of craft beer production and the increasing interest in herbal products market led to a high demand of hops with innovative characteristics, and in particular, to the research of hops endowed with peculiar phytochemical profiles and pharmacologically relevant compounds. This research represents a big challenge not only for hop growers, but also for breeders that, due to the depletion of hop intraspecific agrobiodiversity, caused by mainly its domestication and cultivation process, firstly, need to characterize and, then, increase hop biodiversity.

Therefore, to make the hop culture a solid reality, it is necessary to deepen knowledge on existing hop biodiversity, but also to renew the varietal panorama.

In this context, my PhD thesis project was set in order to valorise and enrich existing hop agrobiodiversity, exploiting the potential of this species.

In the frame of hop agrobiodiversity valorization, two research were carried out. In the first research, the effect of **the adaptability of International cultivar to the Italian climate** on chemical composition variation (bitter acids, oil yield and aromatic profile) in hop cones was evaluated, by comparing hop cones of cultivar Cascade grown in different areas of Italy with those grown in the main producing areas of the United States of America (Oregon and Michigan), Germany (Tettnang region) and Slovenia (Carniola region). Obtained results demonstrated significant differences in bitter acids and xanthohumol content among the analyzed samples. Overall, cone composition resulted notably varied in each growing area, underlining how geography and climatic conditions may affect hop secondary metabolism.

The second research aimed at overcoming limitations due to the lack of hop plants at nursery level, describes two methods of ***in vitro*** **hop propagation,** to obtain a great number of plantlets, in limited space and, independently of the season. Specifically, from hop petioles a remarkable shoot regeneration was obtained, confirming the possibility of using this kind of explants as a method of hop propagation, but, due to the recording of a small percentage of tetraploids, a precise and continuous check of somaclonal variation need to be carried out. To overcome the problem of somaclonal variation and the issues connected to conventional germplasm storage methods, for the first time, in our knowledge, hop microcutting encapsulation technology was tested. Obtained results (up to 82% of conversion) demonstrated the suitability of microcuttings to encapsulation and represents a first step towards the applications of this technology for other purposes, such as hop germplasm conservation.

In the context of agrobiodiversity enrichment three research were carried out.

The first study, that in our knowledge is the first on *in vitro* culture of hop seeds, gives new insights on the mechanisms of **hop seed germination**. The influence of several pre-treatments (timing of chemical scarification, cold stratification, imbibitions in water and in gibberellic solution) on *in vitro* hop (cv. Columbus) seed germination was evaluated. Firstly, a sterilization protocol has been set up, minimizing contamination occurrence. Moreover, the use of gibberelic acid both in the imbibitions solution and in the culture medium allowed to achieve a 33% of *in vitro* hop seed germination. The use of sex-linked molecular markers has made possible the precocious individuation of seedling gender. Results obtained in this investigation are really promising; indeed, thanks to biotechnologies it was possible to obtain a great number of seedlings in winter, independently of the natural germination season.

All in the interest of enriching the variability, other two studies have been carried out to induce new variability, investigating the gametoclonal and the somaclonal variation.

One study aimed at studying **hop male flower biology, microspore and pollen development**. First of all, obtained results provided a detailed description of the different microspore or pollen grain developmental stage present during hop male flower development. Moreover, a correlation between microspore/pollen grain developmental stage and the bud/anther size has been determined. This work filled a gap of knowledge about hop male flower and represents a first step to the use of doubled haploid technology technique in hop breeding, to exploit gametoclonal variation.

In the last study, the effect of different types and concentrations of growth regulators, together with the effect of the time in which the explants were kept in culture was tested on **hop leaf regeneration**. Cytofluorimetric and RAPD marker analysis detected that the 16.8% of the regenerated plantlets were mutated, some of them showed a tetraploid genome, confirming that indirect organogenesis from hop, cv. Gianni, could be considered a valuable method to obtain polyploids. Through *in vitro* tissue culture, it was possible to recover, in a relatively short time, tetraploids, useful in hop breeding, since they can be crossed with diploid to obtain triploids, considered to be superior to both diploids and tetraploids.

Results reported in this Ph.D. thesis give new insights to further deep investigations on hop agrobiodiversity valorization and enrichment, through traditional and biotechnological methods.

## References

Abbo S., van-Oss Pinhasi R., Gopher A. (2014). Plant domestication versus crop evolution: a conceptual framework for cereals and grain legumes. Trends Plant Sci 19:351–360.

Acquaah G. (2012). Principles of plant genetics and breeding. Blackwell, Oxford.

Adams A.N. (1975). Elimination of viruses from the hop (*Humulus lupulus*) by heat and meristem culture. J. Hort. Sci., 50:151-160.

Al-Khayri J.M., Jain S.M., Johnson D.V. (2015). Advances in plant breeding strategies: Breeding, biotechnology and molecular tools, Springer. XII: 707.

Amoriello T. (2019). Indagine sulla filiera brassicola: evoluzione e prospettive per lo sviluppo del settore. In: Atti del II Convegno Nazionale "Criticità e opportunità per lo sviluppo sostenibile di una filiera del luppolo italiano" 16-17 Gennaio 2019, Roma, Italy. pp. 13-24.

Arnold J. P. (2005). Origin and history of beer and Brewing: from prehistoric times to the beginning of brewing science and technology. Alumni Assn. of the Wahl-Henius Institute, 1911. Reprint Edition, BeerBooks.com, 2005, Chicago, IL.

Assobirra (2018). Annual report, Italy, 2018.

Atanassov A., Zagorska N., Boyadjiev P., Djilianov D. (1995). *In vitro* production of haploid plants. World J Microbiol Biotechnol 11:400–408.

Barham E. (2003). Translating terroir: the global challenge of French AOC labeling.*J Rural Stud* 19:127–138.

Barth and Hass (2010). Humulus lupulus and other bitter truths. The secret life of hops. Published in Brauindustrie, Vol. 1.

Barth and Hass. (2017-2018). The Barth Report, Nuremberg, Germany.

Barton K.A., Binns A.N., Matzke, A.J.M., Chilton M.D. (1983). Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T- DNA to R1 progeny. Cell, 32:1033–1043.

Batista D., Sousa M.J., Pais M.S. (1996). Plant regeneration from stem and petiolederived callus of *Humulus lupulus* L. (hop) clone Branganca and var. Brewers's Gold. In Vitro Cell. Dev. Biol. – Plant, 32:37-41

Bayliss, M.W. (1980). Chromosomal variation in plant tissues in culture. Intern. Rcv. Cytol. Suppl. 1 IB, 113-144.

Becker J. (2000). Untersuchungen zur in vitro regeneration und genetischen transformation von hopfen (Humulus lupulus L.). Dissertation, University of Kaiserslautern, Kaiserslautern, Germany.

Behre K.E. (1999). The history of beer additives in Europe—a review. Vegetation History and Archeobotany 8: 35–48.

Benson E.E. (2008). Cryopreservation of phytodiversity: a critical appraisal of theory & practice. Crit Rev Plant Sci 27:141–219.

Bettinger R. (2012). Early steps in agricultural domestication. In: Gepts P, Bettinger R, Brush S et al (eds) Biodiversity in agriculture: domestication, evolution and sustainability. Cambridge University Press, Cambridge, pp 19–20.

Bhat S.R., Chandel K.P.S., Malik S.K. (1995). Plant regeneration from various explants of cultivated. Piper Species 14(6):398–402.

Blakeslee A.F., Belling J., Farhnam M.E., Bergner A.D. (1922). A haploid mutant in the Jimson weed, Datura stramonium. Science 55:646-647.

Blumenthal M. (1998). The Complete German Commission E Monograph: Therapeutic Guide to Herbal Medicines. American Botanical Council, Austin, TX, p. 147.

Bonga J.M., von Aderkas P. (1992). Genetic modification. In: J.M. Bonga and P. von Aderkas (eds) In Vitro Culture of Trees. Kluwer Ac. Publ., Dordrecht, The Netherlands, pp. 156-165.

Burgess A.H. (1964). Hops. Botany, cultivation, and utilization. World Crops Books, Ed. Professor Nicholas Polunin.

Chi H.S. (2002).. The efficiencies of various embryo rescue methods in interspecific crosses of Lilium. Bot Bull Acad Sin 43:139–146

Cistué L., Vallés M.P., Echavarri B. (2003). Barley anther culture. In: Maluszynski M, Kasha K, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer Academic Publishers, Norwell, pp 29–34.

Collard B.C.Y., Jahurfer M.Z.Z., Brouwer J.B., Pang E.C.K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping, and marker-assisted selection for crop improvement: The basic concepts. Euphytica, 142:169–196.

Collonnier C., Fock I., Marie–Christine D. (2003). Somatic hybrids between *Solanum melongena* and *S. sisymbrifolium*, as a useful source of resistance against bacterial and fungal wilts. Plant Sci 164:849–861.

Convention on Biological Diversity (1992). Conference on Environment and Development (the "Earth Summit"), Rio de Janeiro, Brazil, June 1992.

Dantas A.C.M, Boneti J.I., Nodari R.O, Guerra M.P. (2006) Embryo rescue from interspecific crosses in apple rootstocks. Pesq Agrop Bras 41(6):969–973.

Datta S.K. (2005). Androgenic haploids: factors controlling development and its application in crop improvement. Curr Sci 89:1870–1878.

Debbarama C., Khanna V.K., Tyagi W., Rai M., Meetei N.T. (2013). Wide hybridization and embryo-rescue for crop Improvement in Capsicum . Agrotechnology S11: 003. doi:10.4172/2168–9881S11–003.

Deblock M., Herreraestrella L., Vanmontagu M., Schell J., Zambryski P. (1984). Expression of foreign genes in regenerated plants and in their progeny. EMBO Journal, 3:1681–1689.

De Cleene M., De Ley J.(1976). The host range of crown gall. Bot. Rev., 42:389- 466.

Donald C.M. (1968). The breeding of crop ideotypes. Euphytica, 17:385-403.

Dudley J.W., Lambert R.J. (1992). Ninety generations of selection for oil and protein in maize. Maydica 37:81–87.

Duncan R.R. (1997). Tissue culture-induced variation in crop improvement. Adv Agron 58:201–240.

Engelmann F. (1990). Conservation *in vitro* of forest tree genetic resources. In Proc. Training course Mgmt. Forest Genetic Resources and Agroforestry. Bogor, Indonesia.

Engelmann F. (1991). Tropical plant germplasm conservation. in Proc. 4th Conf. IntI. Plant Biotechnol. Network (lPBNet)-Biotechnology for tropical crop improvement in Latin America. San Jose, Costa Rica.

Eri S., Khoo B.K., Lech J., Hartman T.G. (2000). Direct thermal desorption–gaschromatography and gas chromatography–mass spectrometry profiling of hop (*Humulus lupulus* L.). J. Agric. Food Chem. 48:1140–1149.

Evans D.A., Sharp W.R., Medina-Filho H.P. (1984). Somaclonal and gametoclonal variation. Am] Bot. 71: 759-774.

FAO (2019). The state of the world's biodiversity the state of the world's biodiversity for food and agriculture. Rome, 2019.

FAO & PAR (2010). Biodiversity for Food and Agriculture - Contributing to food security and sustainability in a changing world outcomes. pp. 15- 26. Available at: http://www.fao.org/fileadmin/templates/biodiversity_paia/PAR-FAO-book_lr.pdf

Farago J., Psenakova I., Faragova N. (2009) The use of biotechnology in hop (*Humulus lupulus* L.) improvement. Nova Biotechnol 9:279–293.

Faragó J., Nešťáková M. (1998). Kultivácia meristémov v in vitro kultúre a produkcia bezvírusovej sadby pri chmeli obyčajnom (Humulus lupulus L.) [Cultivation of meristems in in vitro culture and production of virus-free stock plants in hop (Humulus lupulus L.)]. Zb. 4. odb. sem. Nové poznatky z genetiky a šľachtenia poľnohospodárskych rastlínPiešťany, 29-31.

Farahani F., Yari R., Masoud S. (2011). Somaclonal variation in Dezful cultivar of olive (Olea europaea subsp. europaea). Gene Conserve 10:216–221.

Fathi H., Jahani U. (2012). Review of embryo culture in fruit trees. Ann Biol Res 3(9):4276–4281.

Fay M.F. (1994). In what situation is in vitro culture appropriate to plant conservation? Biodivers Conserv 3:176–183.

Foroni F., Vignando M., Aiello M., Parma V.P., Squartini M.G., Rumiati A.R.I. (2017). The smell of terroir! Olfactory discrimination between winesof different grape variety and different terroir.Food Qual Prefer58:18–23.

Gao X., Yang D., Cao D., Ao M., Sui X., Wang Q., Kimatu J.N., Wang L. (2010). *In vitro* micropropagation of Freesia hybrid and the assessment of genetic and epigenetic stability in regenerated plantlets 3 Biotech (2016) 6:54 Page 13 of 18 54 123. J Plant Growth Regul 29:257–267.

Gelvin S.B. (1990). Crown gall disease and hairy root disease – a sledgehammer and a tackhammer. Plant Physiology, 92:281–285.

Gepts P., Bettinger R., Brush S., Damania A., Famula T., McGuire P., Qualset C. (2012). Introduction: the domestication of plants and animals: ten unanswered questions. In: Gepts P, Bettinger R, Brush S et al (eds) Biodiversity in agriculture. Domestication, evolution and sustainability. Cambridge University Press, Cambridge, pp 1–8.

Green C.P. (1997). Comparison of Tettnanger, Saaz, Hallertau and Fuggle hopsgrown in the USA, Australia and Europe.J Inst Brewing103:239–243.

Grieve M. (1971). A Modern Herbal. Dover Publications, Inc., New York.

Guha S., Maheshwari S.C. (1964). *In vitro* production of embryos from anthers of Datura . Nature 204(4957):497.

Harding K. (2004). Genetic integrity of cryopreserved plant cells: a review. Cryo Lett 25:3–22.

Hawksworth D. L. (1995). The resource base for biodiversity assessments. In: Heywood V. H. (ed.), Global biodiversity assessment. UNEP, Cambridge University Press, Cambridge. Pp. 545-605.

Henning J.A., Steiner J.J., Hummer K.E. (2004). Genetic diversity among world hop accessions grown in the USA. Crop Sci 44:411–417. doi:10.2135/cropsci2004.4110

Herrera-Estrella L., Depicker A., Vanmontagu M., Schell J. (1983). Expression of chimaeric genes transferred into plant cells using a Ti-plasmid derived vector. Nature, 303:209–213.

Hooykaas P.J.J., Schilperoort R.A. (1992). Agrobacterium and plant genetic engineering. Plant Molecular Biology, 19, 15–38.

Horlemann C., Schwekendiek A., Höhnle M., Weber G. (2003). Regeneration and Agrobacterium-mediated transformation of hop (*Humulus lupulus* L.). Plant Cell Rep., 22:210-217.

Horsch R.B., Fraley R.T., Rogers S.G., Sanders P.R., Lloyd A., Hoffmann N. (1984). Inheritance of functional foreign genes in plants. Science, 223:496–498.

Jain S.M, Brar D.S., Ahloowalia B.S. (eds) (1998). Somaclonal variation and induced mutations in crop improvement, vol 32. Current plant science and biotechnology in agriculture. Springer, Dordrecht.

Jakowitsch J., Metter M.F., van der Winden J., Matzke M.A., Matzke A.J.M. (1999). Integrated pararetroviral sequences define a unique class of dispersed repetitive DNA in plants. Proc. Natl. Acad. Sci. USA 66:13241–13246.

Jarvis D.I., Hodgkin T., (1999). Wild relatives and crop cultivars: detecting natural introgression and farmer selection of new genetic combinations in agroecosystems. Mol. Ecol. 8:S159-S173.

Jiang C., Mithani A., Gan X.. Belfield E.J, Klingler J.P., Zhu J.K., Ragoussis J., Mott R., and Harberd N.P. (2011). Regenerant Arabidopsis lineages display a distinct genome– wide spectrum of mutations conferring variant phenotypes. Curr Biol 21:1385–1390.

Kado C.I. (1994). Promiscuous DNA transfer system of *Agrobacterium tumefaciens*: role of the Virb operon in sex pilus assembly and synthesis. Molecular Microbiology, 12:17–22.

Kaeppler S.M., Phillips R.L. (1993). DNA methylation and tissue culture induced variation in plants. In Vitro Cell. Dev. Biol.–Plant, 29:125-130.

Kalua C.M, Allen M.S., Bedgood D.R., Jr, Bishop A.G. (2007). Prenzler PD andRobards K, Olive oil volatile compounds, flavour development andquality: a critical review.Food Chem100:273–286.

Kartha K.K. (1985). Cryopreservation of plant cell and organs. IAPTC Newsletter 45:2-15.

Kasha K., Maluszynski M. (2003). Production of doubled haploids in crop plants. An introduction. In: Maluszynski M, Kasha K, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer Academic Publishers, Norwell, pp 1–4.

Khan K.H. (2009). Gene transfer technologies in plants: Roles in improving crops. Recent Res Sci Technol 1(3):116–123.

Kralj D., Zupanec J., Vasil, D., Kralj S., Pšenicnik J. (1991). Variability of essential oils of hops, (*Humulus lupulus* L.). J. Inst. Brew. 97:197–206.

Kukharchyk N., Kastrickaya M. (2006). Embryo rescue techniques in *Prunus* L. breeding. J Fruit Orn Plant Res 14(Suppl. 1):129–135.

Laimer M., Ucker W. (2003). Plant tissue culture, 100 years since Gottlieb Haberlandt. Springer, Vienna.

Lambardi M., Benelli C., Ozudogru E.A., Ozden-Tokatli Y. (2006). Synthetic seed technology in ornamental plants. Floricult Ornam Plant Biotechnol 2:347–354.

Larkin J., Scowcroft W.R. (1981). Somaclonal variation- a novel source of variabilità from cell cultures for plant improvement. Theor Appl Genet 60:197–214.

Lenglet F. (2014). Influence of terroir products meaning on consumer's expec-tations and likings. Food Qual Prefer32:264–270.

Maluszynski M., Kasha K.J., Szarejko I. (2003). Published doubled haploid protocols in plant species. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants: a manual. Kluwer, Dordrecht, pp 309–335.

Mez E. (1969). Luppolo. In: Enciclopedia Agraria Italiana. Ed. Ramo Editoriale Degli Agricoltori, Roma 6:1064-1077.

Micheli M., Hafiz I.A., Standardi A. (2007). Encapsulation of *in vitro*-derived explants of olive (*Olea europaea* L. cv. Moraiolo) II. Effects of storage on capsule and derived shoot sperformance. Sci. Hortic. 113:286–92.

Miller K., Allegretti M.A., Johnson N., Jonsson B. (1995). Measures for conservation of biodiversity and sustainable use of its components. In: Heywood V. H. (ed.), Global biodiversity assessment. UNEP, Cambridge University Press, Cambridge. Pp. 915-1061.

Moir M. (2000). Hops—a millennium review. Journal of the American Society of Brewing Chemistry 58:131–146.

Morrison R.A., Evans D.A (1987). Gametoclonal variation. Plant Breed Rev. 5: 359-391.

Nehra S.A., Kartha K.K. (1994). Meristem and shoot tip culture: Requirements and applications. In: Plant cell and tissue culture. (Vasil, I. and Thorpe, T.A. Eds.) Dordrecht, Netherlands: Kluwer Academic Publishers. pp. 37-70.

Neve R.A. (1991). Hops. Chapman and Hall, New York.

Nwauzoma A.B., Jaja E.T. (2013). A review of somaclonal variation in plantain ( Musa spp.): mechanisms and applications. J Appl Biosci 67:5252–5260.

Oberthür T., Läderach P., Posada H., Fisher J.M., Samper L.F., Illera J., Collet L., Moreno E., Alarcón R., Villegas A., Usma H., Perez C., Jarvis A. (2011). Regional relationships between inherent coffee quality and growing environment for denomination of origin labels in Nariño and Cauca, Colombia. Food Policy 36:783-794. https://doi.org/10.1016/j.foodpol.2011.07.005.

Olšovská J., Boštíková V., Dušek M., Jandovská V., Bogdanová K., Čermák P., Boštík P., Mikyška A., Kolař M. (2016). *Humulus lupulus* L. (Hops) - a valuable source of compounds with bioactive effects for future therapies, Mil. Med. Sci. Lett. 85:19–30.

Patzak J., Oriniaková P., Matoušek J., Svoboda P. (1999) .Czech hop characterization using RAPD method and genetic distance analysis of selected genotypes. Plant Soil Environ. 45(4):165–172.

Patzak J., Nesvadba V., Henychová A., Krofta, K. (2010a). Assessment of the genetic diversity of wild hops (*Humulus lupulus* L.) in Europe using chemical and molecular analyses. Biochem. Syst. Ecol. 38:136–145.

Patzak J., Nesvadba V., Krofta K., Henychová A., Inalovic M., Richards K. (2010b). Evaluation of genetic variability of wild hops (*Humulus lupulus* L.) in Canada and Caucasus region by chemical and molecular methods. Genome 53:545–557.

Peredo E.L., Ángeles-Revilla M., Arroyo-García R. (2006). Assessment of genetic and epigenetic variation in hop plants regenerated from sequential subcultures of organogenic calli. Journal of Plant Physiology 163:1071–1079.

Pijut P.M., Beasley R.R., Lawson S.S., Palla K.J., Stevens M.E., Wang Y. (2012). *In vitro* propagation of tropical hardwood tree species—a review (2001–2011). Propag Ornam Plants 12(1):25–51.

Plessis P., Leddet C., Collas A., Dereuddre J. (1993). Cryopreservation of *Vitis vinifera* L. cv 'Chardonnay' shoot tips by encapsulation-dehydration: effects of pretreatment, cooling and postculture conditions. Cryo-Lett 14:309–320.

Proseviçius J., Strikulyte L. (2004). Interspecific hybridization and embryo rescue in breeding of lilies. Acta Univ Latv Biol 676:213–217.

Pšenáková I.; Gašpárková Ľ.; Faragó J. (2009). Polyphenol and flavonoid contents of hop callus and cell suspension cultures. Proc. Sci. Comm. IHGC, León, Spain, 109

Qian W., Liu R., Meng J. (2003). Genetic effects on biomass yield in interspecific hybrids between *Brassica napus* and *B. rapa* . Euphytica 134:9–15.

Roca W.M., Chavez R., Martin M.L., Arias D.I., Mafia G., Reyes R. (1989). *In vitro* methods of germplasm conservation. Genome 31:813-817.

Rodolfi M., Silvanini A., Chiancone B., Marieschi M., Fabbri A., Bruni R., Ganino T. (2018). Identification and genetic structure of wild Italian *Humulus lupulus* L. and comparison with European and American hop cultivars using nuclear microsatellite markers. Genet Resour Crop Evolut 65(5):1405–1422.

Rodriguez-Enriquez J., Dickinson H.G., Grant-Downton R.T. (2011). MicroRNA misregulation: an overlooked factor generating somaclonal variation? Trends Plant Sci 16:242–248.

Roy A.T., Leggett G. Koutoulis A. (2001). Development of a shoot multiplication system for hop (*Humulus lupulus* L.). *In Vitro* Cell. Dev. Biol.–Plant, 37:79-83.

Rybacek V. (1991). Hop production. Ed. Elsevier and Faculty of Agronomy, Agricultural University, Prague, Checz Republic.

Schulz V., Hansel R., Tyler V.E. (2001). Rational Phytotherapy. Springer-Verlag, Berlin Heidelberg, pp. 87–88.

Scowcroft W.R. (1984). Genetic variability in tissue culture: impact on germplasm conservation and utilization. IBPGR, Rome.

Seguí-Simarro, J.M., 2010. Androgenesis revisited. Bot. Rev. 76:377-404.

Sharma N., Chandel K.P.S. (1996). *In vitro* conservation of *Orchis latifolia* : a threatened medicinal terrestrial orchid. Indian J Plant Genet Resour 9:1–5.

Simpson W.J., Smith A.R. (1992). Factors affecting antibacterial activity of hop compounds and their derivatives. Journal of Applied Bacteriology 72:327–334.

Small E. (1978). A numerical and nomenclatural analysis of morpho-geographic taxa of Humulus. Systematic Botany 3: 37–76.

Smith R.D., Linington S.H., Wechsberg G.E. (2007). The millennium seed bank, the convention on biological diversity and the dry tropics. In: Prendergast HDV, Etkin NL, Harris DR, Houghton PJ (eds) Plants for food and medicine: proceedings of the joint conference of the society for economic

botany and the international society for ethnopharmacology. Royal Botanic Gardens, Kew, pp 251–261.

Smykal P. (2000). Pollen embryogenesis—the stress mediated switch from gametophytic to sporophytic development. Current status and future prospects. Biol Plant 43:481–489.

Solberg S.O., Brantestam A.K., Kylin M., Bjorn G.K., Thomsen J.M. (2014). Genetic variation in Danish and Norvegian germplasm collection of hops. Biochem Syst Ecol. 52: 53-59.

Songstad D.D., Somers D.A., Griesbach R.J. (1995). Advances in alternative DNA delivery techniques. Plant Cell Tissue Organ Culture, 40:1–15.

Stajner N., Satovic Z., Cerenak A., Javornik B. (2008). Genetic structure and differentiation in hop (*Humulus lupulus* L.) as inferred from microsatellites. Euphytica 161:301–311. http://dx.doi.org/10.1007/s10681-007-9429-z.

Suštar-Vozlič J., Javornik B., (1999). Genetic relationships in cultivars of hop, *Humulus lupulus* L., determined by RAPD analysis. Plant Breed. 118:175-181.

Svoboda P. (1992). Kultivace izolovaných vrcholů chmele (*Humulus lupulus* L.) in vitro. [Culture of isolated shoot tips of hops (Humulus lupulus L.) in vitro.] Rostl. Výr., 38:523-528.

Szarejko I. (2003). Anther culture for doubled haploid production in barley (*Hordeum vulgare* L.). In: Maluszynski M, Kasha K, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. A manual. Kluwer Academic Publishers, Norwell, pp 35–42.

Takeda S., Matsuoka M. (2008). Genetic approaches to crop improvement: responding to environmental and population changes. Nat Rev Genet 9:444–457.

Taji A.M., Kumar K., Lakshmanan P. (2002). *In vitro* plant breeding. Haworth Press, Inc., New York.

Tan M., Yan M., Wang L. Yan X., Fu C., Wang L. (2013). Replication of pistillate plants of Ricinus communis L. and investigation of the sex stability and genetic variation of the somaclones. Ind Crops Prod 50:50–57.

Thakur A.K., Chauhan D.K., Parmar N., Verma V. (2012). Role of genetic engineering in horticultural crop improvement–a review. Agr Rev 33(3):248–255.

Tukey H.B. (1933). Embryo abortion in early-ripening varieties of *Prunus avium*. Bot. Gaz.94: 433–468.

Tyler V.E. (1987). The New Honest Herbal. A Sensible Guide to Herbs and Related Remedies, 2nd ed. Stickley Co., Philadelphia, pp. 125–126.

van Wordragen M., Dons H. (1992). *Agrobacterium tumefaciens*-mediated transformation of recalcitrant crops. Plant Molecular Biology Report, 10:12–36.

Vaughan D., Balazs E., Heslop-Harrison J. (2007). From crop domestication to super-domestication. Ann Bot 100:893–901.

Vine S.J., Jones O.P. (1969). The culture of shoot tips of hop (*Humulus lupulus* L.) to eliminate viruses. J. Hort. Sci., 44:281-284.

Wang B., Wang R.R., Cui Z.H., Bi W.L, Li J.W, Li B.Q., Ozudogru E.A, Volk G.M, Wang Q.C. (2014) Potential applications of cryogenic technologies to plant genetic improvement and pathogen eradication. Biotech Adv 32:583–595.

Wang M., van Bergen S., Van Duijn B. (2000). Insights into a key developmental switch and its importance for efficient plant breeding. Plant Physiol 124:523–530.

Wilson D.G. (1975). Plant remains from the Graveney boat and the early history of *Humulus lupulus* L. in W. Europe. New Phytologist 75:627–648.

Withers L.A. (1990a). *In vitro* approaches to the conservation of plant genetic resources Pages 261-276 in Withers, L.A.; Alderson, P.G., eds. Plant tissue culture and its agricultural applications.

Withers L.A. (1990b). *In vitro* techniques for the conservation of crop germplasm. In Proc. National Conf. Plant and Animal Biotechnol. Nairobi, Kenya. IBPGR, Rome, Italy.

Withers L.A. (1991). Maintenance of plant tissue culture. In Kirsop, B.E.; Doyle, A., eds. Maintenance of microorganisms and cultured cells: a manual of laboratory methods. 2nd Ed. Academic Press, London.

Wyse Jackson P.S. (1999). Experimentation on a large scale–An analysis of the holdings and resources of botanic gardens. BGCNews. 3(3):27-30.

Zanoli P., Zavatti M. (2008). Pharmacognostic and pharmacological profile of *Humulus lupulus* L. Journal of Ethnopharmacology 116:383–396.

Zhang Z.B., Burgos N.R., Zhang J.P., Yu L.Q. (2007). Biological control agent for rice weeds from protoplast fusion between Curvularia lunata and Helminthosporium gramineum . Weed Sci 55(6):599–605.

Zoratti L., Jaakola L., Häggman H. (2015). Anthocyanin Profile in Berries of Wild and Cultivated Vaccinium spp. along Altitudinal Gradients in the Alps. J. Agric. Food Chem. 63:8641–8650.

Zupan J., Muth T.R., Draper O., Zambryski P. (2000). The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant Journal, 23:11–28.

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