UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

Cycle XXXV

S.S.D. AGR07 Genetica Agraria

Development of rocket hybrids (*Eruca sativa* and *Diplotaxis tenuifolia*) resistant to *Fusarium oxysporum* ff. spp. and *Phytophthora parasitica*

Coordinator:

Ch.mo Prof. Paolo Ajmone Marsan

Candidate: Gabriella Florinda De Angelis

Matriculation n: 4915189

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1.INTRODUCTION

1.1 SPECIES CHARACTERISTICS

1.1.1 Origin of the species and taxonomy

Some species belonging to the genus *Eruca* and *Diplotaxis* are indicated by the common name "rocket". These two *genera* belong to the Brassicaceae or Cruciferae family that contains agricultural and scientific important species (Padulosi et al., n.d.).

The group including cauliflower, broccoli, and sprouting broccoli is designated as inflorescence kales (Sharma et al., 2014). Oilseed rape and swede belong to the taxon *Brassica napus.* The leafy crucifer includes kale (*Brassica oleracea* var. *sabellica*), cultivated rocket (*Eruca vesicaria* subsp. *sativa*) and wild rocket (*Diplotaxis* spp.).

Leaves of this species are used as fresh-cut products or in industrial preparation. The name *Eruca* has probably Latin origin (*uro* or *urere*) and means "burn" for the typical pungent flavour of its leaves. The name Diplotaxis comes from the Greek diplos (double) and taxis (row) referring at the seeds, arranged in a double row inside the siliqua. The Mediterranean Basin and Occidental Asia are accredited as centres of origin and domestication of the species. (Pignone & Martínez-Laborde, 2011)

The garden rocket, *Eruca vesicaria* (L.) Cav., known as cultivated rocket, was already used and appreciated since Egyptians and Latins. The taxonomic limits of the genus *Eruca* have been moved with time, but today is known that the genus *Eruca* contains a single species, *Eruca vesicaria* (L.) Cav., which, in turn, includes three subspecies. All of these can be found in the wild type, but only *E. vesicaria* subsp. *sativa* (Miller) Thell. (syn. *Eruca sativa* Miller) has been domesticated and it occupies a wider geographical area in the world. The second type subspecies, *vesicaria* (Mill.), as well as the third subspecies, *pinnatifida* (Desf.), is only local in the West Mediterranean area.

The genus *Diplotaxis* comprises 32 (Padulosi et al., n.d.; Pignone & Martínez-Laborde, 2011) to 34 species plus several additional subspecies and local taxa, native to Europe, the Mediterranean region, Southwest Asia (up to the Himalayas), and Macaronesia, with the highest diversity in the Iberian Peninsula, Northwest Africa, and Cape Verde. Edible species are *Diplotaxis muralis* (L.) DC, a hybrid species between *Diplotaxis tenuifolia* and *Diplotaxis viminea*, *Diplotaxis erucoides* (L.) DC subsp*. erucoides* and *Diplotaxis tenuifolia* (L.) DC known as wild rocket and widely cultivated.

A few species have become naturalized elsewhere (North and South America, Australia, etc.). The genus displays a considerable degree of heterogeneity in morphology, molecular marker profiles, chromosome numbers (Fig. 1), and geographical amplitude (Pignone & Gómez-Campo, 2011; Pignone & Martínez-Laborde, 2011).

Figure 1 Species and subspecies of Diplotaxis tenuifolia with chromosomes number and geographical area

Species (and subspecies)	$2n$[c]	Geographical area[d]
D. tenuifolia (L.) DC.	22 (Harberd 1972)	Europe, Middle East*
D. cretacea Kotov	22 (Harberd 1972)	Ukraine
D. muralis (L.) DC.		
subsp. *muralis*	42 (Harberd 1972)	Europe*
subsp. *ceratophylla* (Batt.) Mart.-Laborde	–	N Algeria
D. scaposa DC.		Island of Lampedusa
D. simplex (Viv.) Spr.	22 (Harberd 1976)[e]	N Africa
D. viminea (L.) DC.	20 (Harberd 1972)	Europe, N Africa, Middle East*
D. harra (Forssk.) Boiss.		
subsp. *harra*	26 (Gómez-Campo 1980)	N Africa, Middle East
subsp *crassifolia* (Raf.) Maire	26 (Harberd 1972)	Sicily
subsp. *lagascana* (DC.) O. Bolòs & Vigo	26 (Harberd 1972)	SE Spain
D. kohlaanensis A. G. Miller & J. Nyberg	–	Yemen
D. villosa Boulos & Jall.	–	Jordan
D. pitardiana Maire	–	NW Africa
D. nepalensis Hara	–	Nepal
D. antoniensis Rustan	–	Cape Verde
D. glauca (J.A. Schmidt) O.E. Schulz	26 (Harberd 1972)	Cape Verde
D. gorgadensis Rustan		
subsp. *gorgadensis*	–	Cape Verde
subsp. *brochmannii* Rustan	26 (fide Rustan 1996)	Cape Verde
D. gracilis (Webb) O.E. Schulz	26 (Rustan 1996)	Cape Verde
D. hirta (A. Chev.) Rustan & Borgen	26 (Rustan 1996)	Cape Verde
D. sundingii Rustan	26 (Rustan 1996)	Cape Verde
D. varia Rustan	–	Cape Verde
D. vogelli (Webb) Cout.	–	Cape Verde
D. acris (Forssk.) Boiss.	22 (Amin 1972)	N Africa, Middle East (to Iraq)
D. griffithii (Hook.f. & W. Thomps.) Boiss.	–	Afghanistan, Pakistan
D. assurgens (Delile) Grenier	18 (Harberd 1972)	Morocco
D. berthautii Braun-Blanq. & Maire	18 (Takahata and Hinata 1978)	S Morocco
D. brachycarpa Godron	18 (Martínez-Laborde 1988a)	N Algeria
D. catholica (L.) DC.	18 (Harberd 1972)	Iberian Peninsula, N Morocco
D. ollivieri Maire	–	S Morocco
D. siifolia Kunze		
subsp. *siifolia*	20 (Harberd 1972)	Iberian Peninsula, NW Africa
subsp. *bipinnatifida* (Coss.) Mart.-Laborde	–	S Morocco
subsp. *vicentina* (Sampaio) Mart.-Laborde	20 (Fernandes and Queirós 1970–1971)	SW Portugal
D. tenuisiliqua Delile		
subsp. *tenuisiliqua*	18 (Harberd 1972)	N Morocco, NW Algeria
subsp. *rupestris* (J. Ball) Mart.-Laborde	–	S Morocco
D. virgata (Cav.) DC.	18 (Harberd 1972)	Iberian Peninsula, Morocco
D. ibicensis (Pau) Gómez-Campo	16 (Gómez-Campo 1980)	E Spain coast, Balearic Islands
D. brevisiliqua (Coss.) Mart.-Laborde	16 (Martínez-Laborde 1991b)	NE Morocco, NW Algeria
D. ilorcitana (Sennen) Aedo, Mart.-Laborde & Muñoz Garm.	16 (Martínez-Laborde 1991b)[f]	E Spain
D. siettiana Maire	16 (Takahata and Hinata 1978)	Island of Alboran
D. erucoides (L.) DC.		
subsp. *erucoides*	14 (Harberd 1972)	Europe, N Africa, Middle East*
subsp. *longisiliqua* (Coss.) Gómez-Campo	14 (Gómez-Campo 1980)[g]	N Algeria

1.1.2 Botanical aspects of rocket species

Rocket species (*Eruca sativa, Diplotaxis tenuifolia*) have neutral photoperiod and flower induction is stimulated by high temperatures, while in winter the vegetative part of the plant development is favorited. Floral structure (with bright color petals and large pollen grains) is indicative of the fact that these two species are mostly cross-pollinated. There is no scientific evidence regarding the presence of self-pollination for *Eruca sativa*, instead different types of sexual reproduction are found in the genus *Diplotaxis*. Some degree of self-pollination has been found in *D. erucoides subsp. erucoides*. *D. viminea* is a clearly autogamous species, as indicated by its much smaller flowers, apparently sterile lateral anthers, and high degree of ovule fertilization and seed formation, and *D. muralis* is predominantly self-pollinated. (Hall et al., 2012a) (Sharma et al., 2014)

Flower has a pluriovular ovary, as typical of other Brassicaceae species. The fruit of *Diplotaxis* spp. consist of siliques that form and mature in 30-40 days, depending on the climate conditions, until the siliques open (rocket is a dehiscent species) and disperse the seed which is very small and abundant; however, this characteristic of *Diplotaxis tenuifolia*, still make it a weed species rather than a cultivated species. *Eruca sativa* has larger flowers with pluriovular ovary and forms siliques less dehiscent than *Diplotaxis tenuifolia*; this last trait was selected over the years by farmers and breeders to avoid loss of seeds during harvest. Differences between the two most common rocket species are summarized in Tab.1.

Table 1 Botanical differences between Eruca sativa and Diplotaxis tenuifolia

TRAIT	***Diplotaxis tenuifolia***	***Eruca sativa***
Cycle	Perennial	Annual cycle
Leaf aspect	small, dark green, jagged, elongated, long and thin petiole	big, bright green, rounded, small petiole
Leaf dimensions	Length 10 cm, width 2-3 cm	Length 20 cm, width 6 cm
Height	30-40 cm	50-90 cm
Flower	small, yellow	big, white or light yellow, diameter of 3 cm
Impollination	Entomophilic	Entomophilic
Fruit	Siliqua (25-50 mm)	Siliqua (15-50 mm)
1000 seeds	0,2-0,3 grams	1,7- 2 grams
Chromosome number	2n= 22	2n=22

1.1.3 Genome and chromosome number

The whole genus *Diplotaxis* is diploid, but chromosome number isunknown for most taxa. Polyploidy is almost absent within *Diplotaxis genus*, but there is a remarkable degree of disploidy. The series of gametic number ranges from n= 7 in *D. erucoides*, through n= 8, 9, 10 and 11, to n = 13 in the *D. harra* aggregate, whereas *D. muralis* has n=21, the only species in the genus allotetraploid. According to Harberd and McArthur (1972), *D. muralis* would be an amphidiploid probably arisen from *D. tenuifolia* (n= 11) and *D. viminea* (n = 10).

1.1.4 Agronomical aspects

While *Eruca sativa* and other species of *Diplotaxis* genus are annuals, *Diplotaxis tenuifolia* has a perennial growth habit, which makes it more suitable as a crop: its leaves can regrow after each harvest and multiple harvests are possible. For this reason, over the years many farmers have preferred the wild species to the cultivated one.

Eruca sativa is generally cultivated in open field or covered plastic tunnels and sold as a bunch or destined for the processing market. Some *Diplotaxis* species (*Diplotaxis erucoides*, *Diplotaxis muralis*) are collected as spontaneous plants aimed at local traditional consumption.

Diplotaxis tenuifolia is cultivated in open fields or more commonly in greenhouses covered with thermal polyethylene films (cultivation methods showed in Fig.2). The protected environment makes possible to grow wild rocket both in winter and in summer and ensure the production all the year round. Shading through net or roof whitening may be needed under excessive light intensity during cultivation in summer. Perennial wall-rocket can also be grown as a soilless crop, using floating system or nutrient film technique (Caruso et al., 2018a)

Figure 2 Rocket cultivated in covered greenhouses and open field

Rocket cultivation for baby-leaf production, direct sowing is the most common technique, instead of transplanting which is preferred for rocket cultivation in bunches. The density is around 1800 plants/m^2 (that requires approximately 5kg/ha of seeds) as explained in Tab.2. Crop density, climate conditions and irrigation management are the most important parameters for the success of rocket crop cultivation. (Caruso et al., 2018a)

Table 2 Sowing parameters for baby leaf cultivation of Diplotaxis tenuifolia

Species	**Plants/m^2**	**Seeds grams/ m^2**	**Sowing depth**	**Germinability Minimum %**	**Germinability optimal °C**
D.tenuifolia (summer)	2500-2800	0,7-0,8	3-5 mm	80-85	27
D.tenuifolia (winter)	1800-2200	0,5-0,6	3-5 mm	80-85	27

Harvest takes place when the first 3-6 true leaves are completely grown, but the main parameter is the leaf length for the market requests (total height of the plant approximately 12-15 cm). In baby leaf wild rocket, secondary cuts are appreciated more for the greater incision of the leaves compared to the first cuts. Harvest is mechanized and involves cutting the leaves at a height of 5 cm from the ground, so as not to cut off the shoot necessary for the formation of the leaf rosette for subsequent harvesting and to obtain a leaf product of 7-11 cm. There are differences in number of cuts and cycle length based on the location of the cultivation, as described in Tab.3.

Table 3 Cycle length and yield parameters in South Italy and North Italy cultivation of Diplotaxis tenuifolia

Species	**Cycle (gg) spring**	**Cycle (gg) summer**	**Cycle (gg) autumn**	**Cycle (gg) winter**	**Number of cuts**	**Yield first harvest (kg/m^2)**	**Yield other harvest (kg/m^2)**
Wild rocket North of Italy	60-40	30-20	25-40	50-80	2-4	0,8-1,2	0,5-1
Wild rocket South of Italy	30-25	20-15	25-35	35-50	3-5	0,8-1	0,5-0,8

Temperature affects flowering in rocket. This phenomenon can be observed during spring and summer seasons, where temperature shift between day and night can be very low. Tolerance to drought stress is remarkable, in fact the root system is provided with a very developed taproot that grows very deep and ensure resistance to water stress.

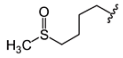
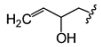
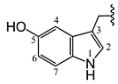
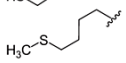
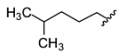
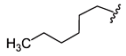
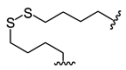
Diplotaxis tenuifolia has a gradual and prolonged flowering. This makes difficult to collect the seeds, which must be carried out when the first flowers (and siliques) at the bottom begin the dehiscence and lose their seeds, when the central part of the plant is almost completely dry. A compromise must be found to reconcile the quality and quantity of production.

Rocket is a species identified as strongly allogamous and self-incompatible, therefore seed production is favored using pollinating insects (pollinators increase productivity by 100%, different insect species can be used like bees, flies and bumblebees). The percentage of anemophilous pollination is negligible, due to the characteristics of the pollen, large and heavy, which disfavour the movement due to the wind.

1.1.5 Glucosinolates and healthy compounds

The genus *Diplotaxis* and *Eruca*, as other Brassicaceae, are major producers of glucosinolates, sulfur-rich thioglucosides present in the vegetative parts of this botanical family. The main glucosinolates that have been found in rocket are described in Fig.3. Isothiocyanates are the volatile products of hydrolysis of glucosinolates, and they are responsible for the characteristic aroma of many crucifers (Hall et al., 2012a) (Pignone & Martínez-Laborde, 2011)

Figure 3 Main glucosinolates found in rocket and their chemical structure (from the web)

Structure of R[a]	R – name	Common name	Elemental composition	Monoisotopic mass (Da)	$[M-H]^-$ (*m*/*z*)	Occurrence[b]
	4-methylsulfinylbutyl	Glucoraphanin	$C_{12}H_{23}NO_{10}S_3$	437.05	436	LEs/SEs
	(*R*,*S*)-2-hydroxy-3-butenyl	Progoitrin/epiprogoitrin	$C_{11}H_{19}NO_{10}S_2$	389.04	388	LEs/CRM
	2-propenyl	Sinigrin	$C_{10}H_{17}NO_9S_2$	359.03	358	LEs
	4-hydroxy-3-indolyl methyl	4-Hydroxyglucobrassicin	$C_{16}H_{20}N_2O_{10}S_2$	464.06	463	LEs/CRM
	5-hydroxy-3-indolylmethyl	5-Hydroxyglucobrassicin	$C_{16}H_{20}N_2O_{10}S_2$	464.06	463	LEs
	4-mercaptobutyl (MB)	MB-GLS	$C_{11}H_{21}NO_9S_3$	407.04	406	LEs
	4-methylthiobutyl (MTB)	Glucoerucin	$C_{12}H_{23}NO_9S_3$	421.05	420	LEs/SEs
	4-methoxy-3-indolylmethyl	4-Methoxyglucobrassicin	$C_{17}H_{22}N_2O_{10}S_2$	478.49	477	LEs/SEs
	4-methylpentyl (logP = 2.9)	Methylpentyl-GLS[c]	$C_{13}H_{25}NO_9S_2$	403.46	402	LEs
	*n*-hexyl (logP = 3)	*n*-Hexyl-GLS[c]	$C_{13}H_{25}NO_9S_2$	403.46	402	LEs
	Dimeric 4-mercaptobutyl	DMB-GLS	$C_{22}H_{40}N_2O_{18}S_6$	812.06	811	LEs
	4-(β-D-glucopyranosyldisulfanyl)butyl	4-GDB-GLS[d]	$C_{17}H_{31}NO_{14}S_4$	601.06	600	LEs

Approximately 120 individual glucosinolates have been isolated chiefly from species of Brassicaceae and related families. In rocket plant, glucosinolates are stored in the vacuoles but, following cell damage and exposure to myrosinase, they are hydrolyzed into isothiocyanates and other products such as sulforaphane. Glucosinolate content is responsible for the flavor and odor in *Brassica* vegetables. In recent years, various glucosinolates have been isolated and identified from the seeds and leaves of rocket salad. Six desulfo-glucosinolates were isolated from rocket salad (Bell & Wagstaff, 2014; Bennett et al., 2007)

Erucin is the main dietary isothiocyanate present in rocket salads (*E. sativa* and *Diplotaxis* sp.). Erucin is potentially capable of protecting cells against oxidative stress via three mechanisms: (i) induction of phase II enzymes (those enzymes that catalyze the conjugation reactions), (ii) scavenging hydrogen peroxide and alkyl hydroperoxides accumulated in cells and peripheral blood, and (iii) acting as a precursor of sulforaphane, a potent inducer of UDP-glucuronosyltransferase, which detoxify electrophiles and increase cellular antioxidant defenses (Bell et al., 2015)

Recently, it has been discovered that Erucin is a promising chemopreventive phytochemical (Bennett et al., 2007).It has been scrutinized that cruciferous vegetables such as cabbage, broccoli, cauliflower, radish, and turnip are rich source of glucosinolates and are associated with lowering the risk of various types of cancers such as lung, stomach, rectum, ovarian and endometrial (Bell et al., 2015).

Other chemical compounds named flavonols and flavonoids are present in rocket. 5000 different flavanoids, flavones, flavonols, flavanols, flavanones, anthocyanins, catechins and isoflavones are highly acceptable and common in human diet. Flavanoids have various properties such as anti-inflammatory, oestrogenic, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumour activity along with antioxidant and free radical scavenging activity It has been observed that flavanoids can act as anticancer agents, where it regulates the cell division in many signal transduction pathways. Moreover, they also plays a role as secondary metabolites and involved in various mechanisms like stimulation of nitrogen-fixing nodules, protection by UV-rays, pigmentation, and disease resistance (Taranto et al., 2016)

All the listed compounds belong to the broad category of allelochemicals: these are compounds that influences growth, health and behaviour of other organisms The interest in allelochemicals is in the relation to their potential use as alternative pest management agents that could possibly reduce the use of synthetic pesticides in agriculture and horticulture (Taranto et al., 2016).

1.2 ROCKET CROP IN THE MARKET

1.2.1 Rocket product destinations

Diplotaxis tenuifolia was converted from a wild species to an interesting crop about twenty-five years ago, thus increasing more and more in terms of farm diffusion. Notably, it has been drawing attention from vegetable operators due to its climatic resilience, successful yield performances, and profitability. The success of this crop on consumers is connected to its typical pungent taste and the excellent nutraceutical properties, depending on the presence of valuable bioactive compounds, such as glucosinolates and the high content of antioxidants. The mentioned features have enhanced spreading of perennial wall-rocket in crop systems even outside the native Mediterranean area, introducing this crop as an interesting option for vegetable growers worldwide.

The use of wild rocket (also known as "perennial wall-rocket") as a crop has been known in Italy and in Europe since 19th century. Rocket has been used for fresh market consumption, as a fresh-cut product (IV range salad) and in industrial use for sauce preparations.

In fresh market, older leaves are not appreciated because of the the high content of glucosinolates which makes them too bitter for consumers (Caruso et al., 2018a). As in other Brassicaceae, breeding may allow for regulating the glucosinolates content to obtain a product which fits best with some consumer taste. However, up today, the number of commercial cultivars is limited if compared to the increasing market demand. Therefore, some efforts have already been made collecting new germplasm throughout the Mediterranean area, in the awareness that breeding might provide very profitable results (Caruso et al., 2018b).

Rocket is the second product consumed among the ready-to-use products (10%), after fresh salad (including lettuce group and endives) that consist in 70% of the leafy product. (Gilardi et al., n.d.; Morais et al., 2020) Ready-to-use are defined as those products prepared and conditioned to provide the consumer with a series of services including cleaning, washing, cutting into units or sub-units ready for use, preserving the characteristics of freshness and authenticity of the product over time.

Other potential uses of perennial wall-rocket concern seeds, from which an oil can be extracted at 36% proportion, containing 38.9% linolenic acid, 22.3% linoleic acid, 12.6% oleic acid, and 11.8% erucic acid.(Egea-Gilabert et al., 2009)

Although lower than the percentages reported in other cruciferous crops, the high concentration of erucic acid in rocket seed oil discourages its use for human consumption. In addition to the previously discussed beneficial effect to human health related to the content in sulphoraphane, glucosinolates and flavonoids, the use of *D. tenuifolia* as a medicinal plant is widely documented in the tradition of several people and cultures for its antioxidant properties.

The success of the most widespread species, which are *Diplotaxis tenuifolia* in Italy and *Eruca sativa* in most of the European states, is due to its adaptability and to the compounds of the species that gives to rocket the typical and spicy flavor, appreciated by consumers.

1.2.2 Rocket's main market

In the context of global markets, higher demand of the product comes from Germany and the United Kingdom, the most demanding countries in terms of quality and controls. Other areas in which interest in this species is developing are Middle East, South Africa, Americas and, indirectly, the countries of Eastern Europe, where national producers export through the fresh market. Another new market to which rocket is beginning to be made known is Japan, attracted by "made in Italy" tastes (https://www.freshplaza.it/).

Nowadays, *Eruca sativa* only partially interested the Italian market. Its market widespread in Central Europe, Northern India, Continental America, South Africa and Australia , where varieties suitable for cultivation have been developed. (Padulosi et al., n.d.) Annual garden rocket is also cultivated in Pakistan and India and used as an industrial oil crop.

The market value of the product for fresh-cut products destination is estimated at around 30-40 million euros for export, to which the value of the fourth internal range must be added. In Europe, wild rocket (*Diplotaxis tenuifolia*) is cultivated mainly in Italy and in Iberian Peninsula, where *Eruca sativa* is also common in open field cultivation. Both autumn and summer cycles are possible for rocket, segments for cultivation are summarized in Tab.4.

Table 4 Segments for rocket cultivation in Europe

Country	**Cultivation**	**Cycle**
Spain	open field, greenhouse	autumn-spring
Portugal	open field, greenhouse	autumn-spring
North Italy	greenhouse, plastic tunnels	spring-summer
South Italy	greenhouse, plastic tunnels	autumn-spring-summer

1.2.3 Rocket market in Italy

Interest in wild rocket in Italy has grown recently because of the progressive spread of ready-to-use salads. Up until two decades ago, *Eruca sativa* was the main rocket species in cultivation, while *Diplotaxis tenuifolia* was harvested and used as a wild herb. Wild rocket then spread thanks to its stronger flavor and more succulent leaves, characteristics appreciated by the consumer, to cover an area of 4000 hectares in Italy (Caruso et al., 2018b).

In Italy there are two main cultivation areas for this crop, highly specialized: the first one in the South of Italy (Campania region), specifically within an area called “Piana del Sele” (province of Salerno), and the second in Bergamo area in the North of Italy, where cultivation under controlled conditions increased during the last 10 years. There are other smaller cultivation areas that produce rocket for ready-to-use destination, Veneto at first and, lately,new cultivations started in Tuscany, Puglia and Lazio. In Tab.5 the major cultivation areas in Italy are shown, with surfaces (where known) and production.

Table 5- Rocket cultivation areas in Italy

Area	2005	2020	Ton/year	% of Italy production	Cultivation
Campania	1800	2500-3000	400000	73	autumn-winter cycle, spring-summer cycle, wild rocket
North Italy (Lombardia, Veneto)	150-200	300-400	No data available	15-20%	Spring-summer cycle
Other regions	50	>100	No data available	No data available	open field for first range product, some experimental cultivation for IV range

Piana del Sele is a fertile alluvial plain in the Salerno province (Campania). The average temperature of 16.8°C and the Mediterranean climate makes this area an important agricultural basin of South Italy. Its productions, especially "baby leaf" supply the whole ready-to-eat salad market from October to April. In this area, rocket cultivation is highly specialized. The presence of technicians and large companies focused on the cultivation of leafy vegetable led to the settlement of traditional cultivation and the request to the European Union of the IGP recognition. Among all the "baby leaves", wild rocket tolerates less the cold season. For that reason, its production has become strategic at warm latitudes like South of Italy in the winter period, when it is not possible to cultivate it in Central and Northern Europe, and also the production areas of Northern Italy meet difficulties in development due to the cold and lack of light. (Clarkson et al., 2005)

In 2021-2022 season, the price for rocket was 2,60 €/kg (sowing October-November) and 1,80 €/kg during the peak of production in Northern Italy during all the summer season.

Starting from February, the market has become very competitive thanks to the presence of other producers (especially Bergamo area in the North of Italy) and the average price drops to 0,70-1,00 €/kg.

For what concerns product yield, first cut harvests produce 800-1000 gr/mq, then the production can decrease to 500-600 gr/mq in the coldest months (January-February). The most specialized companies in winter cycle carry out up to 10-12 cuts with a single sowing, done in October, with an actual average of 7-8 cuts. During summer, given much phytopathology problems and the early bolting of plants, no more than 4 cuts are possible. So, farmers proceed with repeated sowings as the crop cycle shortens considerably.

For all these features, Piana del Sele is the reference market for the request for new traits for breeding.

1.3 BREEDING FOR ROCKET CROP IMPROVEMENT

1.3.1 Breeding methods used for Allogamous plant

Rocket (*D. tenuifolia*) selection methods follow the selection guidelines of allogamous plants. Almost all rocket cultivations are obtained using open pollinated (OP) populations, but first hybrids have been sold by seed companies during last years.

Until the first half of the twentieth century, varieties obtained from mass selection or sib selection represented the basis on which the cultivation of most of the allogamous species has been based.

These varieties still find a wide diffusion for those crops or areas in which high performing varieties, such as F1 hybrids, cannot be proposed for economic or practical reasons, because often they are more expensive and demanding in terms of cultivation practice. Moreover, in leafy vegetables, the advantage provided by vigor of the hybrid is not evident as in other horticultural species.

Intra-population methods are the most common selection methods used for breeding of allogamous plants:

1) Mass selection provides for the choice of plants on a phenotypic basis, both for qualitative and quantitative characteristics before flowering or after flowering, by elimination of off-types. This methodology is more effective for highly heritable traits;
2) Half sib family selections are obtained by the hybridization of single plants derived by a parent plant in common;
3) Full sib family selection in the starting phase of the breeding program, controlled crosses are carried out between individual plants to form the full sib families;
4) Self-pollinated family selection: self-generations are generally two, S1 and S2.

For allogamous plants, it is not always possible to create a pure line using self-pollination. Often problems of inbreeding depression occur, so it is necessary to study different methods to obtain modern varieties in species that are strictly allogamous.

Artificial selection has been integrated often with interspecific and inter-varietal hybridization and with natural selection in the development of varieties. Both approaches are classified as selection methods with the application of intercrossing, historically used to allogamous plants in which

hybridization between different genotypes is favoured by the biology of the species. The hybridization can occur with human intervention, limited to facilitate dissemination and distribution of pollens on genotypes, using insects as pollen vectors or using appropriate schemes to facilitate the crossing plans.

To reproduce single plants, to create new variability and sib-reproduction, different methods are available:

1) Isolation: for male sterile plants and monoic self-incompatible plants isolation is needed to produce seeds from one or both parents;
2) Pollination (pollinating insects) in a cage with a net that prevents the introduction of undesirable pollinators;
3) Manual cross: emasculation of mother plants, pollen harvesting and manual pollination. Pollination should be achieved early in the morning when the flower begins to open. Pollen viability is variable and depends on the species and plant genetic backgrounds.

In rocket, flowers on the racemes open in a scalar manner. A single flower remains open for a few hours, it generally opens in the early hours of the morning and generally the female part becomes receptive first, then the male part with the production of pollen. in the hottest hours the flower generally reaches its maximum opening. the flower closes after about 24 hours and, if pollinated, forms a siliqua in 3-4 weeks.

1.3.2 Main challenges in rocket crop breeding

The most important aspects concerning this crop are linked to the pathologies that destroy or affect leaves before harvesting and the knowledge of its compounds for a future qualitative improvement. Therefore, it is necessary to define the right selection tools into the breeding program, including phytopathological assays, and evaluate qualitative and quantitative differences between selected varieties.

Even if *Diplotaxis tenuifolia* (2n=22) breeding is very recent, the interest of research groups has increased over the past 15 years, providing important information as a starting point. There are still few molecular detailed information and there are still elements to be clarified regarding the categorization of specialized pathogens and the registration of new varieties. (Tripodi et al., 2017a)

On the other hand, *Eruca sativa* (2n=22) has been domesticated before than *Diplotaxis tenuifolia*, the genetic improvement lasted for several decades and now only one domesticated species is identified in this genus. (Pignone & Gómez-Campo, 2011)

In the last 20 years, given the increased interest in rocket species for its usage in salad market, many seed companies have included perennial wall rocket (*Diplotaxis tenuifolia*) and annual garden rocket (*Eruca sativa*) on their breeding programs.

Leaves of both species are usually harvested at the commercial size of 10 to 15 cm in length and used as fresh product. Consequently recent breeding programs focused mainly on uniformity of growth, growth speed, healthiness of the leaf, stress and drought resistance and yield)(Hall et al., 2012b)

The cultivation of perennial wild rocket is concentrated in the Italian and Iberian Peninsula markets; therefore the efforts of breeding programs are focused on the requirements of these markets. Given the climate change and the pressing demand for this product, farmers are looking for varieties that allow them to complete production without running into phytopathological and quality problems.

The use of intensive cropping systems in South Italy, North Italy and Spain, the evolution of rocket's pathologies was favored. Up today, rocket plants results susceptible to several diseases, including rotting damping-off, *Fusarium* wilt and bacterial leaf spot, which constitute a limiting factor for both the quantity and the quality of yields. In addition, *Rhizoctonia* rotting and *Sclerotinia*, the two most destructive broad-spectrum soil-borne diseases affecting vegetables (Pane et al., 2017) are particularly aggressive and epidemics in the presence of high relative humidity and mild temperature (Gilardi et al., n.d.)

The most widespread and destroying pathogen in Italian protected crops is *Fusarium oxysporum* ff.spp., which can cause significant production losses especially in summer season, favored by high temperatures and humidity.

In the Iberian Peninsula and Italy, infection of *Hyaloperonospora parasitica* is reported in open field and its infection compromise the quality of the final product. In Italy, infection of *Hyaloperonospora parasitica* is mainly reported during autumn-winter cycle, when high thermal excursions and high humidity in early morning occur.

Pane et al., 2017 analyzed a germplasm collection made up of different *Eruca* and *Diplotaxis* accessions from different areas to evaluate resistance to other pathogens with phytopatological assays: *Rhizoctonia solani* and *Sclerotinia minor* (responsible for the damping-off), *Fusarium oxysporum* ff. spp. *raphani* and *conglutinans*, (responsible of the wilting of plants) and *Xanthomonas campestris* pv. campestris race 1 (that causes leaf spot disease). All these pathologies affect plants in field, with severe symptoms on leaves, making the final product unacceptable to the market.

1.3.3 Breeding for quality and post-harvest

Leaves quality and post-harvest conditions are two other aspects that interest breeders and research groups who want to improve the varieties of rocket and leafy crops.

Tripodi et al., 2017 published a work including different species of *Diplotaxis*, classified according to the number of chromosomes, reporting the main leaves metabolites and their chemical and nutritional functions, highlighting how the crop may be interesting from a nutritional point of view and how different compounds could play a role in the taste and shelf life.

Another important research on the composition of rocket were published by Taranto et al., 2016. A germplasm collection of 40 accessions of *Diplotaxis tenuifolia* (16 accessions), *Diplotaxis muralis* (2 accessions), *Eruca sativa* (21 accessions) and *Erucastrum* spp. (1 accession) obtained from different European germplasm banks and seed companies was analyzed, outlining the molecular profile of the single accessions, the content in glucosinolates (GLS), flavonols, phenols and other compounds, grouping the species in a dendrogram that shows genetic diversity. There are many factors that play an important role in the final quality of a fresh-cut product: variety genetic background, environment, cultivation techniques adopted, stage of maturation at harvest, conditions of the phase between harvesting and processing, process method, transport system.

In leafy species, the phenological stage at the time of harvest influences the content of dry matter and the content of antioxidants. The first parameter is related directly to the mechanical resistance to processing and to the visual quality of the final product. Antioxidant compounds, such as the reduced form of vitamin C, are useful in counteracting damage from ROS (Reactive Oxygen Species).

To summarize, important characteristics for a variety for fresh-cut market are (Collelli and Elia, 2009) :

- uniformity of size and maturation, variety adaptable to mechanical harvesting
- organoleptic and sensory characteristics improved (shape, color, flavor)
- good content of dry matter (greater consistency, mechanical resistance to processing)
- low sensitivity to low temperatures, to perform multiple sowing during the year
- high genetic resistance to diseases typical of this type of intensive cultivation
- low level of activity of enzymes that contribute to degradation processes (browning, oxidation)
- low breathing activity (maintenance of green color)
- slower ripening after harvesting

Currently, seed companies are focusing on the selection of varieties mainly for fresh-cut market (e.g. baby leaves). For the planting, on spinach, lettuce and other baby leaf species it may be useful to increase the planting density up to 1000 plants *per* square meter to ensure that the plant grows straight, already prepared for mechanical harvesting, allowing less stagnation of morning moisture on leaves (Elia 2006).

CHAPTER 2- BREEDING

2.1 ISI SEMENTI BREEDING PROJECT

ISI Sementi is an Italian seed company that has been producing seeds of hybrid and open-pollinated horticultural varieties since 1981. Initially family-run, then becoming part of the Japanese group Mitsui & co. in 2022, ISI Sementi is the first Italian seed company with its own molecular quality laboratory and with a research on breeding based in Italy. Since its foundation, it has been dealing with horticultural species and in particular onion, processing and indeterminate tomato. In its forty years of history, several species have been added in the company's sales network, including squash, lettuce and minor crops such as rocket, which was the subject of this phD study.

2.1.1 Starting point of the project

The breeding program of ISI Sementi is mainly focused on Italian and Iberic peninsula markets, where the most widespread species of rocket is *Diplotaxis tenuifolia*, also known as "perennial rocket" or "wild rocket". In these markets, also *Eruca sativa* species is cultivated even if on inferior extensions. ISI Sementi commercial range included 2 wild rocket varieties when the PhD project started:

- ATLANTA (Fig.4 on the left), with a medium-late cycle (positioning from March to September), characterized by dark colored leaves, very thick and smooth consistence. Leaf shape is elongated with marked and evident jaggedness from the first cut. The leaf has an erect posture and a green petiole. Good tolerance to pre-flowering and yellowing.
- DALLAS (Fig.4 on the right), with a medium-early cycle (positioning from September to February) characterized by a consistent, healthy and very rustic leaf; the variety has an intense green color and a very elegant indentation. Excellent growth capacity in the presence of low temperatures and low light; good yellowing tolerance.

Figure 4 Atlanta and Dallas, from ISI Sementi catalogue

These varieties present uniformity and good qualities of leaves but low productivity. In addition, they were not bred considering selection for resistances to pathogens and quality of the final product. For these reasons, they are in the final stages of production and sale.

A primary objective of the project was to carry out a breeding program targeted to the crop weaknesses described in the previous paragraphs.

Given the economic convenience for species such as rocket, which seeds are not very profitable, the most frequently used breeding methods are bulk or full-sib selection. In rocket, a strongly allogamous species, it is not possible to create a pure line as in other species because it does not tend to pollinate itself and create a real F1. The intention of ISI Sementi breeding to create a hybrid of rocket must follow other paths than the traditional ones, for example forcing the self-pollination of the plants or using methods for introducing male sterility. On the seed company point of view, hybrid commercialization is advantageous to avoid illegal seed reproduction, so have a greater economic return on the market. For this reason, various paths have been followed.

2.2.2 New breeding project goals

Visiting the Italian cultivations and processing areas of rocket (*Diplotaxis tenuifolia*) during the first months of the PhD project, allowed to clarify the agronomical requirements and the market target to deal with. Wild rocket is cultivated all year long in greenhouses with very short cycles, especially in the summer. The supply chain demand for the product reaches its peaks in winter, when the product is less present on farms. This fact leads farmers to shorten production cycles and often make them continuous.

The continuous cycles without rest of the soil, leads to problems related to fungal and bacterial diseases, due to high humidity under greenhouses and high transpiration of plants. The breeding strategy defined the resistance to phytopathological disease as priority, in particular the resistance to the root pathogen *Fusarium oxysporum* ff.spp. The pathogen is endemic and is present in tunnel and open field crops, especially where monocultures are carried out with few rotations, which allow the fungus to adapt and become more aggressive, sometimes changing and overcoming the genetic and physical defences of the plant. At the same time, bibliographic research and collaborations were carried on to obtain information on downy mildew disease in field. Strains collected in the field were used to define a protocol of infection useful for breeding

purposes. The final goal was to select plants genetically resistant to *Fusarium oxysporum* ff.spp. and *Hyaloperonospora parasitica*, combining the two resistances in the same genotype.

Furthermore, the production of nitrates into the leaves occur and reaches its peak during the winter cycle, accumulating toxic quantities beyond thresholds defined by laws and making the product not acceptable by the large distribution. Accumulation is conditioned by light, nitrogen fertilization, irrigation and other agronomic practices and is particularly a problem in baby leaf leaves because they are eaten fresh (ready-to-use products). (Santamaria et al., 2001)

Breeding efforts were also directed on nutraceutical compound viability and sustainability, fundamental aspects for next generation farming and the supply chain.

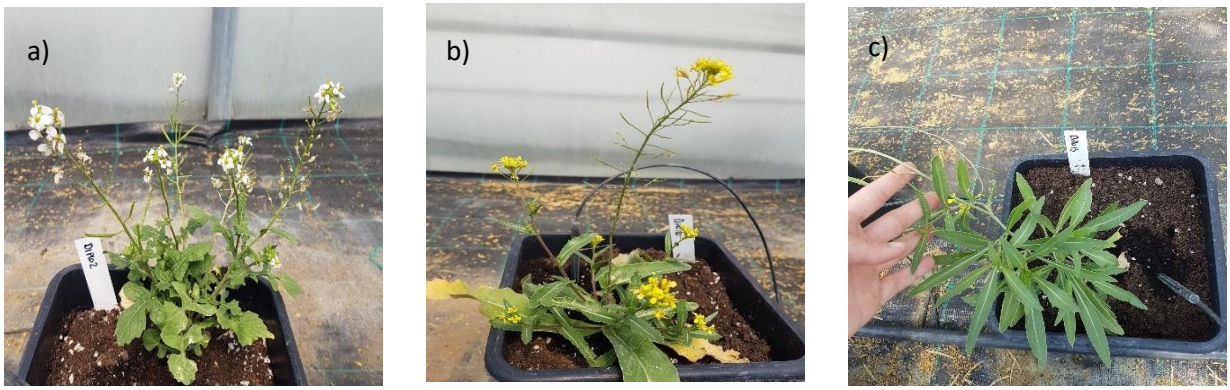
The traits deepened and improved related with consumer trends were: shelf life, growth rate, uniformity of germination, bolting, Brix degree, acidity, conductivity, quantity of chlorophyll, molecular profiling of the different molecules characterizing the species (Vitamins, Glucosinolates, Flavonols) .

The creation of two-way F1 hybrids for their economic advantage and their improved stability characteristics has been an aspect studied during the PhD project. The introgression of male-sterility in *Diplotaxis tenuifolia* has been proven, through interspecific crosses with *Brassica oleracea* male-sterile variety, as it will be discussed in the next chapters. The creation of hybrids would allow to easily introgress resistance and traits of interest.

The leader marketed varieties were phenotyped, and new product profiles were defined. The first evaluation of competitor varieties and the accessions given by seed banks was made between 2019 and 2020. Seed bank accessions were reproduced to obtain a greater seed stock for further phenotypical evaluations in commercial agronomic contexts. To increase the variability of the germplasm, several cross pollinations were carried out (cycle in spring and in late summer) between populations of different origin.

Variability and new phenotypes were obtained also from genebanks accessions: 18 accessions of different *Diplotaxis* species *(tenuisiliqua (Fig.5b), muralis (Fig.5a), tenuifolia, erucoides (Fig.5c)*) collected in different areas of the world were requested from IPK genebank (Leibniz Institute of Plant Genetic and Crop Research). Moreover, seed of *Eruca sativa* species were collected in different Italian regions and classified for agronomical traits.

Figure 5 a) Diplotaxis erucoides, b) Diplotaxis tenuisiliqua and c) Diplotaxis muralis cultivated in pot in greenhouse in ISI and used for breeding.



Accessions were transplanted in the field using cages protected with anti-insect nets and multiplied using pollinating flies, used for two to three weeks during full bloom.

Furthermore, lines present in the germplasm company from previous breeding activities were phenotyped and selected for single seed descent (SSD) by phenotypic selection up to generation F4 and F5. Populations were stabilized eliminating off-types and selecting for uniformity and common characteristics of earliness, flowering and development. In Tab.6 a summary of the starting material of the project or breeding, including material acquired externally and already in possession of ISI.

Table 6 Starting rocket germplasm for the project

PLANT MATERIAL	NUMBER OF POPULATIONS
Old ISI Sementi varieties	2
Benchmark competitor varieties	30
Wild accessions collected from seed bank	18
Local accessions of *Eruca sativa* and *Diplotaxis tenuifolia*	50

After selecting all plant material with classic breeding techniques, further analyzes were performed on the most interesting and advanced breeding lines to measure part of their biochemical composition and tolerance to abiotic stress. This latter was focused on salt stress. The aim for biochemical studies was to analyze the varieties in advanced breeding stage in different

areas of cultivation for secondary metabolites, presence of nitrates and water content. For salt stress assay, the aim was to evaluate the germination and root development of 5 varieties of *Diplotaxis tenuifolia*, identify the most resilient to salt stress conditions and, in the end, to perform a correlation test to study the relationship between traits studied.

2.1.3 Objectives of rocket breeding project:

For the reasons discussed in the previous paragraphs, the objectives of the breeding program can be summarized as follows:

- Observation and selection of old varieties, local populations, all the germplasm present in ISI Sementi seed stock, acquisition of germplasm from seed bank;
- Collection of important characters for selection (application of official DUS test, agronomical traits defined with the technical operator in ISI Sementi, other appreciable characteristics of the leaf);
- Creation of new germplasms to create variability and select for new characteristics in future generations through the definition of an effective manual crossing method (obtaining F1 and F2 populations);
- Reproduction of populations in field isolating them with insect nets to avoid cross-hybridization;
- Experimentation of new selection tools (for quantitative traits and qualitative analysis);
- Development of phytopathological assays (*Fusarium oxysporum* resistance assay test on plants and *Hyaloperonospora parasitica* on younger leaf) to select only resistant genotypes and obtain improved varieties.

2.2 MATERIALS AND METHODS

2.2.1 Plant material used for breeding

The first sowing which allowed to start the rocket breeding program included genetic material from different sources (Tab. 7).

Table 7 List of genotypes used for breeding with source

SOURCE	**NAME**	**SPECIES**
ISI Sementi	Runway	*Eruca sativa*
ISI Sementi	Atlanta	*Diplotaxis tenuifolia*
ISI Sementi	Dallas	*Diplotaxis tenuifolia*
T & T	Marte	*Diplotaxis tenuifolia*
Tokita	Red Dragon	*Diplotaxis tenuifolia*
Tokita	Discovery	*Diplotaxis tenuifolia*
Tokita	Gourmet	*Diplotaxis tenuifolia*
Ortis	Extrema	*Diplotaxis tenuifolia*
Adua seeds	Virtus	*Eruca sativa*
Adua seeds	Victum	*Eruca sativa*
Local accession Spain	Charisma	*Diplotaxis tenuifolia*
Cora seed	Celebris	*Diplotaxis tenuifolia*
Local accession Italy	Captain	*Diplotaxis tenuifolia*
Enza Zaden	Bellezia	*Diplotaxis tenuifolia*
Enza Zaden	Grazia	*Diplotaxis tenuifolia*
Enza Zaden	Prudenzia F1	*Diplotaxis tenuifolia*
Enza Zaden	Letizia	*Diplotaxis tenuifolia*
Enza Zaden	Tricia	*Diplotaxis tenuifolia*
Maraldi sementi	Sele F1	*Diplotaxis tenuifolia*
Maraldi sementi	Rubicone F1	*Diplotaxis tenuifolia*
Vilmorin	Celebris	*Diplotaxis tenuifolia*
Vilmorin	Rapida	*Diplotaxis tenuifolia*
Vilmorin	Charisma	*Diplotaxis tenuifolia*
IPK GENEBANK	DIPLO02	*Diplotaxis erucoides*

IPK GENEBANK	DIPLO05	*Diplotaxis muralis*
IPK GENEBANK	DIPLO13	*Diplotaxis muralis*
IPK GENEBANK	DIPLO16	*Diplotaxis muralis*
IPK GENEBANK	DIPLO01	*Diplotaxis tenuifolia*
IPK GENEBANK	DIPLO03	*Diplotaxis tenuifolia*
IPK GENEBANK	DIPLO04	*Diplotaxis tenuifolia*
IPK GENEBANK	DIPLO06	*Diplotaxis tenuifolia*
IPK GENEBANK	DIPLO08	*Diplotaxis tenuifolia*
IPK GENEBANK	DIPLO09	*Diplotaxis tenuifolia*
IPK GENEBANK	DIPLO10	*Diplotaxis tenuisiliqua Delile*
Accession collected in South Italy	AP001	*Eruca sativa*
Accession collected in South Italy	AP002	*Eruca sativa*
Accession collected in South Italy	AP003	*Eruca sativa*
Accession collected in South Italy	AP004	*Eruca sativa*
Accession collected in South Italy	AP005	*Eruca sativa*
Accession collected in South Italy	AP006	*Eruca sativa*
Accession collected in South Italy	AP007	*Eruca sativa*
Accession collected in South Italy	AP008	*Eruca sativa*
Accession collected in South Italy	AP009	*Eruca sativa*
Accession collected in South Italy	AP010	*Eruca sativa*
Accession collected in South Italy	AP011	*Eruca sativa*
Accession collected in South Italy	AP012	*Eruca sativa*
Accession collected in South Italy	AP013	*Eruca sativa*
Accession collected in South Italy	AP014	*Eruca sativa*
Accession collected in South Italy	AP015	*Eruca sativa*
Accession collected in South Italy	AP016	*Eruca sativa*
Accession collected in South Italy	AP017	*Eruca sativa*
Accession collected in South Italy	AP018	*Eruca sativa*
Accession collected in South Italy	AP019	*Eruca sativa*
Accession collected in South Italy	AP020	*Eruca sativa*
Accession collected in South Italy	AP021	*Eruca sativa*

Accession collected in South Italy	AP022	*Eruca sativa*
Accession collected in South Italy	AP023	*Eruca sativa*
Accession collected in South Italy	AP024	*Eruca sativa*
Accession collected in South Italy	AP025	*Eruca sativa*
Accession collected in South Italy	AP026	*Eruca sativa*
Accession collected in South Italy	AP027	*Eruca sativa*
Accession collected in South Italy	AP028	*Eruca sativa*
Accession collected in South Italy	AP029	*Eruca sativa*
Accession collected in South Italy	AP030	*Eruca sativa*

2.2.2 List of genotypes used for quality test and salt stress test

For quality and salinity tolerance experiments, the genotypes are listed in Tab.8

Table 8 Genotypes used in salt stress and quality experiments

GENOTYPE	**PHASE**
ISI 1	experimental breeding stage (SC)
ISI 2	experimental breeding stage (SC)
ISI 3	experimental breeding stage (SC)
ISI 4	Benchmark variety
ISI 5	Benchmark variety

2.2.3 Cultivation methods

First breeding selection of rocket population was carried out on polystyrene trays. 50-100 seeds for each population were sown (depending on the seeds stock). During growth phase, trays were placed in nursery with controlled temperature (20-25°C). After one month, single plants were selected for uniformity, leaf shape, leaf color, growth habit, labeled and transplanted in open field for reproduction, isolating them with an anti-insect net (Fig.6a), or in larger pots placed in controlled greenhouse, for crossings and reproduction.

2.2.4 Manual cross methods

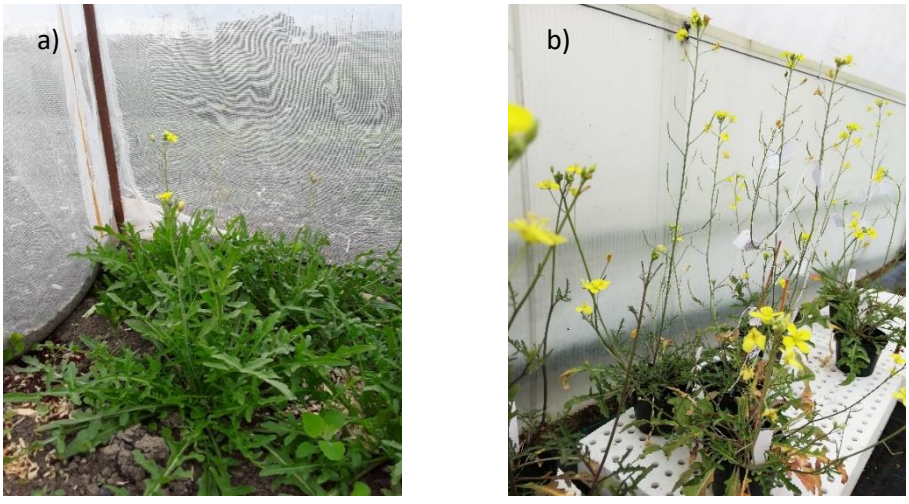
Plants for planned crossings were transplanted in pots and placed in a greenhouse with a temperature of 25°C and anti-insect net to prevent the entry of pollinators. When the flowering phase occurred, crossings were made manually. Female candidate plants were emasculated, then pollen obtained from the plant selected as male parent was used to pollinate the ovary of the female parent. The emasculation consisted in the removal of the anthers with laboratory tweezers. After pollination, flowers were labeled with tags indicating the cross combination and the date of crossing (Fig.6b). After 21-28 days, the formation of the seeds occurred, and seeds were harvested.

2.2.5 Reproduction of populations

Accession multiplication and seed production were carried out from selected populations transplanting them in open field, in the experimental fields in ISI Sementi (Fidenza, see Fig.6a). Cultivation in open field generally took place from April (transplant) to August (seed harvest). A second sib reproduction cycle was possible transplanting plants in late spring-early summer with harvest in early autumn. However, during this cycle, phytopathological problems occurred due to high humidity and rain during the harvest phase.

Generally, five to ten plants (two replicas) of each population were transplanted and isolated. To isolate populations and prevent cross breeding, the groups of plants were placed at a distance of 2 meters from one to another and insect nets were closed to delimitate the plot.

Figure 6 a) Reproduction of plants under cage in the experimental field of ISI Sementi; b)crosses on plant labeled with tags



Considering the self-incompatibility of rocket, pollination between sister plants must be favored by using pollinating insects. Flies were used as pollinators for their easiness of use. Flies are sometimes used for this aim instead of bees and bumblebees, with satisfactory results in terms of seeds produced. The larvae were thrown under the insect nets once a week for a month. The first launch of pollinators is usually done when at least 60 percent flowering has been achieved. Larvae in summer condition usually mature in 24-48 hours. Adult flies have pollinator function.

2.2.6 Phenotyping and selection of plant material

Every year, new F1 populations were evaluated and selected. Within the F1 generations,3 to 15 plants were selected choosing the most uniform among them and those with the best characteristics. These sister plants from same F1 were isolated by nets in the field and cross reproduced by pollinating insects to obtain F2 generations. Before reproduction in the field for the advancement of generation, the plants from generation F2 onwards were first sown and evaluated in containers. In a first phase the off type plants were eliminated and the plants most similar to each other were reproduced with the same method used in F1. Generations after F2 were obtained crossing sib-plants up to the most advanced generation, generally up to the maximum of the sixth generation because the population was already stable due to the most important agronomic characteristics.

In the different generations, selections were carried on following the product profile designed by the marketing manager of ISI Sementi, profile described in general lines in the introductory part in chapter 2 Breeding. Several traits were scored in the first selection phases, by the elimination of the off-types: leaf jaggedness, uniformity and leaf color. The comparison with the market benchmarks and agronomical selection made possible to obtain more stable populations with darker and jagged leaves. In parallel with the agronomic phenotyping, a selection was also carried out by means of pathological tests (as it will be described in the next chapters).

Once a certain phenotypic stability has been reached in the population (at least in F4 generation), a phenotyping of the selected populations has been carried out. Plants characters were classified using CPVO (Community Plant Variety Office) official guidelines, from the last update of April 2022 of the PROTOCOL FOR TESTS ON DISTINCTNESS, UNIFORMITY AND STABILITY Diplotaxis tenuifolia (L.) DC. WILD ROCKET, UPOV Code: DIPLO_TEN (Fig.7,8,9)

The Community Plant Variety Office (CPVO) is an agency of the European Union established in 1994 with headquarters in Angers (France). Its task is to administer the system of horticultural

rights also known as plant variety rights, a form of intellectual property relating to plants: it grants intellectual property protection for new plant varieties. These rights are valid for a period of 25 or 30 years.

Figure 7 (a,b,c,d) Table of characters described in CPVO (last CPVO update published in April 2022)

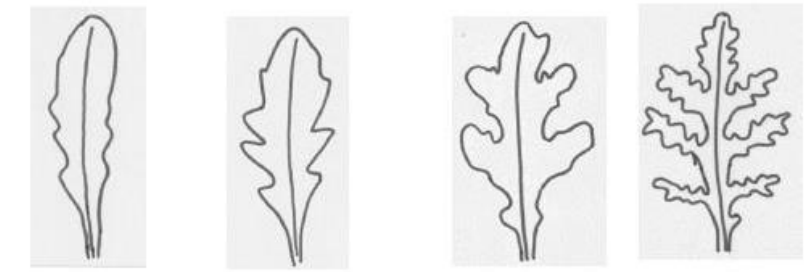
CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
1.	**1.**	**VG**	**Leaf: attitude**		
QN		**(a)**	erect	Olivetta	1
			erect to semi erect		2
			semi erect	Discovery	3
			semi erect to horizontal		4
			horizontal	Nature, Tiger	5
2.	**2. (*)**	**VG**	**Leaf: colour of blade**		
QL		**(a)**	green	Nature	1
			grey green	Tiger	2
3.	**3.**	**VG**	**Leaf: intensity of colour**		
QN		**(a)**	very light		1
			very light to light		2
			light	Wildfire	3
			light to medium		4
			medium	Nature	5
			medium to dark		6
			dark	Anastazia, Dragons Tongue	7
			dark to very dark		8
			very dark		9
4.	**4. (*)**	**VG**	**Leaf: anthocyanin coloration of veins**		
QL		**(a)**	absent	Nature, Tiger	1
G			present	Bloody, Dragons Tongue	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
5. (+)	**5. (*)**	**VG/MS**	**Leaf: length**		
QN		**(a)**	very short		1
			very short to short		2
			short	Olivetta	3
			short to medium		4
			medium	Tiger	5
			medium to long		6
			long	Nature	7
			long to very long		8
G			very long		9
6.	**6. (*)**	**VG/MS**	**Leaf: width**		
QN		**(a)**	very narrow		1
			very narrow to narrow		2
			narrow	Olivetta	3
			narrow to medium		4
			medium	Tiger	5
			medium to broad		6
			broad	Nature	7
			broad to very broad		8
G			very broad		8

7. **(+)**	**7.** **(*)**	**VG**	**Leaf: division**		
QN		**(a)**	absent or very weak	Olivetta	1
			very weak to weak		2
			weak	Tiger	3
			weak to medium		4
G			medium	Nature	5
			medium to strong		6
			strong		7
			strong to very strong		8
			very strong		9
8. **(+)**	**8.**	**VG**	**Leaf: width of primary lobes**		
QN		**(a)**	very narrow		1
			very narrow to narrow		2
			narrow	Athena, Dragons Tongue, Themisto, Gourmet	3
			narrow to medium		4
			medium	Tiger, Venicia	5
			medium to broad		6
			broad		7
			broad to very broad		8
			very broad		9
9. **(+)**	**9.** **(*)**	**VG**	**Leaf: secondary lobing**		
QN		**(a)**	absent or weak	Tiger	1
			medium	Nature	2
G			strong	TT Marte	3

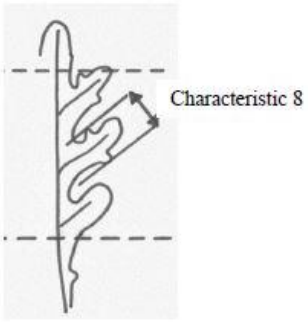
CPVO N°	UPOV N°	Stage, Method	Characteristics	Examples	Note
10. (+)	**10. (*)**	**MG**	**Time of flowering**		
QN			very early		1
			very early to early		2
			early		3
			early to medium		4
			medium	Tiger	5
			medium to late		6
			late	Nature	7
			late to very late		8
			very late	Olivetta	9
11.	**11.**	**VG**	**Plant: height at flowering stage**		
QN			very short		1
			very short to short		2
			short	Tiger	3
			short to medium		4
			medium	Nature	5
			medium to long		6
			long	Verdia, Voyager	7
			long to very long		8
			very long		9

Figure 8 Particular of leaf shape in CPVO schede



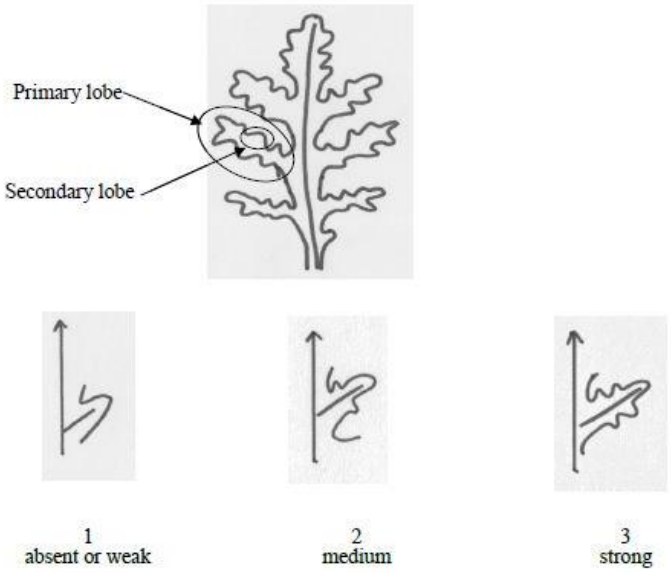
The division of the leaf should be observed in the middle third of the leaf.

Figure 9 Particular of leaf lobation in CPVO schede



The width of the primary lobes should be observed in the middle part of the leaf.

Ad. 9: Leaf: secondary lobing



2.2.7 Seed harvesting

Seeds collection was carried out starting about 90 days after the transplant in summer cycle. Cotton bags with very dense meshes were used to harvest seeds to avoid high humidity rate, which decrease seed viability. A different bag was used for each population. Plants were cut at the base and placed in cotton bags with a very dense mesh to avoid excessive losses of seeds. The bags were stored in a dry place for a week and during which the seeds fell naturally into the bag because of the opening of the dry siliques.

Siliques were threshed, and filters of different meshes were used to separate the seeds from plant residues. Collected seeds were stored in paper bags, weighed, classified with a code referring to every year of harvest and stored in the seed warehouse of ISI Sementi.

2.2.8 Screening phase evaluation

The market lifecycle of a variety starts with the breeding of the variety itself and ends when the variety is no longer sold, and so retired for commercialization, because considered obsolete being no longer adapted to market requirement. All the different stages in the lifecycle of a variety are encoded to make more efficient the activity inside the breeding company. All the different stages with the corresponding code are reported in Tab.9; the codes will be used subsequently in the result section for addressing specific stages of the creation of new varieties.

After reaching the phenotypic uniformity, the populations selected in the breeding is advanced to the screening phase where they are evaluated in open field or greenhouses in standard cultivation conditions to observe if they are suitable for the market.

Table 9 Phase code used in breeding to separate different stages for the creation of a new variety

PLC 1	SP phase (creation of stable varieties in advanced generations)
PLC 2.1	SC (screening phase, different cultivation areas in small plot)
PLC 2.2	SCA (screening phase in larger plots in different locations)
PLC 3	VS (varieties are evaluated in locations and clients)
PLC 4	PC (pre commercial phase)
PLC 5	CO (commercial phase)
PLC 6	PO (phase out, varieties are no longer sold)

To evaluate the performance of a variety in the market, some agronomic parameters and characteristics are evaluated testing varieties in different locations. The evaluation of the screening fields (SC) includes:

- Health of the variety: presence of pathogens and disease, general appearance of the leaf;
- Suitability of the variety: adaptability of the variety in a given sowing period or in a specific growing area;
- Yield: measured in kg of product per mq

- Other characters measured are: cycle, bolting, characteristics of the leaf, as showed in the table below (Tab.10).

Table 10 Traits recorded in phenotyping of Screening trials

		SCORE				
KEYS		**1**	**3**	**5**	**7**	**9**
Cyc.	**Cicle**	Very early	Early	Medium	Late	Very Late
Bolting	**Bolting**	<10%	10-30%	30-50%	>50%	> 80%
Col	**Color (intensity)**	Very light green	Light Green	Medium Green	Dark Green	Very Dark Green
Thi	**Thickness**	Vey Thin	Thin	Medium	Thick	Very Thick
Leaf Width	**Leaf Width**	Very tight	Tight	Medium	Large	Very large
Leaf In	**Leaf Margin Incision**	Not present	Week	Medium	Strong	Very Strong
Score	**Score**	Drop	Insufficient	Medium	Good	Optimal

Three locations were selected in Italy for screening phase: Veneto in the North of Italy, Bergamo area in the North of Italy, Salerno area in the South of Italy. Recently, other two screening platform has been added: one location in the south of Spain and one in Portugal.

During the project, the screening phase (PLC 2.1- SC) was conducted in 2021 in summer cycle, both in North and South of Italy and at the end of 2021 for autumn stage in Piana del Sele area. The next phase (PLC 2.2.- SCA) was conducted in 2022 in larger cultivation plot in various companies located in Southern Italy and in the Iberian Peninsula.

2.2.9 Methods for quality traits

For the analysis and selection of qualitative traits, data were collected with different methods described in the table below (Tab.11)

Table 11 List of quality traits measured for 5 varieties in 2 locations (North and South of Italy) from external laboratories (external service for ISI Sementi)

PARAMETER	UNIT OF MEASURE	MEASUREMENT DESCRIPTION
FRESH WEIGHT	g	Product weighted after harvest
DRY WEIGHT	g	Product dried in a heater for 24 h and weighted
H2O%	mL	Extractor
pH	pH	pH meter
°BRIX	°BRIX	Refractometer
CONDUCIBILITY	uS	Conductivity meter
CHL (SPAD) and API INDEX	Values from 0 to 99 (unit)	AT Leaf instrument
ISOQUERCITIN	mg/kg	Gas chromatography
QUERCITIN	mg/kg	Gas chromatography
ASCORBIC ACID	mg/kg	Gas chromatography
GLUCORAFANIN	mg/kg	Gas chromatography
ASTRAGALIN	mg/kg	Gas chromatography
GLUCOERUCIN	mg/kg	Gas chromatography
GLUCOSATIVIN	mg/kg	Gas chromatography
PROTEINS	g/100 gr	Titrimetry/Gravimetry
TOTAL FAT g/100 g	g/100 gr	Titrimetry/Gravimetry
FIBER g/100 g	g/100 gr	Titrimetry/Gravimetry
ASHES g/100 g	g/100 gr	Titrimetry/Gravimetry
CARBOHYDRATES g/100 g	g/100 gr	Titrimetry/Gravimetry
DRY SUBSTANCE g/100 g	g/100 gr	Titrimetry/Gravimetry
NITRATES mg/kg ($NO3^-$)	mg/kg	Titrimetry/Gravimetry
NICHEL mg/kg	mg/kg	Titrimetry/Gravimetry

2.2.10 Salt stress analysis

For salt stress resistance assay, media with increasing NaCl concentration (0 mM, 40 mM and 80 mM, described in Tab.12) have been prepared and used for the screening of experimental ISI Sementi varieties in SC phase and benchmark varieties.

Table 12 Recipe for salt media for salinity assay

Recipe	0 (A)	40mM (B)	80mM (C)
Macro salts BDS (ml)	100	100	100
Micro salts B5 (ml)	10	10	10
FeNa EDTA (ml)	10	10	10
Vitamins (ml)	1	1	1
NaCl (gr)	0	2,33	4,66
Mesoinositol (mg)	500	500	500
Sucrose (g)	30	30	30
Agar (g)	6,8	6,8	6,8
H_2O	a 1000 ml	a 1000 ml	a 1000 ml
PH 5,7			

Protocol for disinfection of the seeds for the salt stress experiment:

For the disinfection, gauze filters were used (two layers because rocket seed is very small) tied with a thread. Seeds of a single variety were put in a different gauze.

The gauzes containing seeds were soaked in 10% NaCl solution for 10 minutes and then rinsed in sterile water; two repetitions of this step were made. After this passage, gauzes were cut with sterile clippers and the seeds contained were dried under laminar flow hood.

Sowing of varieties *in vitro* culture tubes:

Each tube contained 20 ml of medium. Seeds was inserted into the substrate using laboratory forceps. A single seed per tube was sowed.

After sowing, tubes of different concentration medium were placed in a growth chamber with controlled temperature of 23°C and a long photoperiod (16 h days/ 8 h night).

Evaluation of germinability and final weight evaluation:

After 5,7 and 10 days germinability was scored counting how many seeds per tube had germinated and they had first developed the radicle and then the cotyledons.

For the final evaluation, plants were removed from the tubes and dried. Weight of whole plants were measured and compared.

2.3 RESULTS AND DISCUSSION

2.3.1 Breeding

In the present study, the development of the new varieties started in 2019 with the making of first crosses among selected individuals (Tab.13) with the desired characteristics for producing new genetic variability. Following the first crosses, the selection of the plants in the F1 progeny was based on the application of a mass and recurrent selection approach that was carried on during both summer and autumn-winter periods to increase the number of generations per year, speeding up the process, starting from 2020. During summer, rocket was multiplied in open field with insect net isolators while, during the winter period, plants were multiplied using isolators or single pots in greenhouse.

Table 13 Advancement in breeding from 2019 to summer 2022 (GEN=generation in breeding, PLC1,PLC2 and PLC3=different stages of pre-commercial varieties

	GEN	2019	2020 summer	2020 autumn	2021 summer	2021 autumn	2022 summer
Breeding phase	new F1	60	165	50	0	0	60
	2		40	50	35	10	18
	3	84			20	20	10
	4	10			1	1	10
	5		10			1	1
	6						1
Commercial phase	PLC1		5				
	PLC2				5		
	PLC3						2

In subsequent years, 2021 and 2022, other cross pollinations were carried out to add further variability and to seek for new phenotype of interest on which to base the continuous breeding for new varieties.

Up to 2022, 5 varieties were obtained from recurrent selection and they reached the pre-commercial phase. Other populations in F3 and F4 generation are going to follow the same selections in the upcoming years. In Tab.13 it is also shown how these 5 varieties have gone through the different PLC stages (indicated with PLC1, PLC2 and PLC3) starting from 2020 to be then evaluated the last time during this year to select which of these would become new commercial varieties.

Meanwhile, wild accessions of IPK and *Eruca sativa* have been characterized and the ones considered interesting for their morphological traits have been reproduced under cages with pollinators. Others were used for interspecific crosses to create variability and a new germplasm useful for breeding.

2.3.2 Screening phase and agronomy

After a large-scale reproduction of selected populations, the screening (PLC2) phase started. The varieties mentioned before (see Table 9 for PLC stages description) were reproduced with large quantities of seeds to be screened in different locations. During the Screening, also two benchmarks were observed, to compare performances of experimental varieties with the commercial ones already in the market, one belonging to the current ISI Sementi commercial range and the second by another company (Tab.14). These ones were used for phenotypic comparison.

Table 14 List of rocket varieties in Screening platforms with the corresponding ISI code

CODE	COMPANY	BREEDING PHASE
ISI 1	ISI Sementi	SC
ISI 2	ISI Sementi	SC
ISI 3	ISI Sementi	SC
ISI 4	T&T	Commercial
ISI 5	ISI Sementi)	Commercial
ISI 6	ISI Sementi	SC
ISI 7	ISI Sementi	SC

The ISI populations that obtained the highest evaluation score based on the traits for baby leaf in Screening phase described in Material and Methods were ISI 2 (R04) and ISI 3 (R05), both in the evaluation taken in the experimental field in Bergamo (Tab.15) and in Battipaglia (Tab.16)

Table 15 Evaluation with relative score in the screenings field carried out in Bergamo

CODE	STB	SPD	HAB	1LF	LVE	LCO	LDI	LLO	LLG	LWD
ISI 1	7	5	6	3	6	6	5	5	5	6
ISI 6	7	7	7	6	6	6	7	7	7	7
ISI 2	7	6	7	6	6	7	6	6	6	7
ISI 3	8	8	8	5	8	8	7	8	8	7
ISI 7	6	6	7	6	8	7	7	7	7	5
ISI 4 Marte	8	8	8	6	7	7	7	7	7	7
ISI 5 Dallas	6	5	6	3	4	6	6	6	6	6

Table 16 Evaluation with relative score in the screenings field carried out in Battipaglia

CODE	STB	SPD	HAB	1LF	LVE	LCO	LDI	LLO	LLG	LWD
ISI 1	6	6	5	5	6	7	7	6	7	6
ISI 6	7	7	7	6	7	8	7	7	7	7
ISI 2	8	6	7	6	6	8	9	8	7	8
ISI 3	8	8	8	6	7	8	8	7	6	6
ISI 7	6	4	7	6	6	7	7	7	5	6
ISI 4 Marte	8	8	8	7	7	7	8	8	8	6
ISI 5 Dallas	6	7	6	6	6	6	6	6	5	5

ISI 2 in the evaluations in the two locations obtained the highest scores for the breeding characters evaluated; moreover, ISI 2 had intermediate tolerance to *Fusarium oxysporum*, from observations in fields generally infected with this pathogen where the screening took place. This variety is indicated for spring and summer cycles, has very jagged leaves with a dark green color.

ISI 3 had the fastest growth rate, (even compared with the benchmark Marte) high yield and brilliant green color. Suitable for spring and summer cycle and more tolerant to *Fusarium oxysporum* than ISI 2, ISI 3 had good qualities in autumn cycle too. For this variety, the tolerance observed to *Fusarium oxysporum* in infected fields has been confirmed by laboratory tests that will be described in the following chapters.

ISI 1, ISI 6 and ISI 7, already in the first sowings, showed not optimal performances, susceptibility to *Fusarium oxysporum* during the summer cycle and slow growth. It was decided to propose varieties for autumn cycles and review them in other subsequent tests. In autumn sowing ISI 1 proved to be much more suitable, with a higher degree of tillering and rapid growth not negatively affected by colder temperatures.

In summer 2021, the varieties that obtained the highest scores compared to the benchmark in the screening phase (Tab.17), were sampled and analyzed for agronomical traits and quality traits (shelf life, biochemical compounds, …) to add other data to the characteristics of these varieties and to guide the choice of the one that would pass into the commercial phase.

Table 17 List of varieties sampled for qualitative analysis in 2021

CODE	COMPANY
ISI 2	ISI Sementi (SC)
ISI 3	ISI Sementi (SC)
ISI 4	Marte, T&T
ISI 5	Dallas, ISI Sementi

For each variety, 500 grams in two replies in two locations were sampled. The subsequent season, ISI 1, ISI 2 and ISI 3 were advanced to SCA phase to confirm varieties performances in comparison with competitors and their suitability for key markets. ISI 1 was not sampled for the quality analysis because initially rejected for summer cycle, due to its negative performances based on the observed characters.

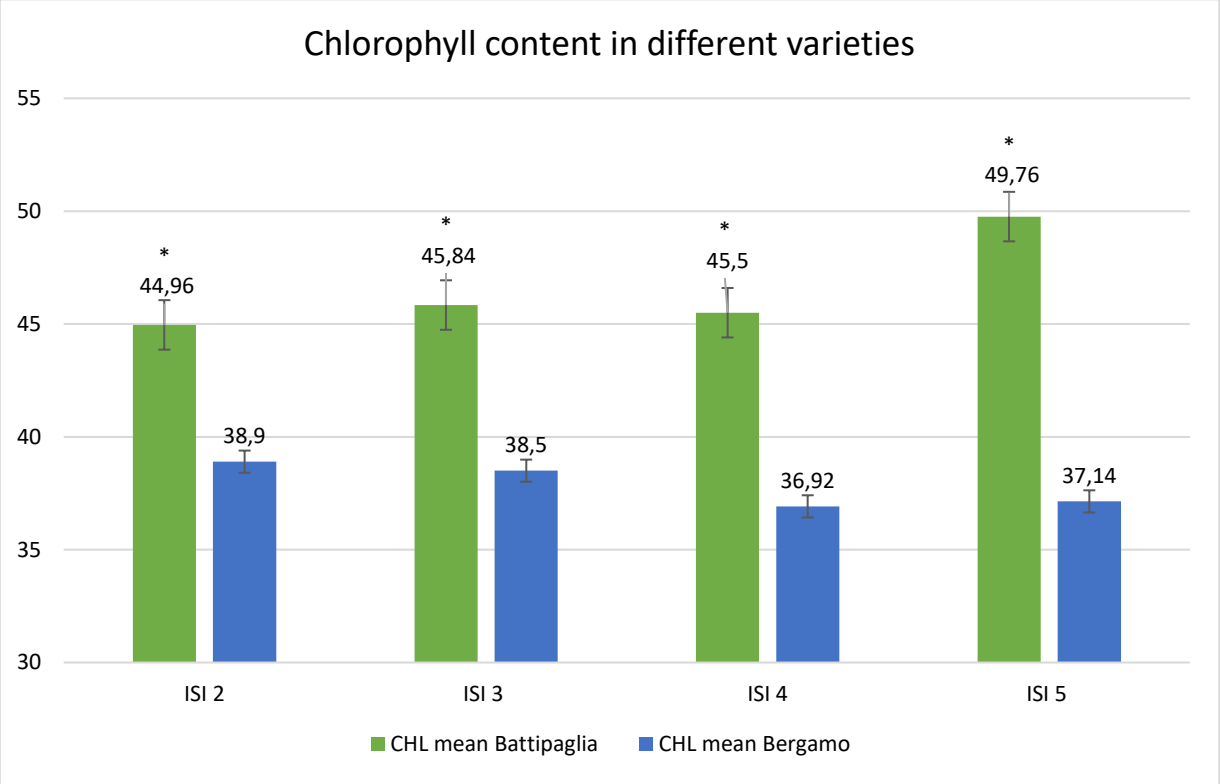
2.3.3 Quality traits results

The analysis done in external laboratories using gas chromatography and gravimetry gave information on the composition of the ISI varieties passed in the screening phase. This made it possible to get to know the varieties in greater depth from a qualitative point of view, as a research basis for future investigations related to taste and shelf life.

The most significant parameters are shown in the subsequent graphics and the complete correlation analysis among parameters is shown at the end of the chapter.

In Fig.10 Chlorophyll content is reported for ISI 2, ISI 3, ISI 4 and ISI 5 in two locations, Battipaglia (SA) and Bergamo, where screening phase took place.

Figure 10 Chlorophyll mean content expressed in SPAD and measured with AT Leaf; Mann-Whitney non parametric statistic test performed for each variety (=p≤0.05).*



SPAD value is a non-destructive measurement of chlorophyll content from the last expanded leaf. The AT Leaf instrument calculates a numerical SPAD value which is proportional to the amount of chlorophyll present in the leaf. Chlorophyll content is reported for both the trials in Battipaglia and Bergamo. The difference, for the same variety in different location, have been analyzed with Mann-Whitney non parametric statistic test; in fact the number of samples did not allow to use another statistical test and further data will be collected in the future to confirm the preliminary results. Mann-Whitney test highlighted significant differences (p value ≤ 0.05 in all the four test performed) in the same variety in two different localities. There is an overall higher chlorophyll mean for all varieties in Battipaglia SC field, due to climatic reasons of light exposition and intensity. The varieties measured showed similarities in chlorophyll content in the same location, except for ISI 5 (with a higher value of 49,76 in Battipaglia). ISI 2 had the highest Chlorophyll mean in Bergamo, with a value of 38,9; however it was very similar to the others, so no real difference in

varieties can be defined. The difference in chlorophyll content found in ISI 5 could origin the darker leaves color of this variety showed in South of Italy.

Concluding, as far as chlorophyll is concerned, all the data gathered are not sufficiently enough to define a statistically significant difference among rocket varieties. However, a difference between the two field locations (based in North of Italy and in South of Italy) has been measured, due to different climatic conditions and solar exposure.

The varieties for which chlorophyll was measured were also sampled during the trial and analyzed by two external laboratories that provided qualitative data reported in Tab.18, which are interesting to highlight differences between varieties and locations.

Table 18 Quality parameters provided for ISI varieties by external quality labs (BAT=samples collected in Battipaglia and BER= samples collected in Bergamo)

	ISI1		ISI2		ISI3		ISI5	
	BAT	BER	BAT	BER	BAT	BER	BAT	BER
DRY WEIGHT (g/100 g)	7,81	7,17	7,55	8,28	7,52	7,32	7,65	6,74
H2O extract/100 grams	23,85	30	23,02	34,05	23,8	31,6	29,65	41,85
pH	5,5	5,85	5,52	5,88	5,53	5,92	5,49	6,14
CONDUC (uS)	5833	4970	6280	5620	6190	5840	5641	7250
CHL_MEAN	49,76	37,14	44,96	38,9	45,84	38,5	45,5	36,92
ISOQ (mg/Kg)	1,35	0,926	1,03	1,433	1,64	3,877	3,16	0,35
QUERCITIN (mg/Kg)	0,002	0,141	0,005	0,228	0,012	0,206	0,011	0,175
ASC.ACID (mg/Kg)	1,59	20,549	4,77	22,267	4,07	17,59	3,32	16,99
GLUCOR (mg/Kg)	21,99	6,162	16,45	13,654	13,04	10,12	13,72	11,47
ASTRAG (mg/Kg)	0,153	0,321	0,052	0,057	0,193	0,212	0,238	0,114
GLUCOER (mg/Kg)	68,28	53,04	87,07	141,79	100,26	205,99	54,51	983,26
GLUCOSAT (mg/Kg)	233,51	297,22	196,07	505,23	204,62	631,44	234,57	412,69
PROTEINS g/100 g	2,76±0,18	2,62 ± 0,17	3,43±0,22	3,03±0,19	2,95±0,19	2,62± 0,17	2,51±0,16	2,56 ± 0,17
TOTAL FAT g/100 g	< LoQ	0,19	0,580±0,048	0,34	0,180±0,035	0,22	0,430±0,042	0,22
FIBER g/100 g	2,30±0,41	1,74	2,18±0,40	2,62	2,14±0,40	1,87	1,78±0,38	2,1
CARBS g/100 g	0,01±0,61	0,330±0,904	0,830±0,60	0,090±0,70	0,030±0,62	0,400±0,980	0,530±1,06	0,110±0,697
DRY MATTER g/100 g	7,00±0,38	6,53	9,00±0,38	7,74	7,18±0,38	6,71	6,80±0,38	6,51
NITRATES (mg/kg)	4480	3670	5960	3740	5050	4650	3770	4240
NICHEL mg/kg	0,025	0,05	0,027	0,229	0,033	0,045	0	0,099

The data do not allow to conclude differences statistically significant, however some observations can be made on the data received.

Concerning fiber content, the four varieties had higher values of fiber in Battipaglia trials, except for ISI 2 variety, which showed high fiber levels (the highest of the varieties) with high values both in Battipaglia and Bergamo. This data could be indicative of a different adaptability of a variety to different environments.

Concerning dry matter, all the varieties accumulate more dry matter in Battipaglia trial than in Bergamo. The variety ISI 2 had the higher dry matter value in both locations. Dry matter and fiber content are parameters that can be used to obtain information on yield and quality. Dry matter is inversely proportional to water content, that generally accelerates the degradation of leafy products. The lower the dry matter content, the higher the water content in the leaves. Furthermore, the fiber content is also interesting as a nutraceutical characteristic.

For this reason, ISI 2 could be a variety with longer shelf life and resistance to oxidation and tissues degradation.

Concerning ascorbic acid, it is clearly evident that in Bergamo it accumulates at higher concentration than in Battipaglia. As for chlorophyll content, this behavior can reflect a strong environmental influence on the manifestation of this character. Ascorbic acid is the reduced form of vitamin C. It is studied as a parameter because it is a powerful anti-oxidant and therefore extend the shelf life of leafy vegetables.

Long shelf life is a highly desired characteristic in leafy vegetables such as rocket. With the term “shelf life” we mean something that refer to several characteristics that are difficult to study from a genetic point of view depending not only to the genotype but also to the processing, the storing conditions and the interaction between genotypes, processing and storing. Concerning this point, in breeding, there is still a lot of work to do to understand if these differences are inheritable in generations. It is however interesting to note that ISI 2 shows high values of ascorbic acid in both locations where the sampling took place.

The nitrates accumulated in the leaves are a problem in the cultivation of “baby leaf” for toxicity to humans. For this reason, farmers can have problems in the acceptance of the product by large-scale distribution.

Nitrates are known as food additives and can be found in nature as part of the nitrogen cycle: they play an important role during nutrition, growth and development of plants. Because of their cumulative properties, they are an important part of vegetables (Caruso et al., 2018a; Crescenzi et al., n.d.; Morais et al., 2020)Absorption of nitrate occurs most often from natural sources, but vegetables accumulate a significant portion of nitrate from nitrogen-based fertilizers, which are used for fertilizing plants for faster and bigger growth. Being leaves the main site of accumulation of nitrates in plants, leafy vegetables, such as lettuce, rocket and spinach, can contain the highest concentrations of nitrate if compared to other vegetables harvested for different parts (Santamaria et al., 2001)

There are several methods to lower the level of nitrates absorbed by leaves, all concerning crop management (water, fertilizers) or research on which varieties tend to accumulate less nitrates in the leaves.

The parameters measured were therefore important to better understand the characteristics of our varieties and to choose the potential commercial varieties among these the candidates.

During the project, the nitrates contained in the leaves at the time of harvest were measured on the five varieties tested. Observing Tab.18, it is possible to note a variable difference in the accumulation of nitrates in the different varieties sampled. However, nitrates can be very influenced by the environmental conditions, field fertilization and different irrigation management up to harvest, making it very variable and difficult to study from a genetic point of view. Even in this parameter studied, which is very interesting also for the fresh market and the residual issue, more statistically representative sample should be studied, eliminating the differences in terms of fertilization and irrigation of the crops.

2.3.4 Salt stress tolerance results

Salt stress assay was performed in soil and *in vitro*, giving a primary view of rocket crop behavior in salt stress conditions.

Three scorings were done during the experiments, starting one week after sowing, day 7, and repeating the scoring at days 14 and 21. From the first evaluation, the number of germinated plants was marked, and the development of seedlings was monitored, noting if the seedlings were developing normally or had abnormalities.

The preliminary results obtained from the two experiments are reported below, which constitute a starting point for future experiments on salinity. Many aspects of the experiment will have to be improved, for example the number of biological replicates will have to be increased, the presence of other salts which interfere with plant growth (especially for soil evaluation) will have to be measured and the significant difference among varieties and not just between groups of different salt concentrations.

In-vitro experiments

In vitro germinability at both salt concentrations was comparable to the medium with no salts. However, with soil B (40 mM) the development of the seedling and roots is clearly superior to both soil A (no salt) and soil C (80 mM).

Table 19 Germination (%) of the varieties tested at 7 days after sowing

Variety	Percentage of germination at 0 mM	Percentage of germination at 40 mM	Percentage of germination at 80 mM
ISI1	66%	100%	66%
ISI2	80%	75%	84%
ISI3	37,5%	87%	85%
ISI4	73%	84%	100%
ISI5	100%	50%	92%

Table 20 Germination (%) of the varieties tested at 14 days after sowing

Variety	Percentage of germination at 0 mM	Percentage of germination at 40 mM	Percentage of germination at 80 mM
ISI1	66%	100%	66%
ISI2	80%	75%	100%
ISI3	62%	87%	85%
ISI4	90%	84%	100%
ISI5	100%	80%	92%

In the last observation, at day 21 post sowing, all plants (especially in concentrations of 0 mM and 40 mM) showed yellow hues probably due to light in the growth chamber, probably due to not due to the use of lights not specific to the growth of rocket *in vitro*.

ISI 2 had the highest growth rate at 40 mM and 80 mM compared to the other varieties, showing more adaptability to salt soils, even at high concentrations.

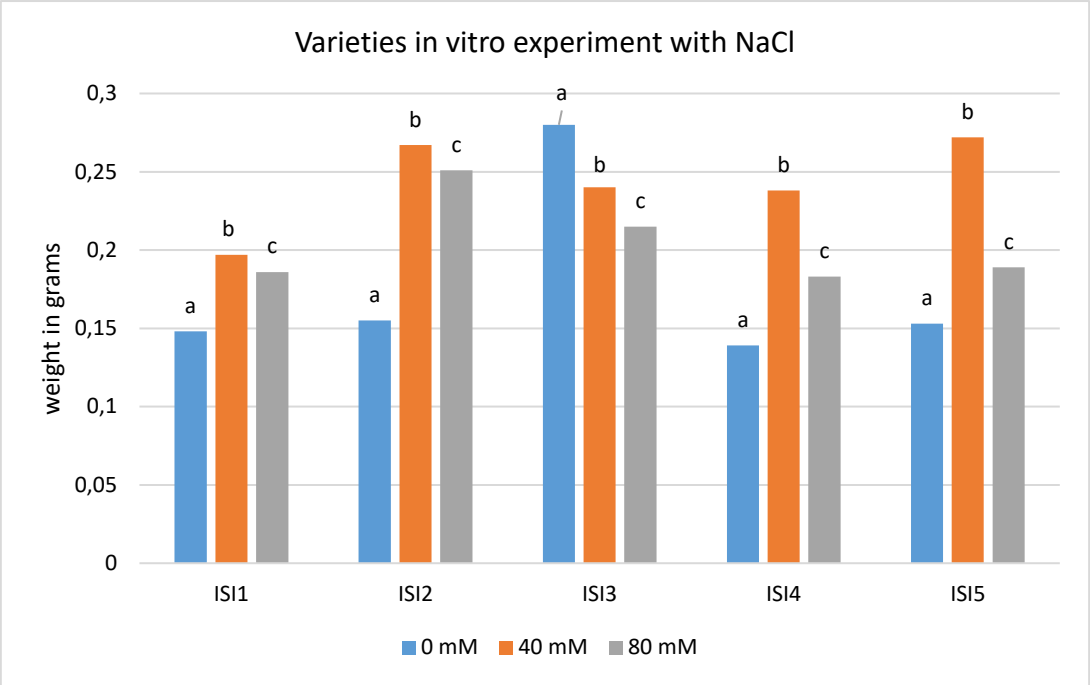
All the varieties showed better development (based on average weight) at 40 mM and a general reduction at 80 mM. A minimum level of salt seems to be useful for rocket growth, in fact all the varieties, with the exception of ISI 3, showed greater growth from 0 to 40 mM (Tab.21).

In Fig.10 weight of the four varieties is reported, comparing the three salt concentrations. The Mann-Whitney statistical test carried out highlighted significant differences between the three groups (in the graphic indicated with different letters).

Table 21 Average (avr) weight in grams of the varieties in three different NaCl concentration media

Variety	avr weight (g)	avr weight (g)	avr weight (g)
	0 mM	**40 mM**	**80 mM**
ISI1	0,148	0,197	0,186
ISI2	0,155	0,267	0,251
ISI3	0,28	0,24	0,215
ISI4	0,139	0,238	0,183
ISI5	0,153	0,272	0,189

Figure 11 Varieties average weight in grams in three different NaCl concentrations (o,40 and 80 mM of NaCl); letters a,b,c means statistical differences among groups performing Mann-Whitney non parametric test with a p value≤0.05



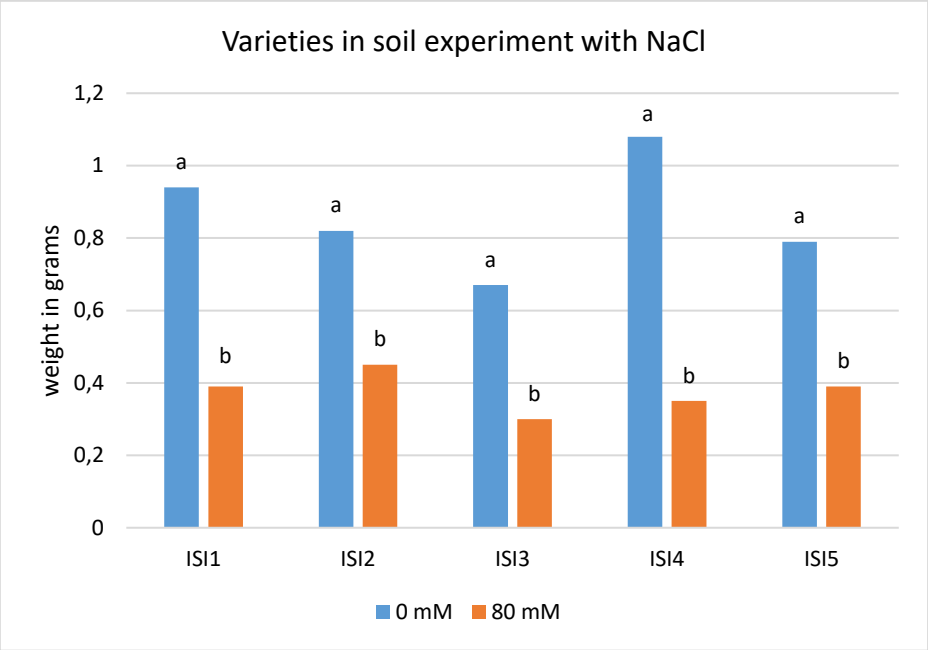
In-soil experiments

The experiment in soil was carried out with only two soil types: A) no salt added to the soil (normal peat irrigated with water) and B) soil irrigated with water with NaCl at the concentration of 80 mM. In soil, the reduction in weight was noticeable passing from 0 mM to 80 mM for all the varieties in the experiment, as shown in Fig.11. To observe varietal behavior, the difference in weight of the seedlings the evaluation day at 0 and 80 mM is also shown in the Tab.22.

Table 22 Weight variation (in grams) of varieties from soil without NaCl and at 80 mM of NaCl

Variety	Weight variation (g)
ISI 1	0,552
ISI 2	0,363
ISI 3	0,370
ISI 4	0,741
ISI 5	0,401

Figure 12 Average weight expressed in grams for rocket varieties in soil experiment at 0mM and 80 mM; a,b means statistical differences between the concentrations (Mann-Whitney non parametric test, p value≤0.05)



Observing the differences in weight of the varieties passing from 0 to 80 mM in the soil (Fig.11), ISI 4 was the most susceptible to high salt concentration (with a weight variation of 0,741 grams passing from 0mM to 80mM, see Tab.22) while ISI 2 and ISI 3 were more tolerant to the increasing salt concentration, with the lowest reduction in average weight (0,363 and 0,370 grams). The two groups show evident differences in the development of the seedlings, also highlighting that NaCl concentration of 80 mM can be critical for the normal development of the variety. The soil experiment, compared to the *in vitro* one, is more complex and less controllable due to the presence of other salts in the substrate that can interfere with plant roots. Probably, the experiment will only be repeated *in vitro* because it is more easy to reduce the environmental variables and to enlarge the sample to a more representative number of seedlings per variety.

P value, correlation between parameters

All the parameters measured were subjected to the correlation test, to study direct or indirect correlation among variables. Some values were particularly significant (close to 1) and they are reported below. The values are represented in the correlation table below (Tab.23).

Interesting observations are for example:

- Ph correlated with Astragalin and Glucoraphanin principally (but also show high value with other compounds);
- Glucosativin correlated with Glucoraphanin, Astragalin and Glucoerucin;
- Humidity correlated with dry matter;
- Brix correlated with the sulfur biochemical compounds present in rocket;
- Nitrates correlated with protein content.

However, these examples are interesting observations, but do not explain the cause and effect relationship behind these compounds. These correlations should be explored to understand the underlying biochemical mechanism at the base of the taste and quality of rocket crop. The next step would be to investigate genetic differences between varieties .

*Table 23 Correlation value among all the measured quality parameters (see pag.57-58); the value with * show significant correlation (next to 1 or -1)*

	DRY_W	H2O_	pH	BRIX	CONDUC	CHL_MEAN	API_MEAN	ISOQ	QUERC	ASC_AC
DRY_W	6,65E-64	1,37E-01	3,86E-01	1,39E-01	1,16E-01	2,02E-01	1,93E-01	4,19E-01	4,08E-01	9,45E-01
H2O	-0,505	1,73E-93	7,00E-02	5,62E-01	5,14E-01	8,02E-01	8,05E-01	6,19E-01	5,96E-01	8,66E-01
pH	-0,309	-0,498	0	1,48E-04	8,14E-03	4,72E-04	4,69E-04	5,02E-02	5,70E-01	6,74E-02
BRIX	0,502	0,170	-0,844	0	1,34E-02	1,75E-04	1,65E-04	1,06E-02	3,21E-01	3,94E-02
CONDUC	-0,529	-0,191	0,675	-0,641	0	4,64E-02	4,33E-02	5,96E-03	5,14E-01	2,83E-01
CHL_MEAN	0,441	0,074	-0,808	0,839*	-0,540	0	1,57E-24	1,74E-01	9,29E-01	1,18E-03
API_MEAN	0,449	0,073	-0,808	0,840*	-0,546	0,999*	0	1,70E-01	9,43E-01	1,26E-03
ISOQ	0,288	0,146	-0,532	0,658	-0,693	0,385	0,388	0	4,57E-01	2,24E-01
QUERC	0,295	0,155	-0,166	0,286	-0,191	-0,026	-0,021	0,217	2,70E-95	3,71E-02
ASC_AC	0,025	0,050	0,502	-0,555	0,309	-0,773	-0,771	-0,347	0,561	0
GLUCOR	0,117	0,601	-0,965	0,752*	-0,593	0,770	0,769*	0,360	0,149	-0,432
ASTRAG	-0,002	0,553	-0,976	0,863*	-0,724	0,736	0,737*	0,613*	0,253	-0,439
GLUCOER	-0,664	0,548	-0,692	0,510	-0,110	0,433	0,430	0,110	0,154	-0,194
GLUCOS	-0,263	0,583	-0,927	0,660*	-0,509	0,724*	0,722*	0,269	0,122	-0,370
PROT	0,362	-0,534	-0,463	0,396	-0,062	0,351	0,348	-0,143	-0,312	-0,358
FAT	0,140	-0,116	-0,245	0,306	-0,042	0,007	0,005	0,164	-0,175	-0,168
FIBER	-0,026	0,335	0,081	-0,183	0,532	-0,102	-0,107	-0,548	-0,126	0,071
CARBS	0,304	-0,453	-0,153	0,489	-0,404	0,171	0,176	0,425	0,260	-0,019
DRY SUBST.	0,407	-0,426	-0,343	0,512	0,093	0,296	0,294	-0,207	-0,025	-0,169
HUMIDITY	-0,398	0,410	0,323	-0,503	-0,092	-0,265	-0,264	0,197	0,014	0,148
NITRATES	-0,201	-0,433	-0,502	0,430	0,297	0,391	0,380	-0,020	-0,558	-0,631
NICHEL	0,439	0,317	0,654	-0,137	0,070	-0,405	-0,399	-0,247	0,854	0,826*

	GLUCOR	ASTRAG	GLUCOER	GLUCOS	PROT	FAT	FIBER	CARBS	DRY SUBST.	HUMIDITY	NITRATES	NICHEL
DRY_W	7,48E-01	9,95E-01	3,62E-02	4,62E-01	3,04E-01	7,00E-01	9,43E-01	3,93E-01	2,43E-01	2,55E-01	5,78E-01	2,04E-01
H2O	2,29E-02	4,04E-02	4,24E-02	2,87E-02	1,12E-01	7,49E-01	3,44E-01	1,88E-01	2,19E-01	2,39E-01	2,12E-01	3,73E-01
pH	2,44E-08	2,85E-09	6,05E-03	1,85E-06	1,78E-01	4,95E-01	8,25E-01	6,73E-01	3,32E-01	3,62E-01	1,39E-01	4,03E-02
BRIX	1,88E-03	6,94E-05	6,27E-02	1,01E-02	2,57E-01	3,90E-01	6,13E-01	1,52E-01	1,30E-01	1,39E-01	2,15E-01	7,07E-01
CONDUC	2,55E-02	3,42E-03	7,09E-01	6,30E-02	8,65E-01	9,09E-01	1,14E-01	2,47E-01	7,99E-01	8,01E-01	4,05E-01	8,48E-01
CHL_MEAN	1,27E-03	2,68E-03	1,22E-01	3,41E-03	3,20E-01	9,86E-01	7,79E-01	6,36E-01	4,06E-01	4,59E-01	2,64E-01	2,46E-01
API_MEAN	1,29E-03	2,60E-03	1,24E-01	3,53E-03	3,24E-01	9,89E-01	7,69E-01	6,26E-01	4,09E-01	4,61E-01	2,78E-01	2,54E-01
ISOQ	2,06E-01	1,98E-02	7,07E-01	3,53E-01	6,93E-01	6,51E-01	1,01E-01	2,21E-01	5,67E-01	5,85E-01	9,57E-01	4,92E-01
QUERC	6,10E-01	3,83E-01	5,99E-01	6,79E-01	3,80E-01	6,29E-01	7,29E-01	4,68E-01	9,46E-01	9,69E-01	9,38E-02	1,65E-03
ASC_AC	1,23E-01	1,16E-01	5,05E-01	1,93E-01	3,09E-01	6,42E-01	8,45E-01	9,59E-01	6,41E-01	6,83E-01	5,02E-02	3,23E-03
GLUCOR	0	1,72E-06	3,32E-03	5,18E-10	2,73E-01	9,75E-01	1,45E-01	3,37E-01	3,59E-01	4,20E-01	1,23E-01	1,77E-01
ASTRAG	0,927*	0	7,68E-03	8,51E-05	2,00E-01	5,13E-01	2,67E-03	3,06E-01	7,04E-02	7,67E-02	2,53E-01	5,93E-01
GLUCOER	0,725*	0,678	0	2,91E-03	3,82E-01	7,16E-01	5,80E-01	3,37E-01	3,78E-01	3,93E-01	9,35E-01	7,80E-01
GLUCOS	0,981*	0,858*	0,732*	0	3,48E-01	6,26E-01	1,78E-01	1,75E-01	5,05E-01	5,21E-01	5,86E-01	4,68E-01
PROT	0,384	-0,442	-0,311	-0,332	0	9,92E-02	6,68E-01	3,89E-01	9,10E-04	9,59E-04	1,93E-02	7,78E-01
FAT	-0,011	-0,235	-0,132	-0,177	0,550	0	7,13E-01	5,85E-02	4,50E-02	3,42E-02	3,19E-01	7,76E-01
FIBER	0,496	-0,835	0,200	0,462	0,156	-0,134	0	1,58E-02	3,88E-01	4,15E-01	7,31E-01	5,63E-01
CARBS	-0,340	0,361	-0,339	-0,466	0,306	0,615	-0,733	0	3,08E-01	2,83E-01	7,33E-01	9,61E-01
DRY SUBST.	0,325	-0,594	-0,313	-0,240	0,875	0,643	0,307	0,359	0	1,22E-11	7,88E-02	7,35E-01
HUMIDITY	-0,288	0,583	0,304	0,231	-0,873	-0,670	-0,291	-0,377	-0,999	0	8,30E-02	7,14E-01
NITRATES	0,521	-0,400	-0,030	-0,196	0,718*	0,352	0,125	0,124	0,580	-0,574	0	8,54E-02
NICHEL	-0,463	-0,193	0,102	0,260	-0,103	-0,104	0,209	0,018	0,123	-0,133	-0,570	0

2.4 CONCLUSIONS FOR BREEDING

All the surveys and methods used in breeding have led to the collection of many important data for rocket breeding.

The project had, as its initial objective, the setting up of a research and a breeding program on this crop at ISI Sementi. At the end of these three years, good results have been achieved in breeding, starting with the obtaining of two commercial varieties, named RB013 and RB015 (ISI code, not commercial name) obtained with mass and agronomic selection, as well as obtaining finer tools for selection within this species.

The research part on qualitative traits must certainly be implemented, setting up a specific research program on those characters considered important for the ready-to-use products and the post-harvest conditions.

CHAPTER 3 - MOLECULAR MARKERS IN ROCKET

3.1 INTRODUCTION

In the last decades, the discovery of molecular markers allowed to support the modern breeding through the application of Marker Assisted Selection (MAS), a powerful tool able to speed up the breeding process selecting DNA loci associated with the phenotypical variance of traits of agronomical value. Through specific markers (the most used are generally those associated with resistance to disease resistance traits and morphological traits) the breeder selects which plants to keep from one generation to the next.

Despite the widespread use of rocket in agronomy, biochemistry and pharmaceutical fields, only few DNA sequence information are available in *Diplotaxis* spp. and *Eruca sativa*. Instead, for other species of the Cruciferae family, genomes have been sequenced. Molecular markers have been developed in several species and they are used in modern breeding to develop new Cruciferae varieties more efficiently (Stockenhuber et al., 2015).

A high degree of synteny is known to exist between closely related species in Brassicaceae, allowing the cross transferability of markers among the species. Comparative genetic studies within the Brassicaceae family have revealed significant conservation of genes and markers representing an advantage in *Diplotaxis tenuifolia* research (Atri et al. 2016). These studies allowed the identification of putative orthologous loci controlling agronomic traits, and permitted the extension of genetic information from model species to more complicated species. SSR information is publicly available for *Brassica napus* L., *B. nigra* (L.) Koch*, B. oleracea* L., and *B. rapa* L.

3.2 OBJECTIVES OF THE STUDY

The objective of the present study on molecular markers in *Diplotaxis* spp. and *Eruca sativa* was to explore ISI Sementi genetic material by testing sequences from other species of the Brassicaceae family exploiting sinteny and testing sequences designed on *Diplotaxis tenuifolia*. The use of molecular markers for the identification of effective F1 is of great importance in the breeding of this species, where there is not much molecular information available.

3.3 MATERIALS AND METHODS

In 2020, the full genome sequence of *Diplotaxis tenuifolia* was not available. To begin exploring the genetics of this species, the study on rocket genetics started by using cauliflower markers and then using markers designed on gene sequences deposited on NCBI. These markers have been tested on the germplasm in possession of ISI Sementi for breeding. In the summer of 2020, ad hoc crossings have been planned for the control and the study of ISI molecular markers on rocket populations. In 2021, other sequences have been used for testing germplasm (Taylor, 2015) to confirm the efficiency of ad hoc crosses.

3.3.1 DNA extraction method

DNA extraction of fresh leaves samples was carried out using Chelex® 100 Resin (Bio-Rad Laboratories, Hercules, CA, USA). Chelex® is an ion exchange chelating resin which bind divalent ions such as magnesium and polar cellular components, leaving the supernatant containing the purest DNA.

For the extraction a small portion of a leaf, 150 µl of 5% Chelex® 100 Resin (Bio-Rad) and a single ball of lead to help with the grinding were placed in a 1.2 ml tube (96-tube sample plate). The vegetable tissue was grinded for 10 minutes by mechanical stirring. After stirring, 80 µl of extract was transferred to a new 96-well plate and placed in the Euroclone® thermal cycler at 98 ° C for 3 minutes and stirred on a Hamilton Microlab StarLET shaker for a few minutes. The plate was placed in a Thermo Scientific ™ Heraeus ™ Labofuge ™ 400 centrifuge at 3000 rpm (1300 g) for 3 minutes. The final extract obtained was diluted and used as a template DNA for PCR.

3.3.2 Manual cross method

Rocket is an allogamous plant with the presence of self-incompatibility systems, therefore cross pollination is highly favored. For this reason, it is necessary to prevent the intrusion of pollinating insects that can carry alien pollen after the opening of the flowers. In fact, rocket pollination is mainly entomophilous. The cross pollinations were made in a temperature-controlled greenhouse (temperatures between 25-28° C). Emasculation of female plant was obtained with the manual removal of the anthers using tweezers. At the same time the pollen of the young flowers was collected from the male plants as soon as it was produced and placed on the most exposed part of the female stylus.

If crosses are performed in a greenhouse or in a tunnel protected by an anti-insect net, it is not necessary to cover the flower with a bag. Alternatively, the pollinated flower must be covered at least for the first 24 hours with a paper bag to prevent the deposit of external pollen by pollinator insects.

In this phase, it is important to ensure an optimal humidity level (around 60-70%), to favor fertilization. Higher humidity (>70%) wets pollen and makes it inactive, very high and dry temperatures lead to tissue death or pollen melting.

During spring (the most favorable season for the reproduction of the species), the formation of the embryo takes place in 21-28 days after flower setting.

The crossing scheme (female and male parents are reported in Tab.24) was constituted to obtain different combinations and to observe the biggest variability in the F1 and to verify the applicability of the molecular markers designed on NCBI sequences.

Table 24 Combination scheme for crossings in 2020

FEMALE	MALE
MARTE	CAPTAIN
MARTE	GRAZIA
MARTE	TRICIA
MARTE	EXTREMA
MARTE	ATLANTA
MARTE	DALLAS
CAPTAIN	LETIZIA
GRAZIA	LETIZIA
TRICIA	LETIZIA
CAPTAIN	BELLEZIA
GRAZIA	BELLEZIA
TRICIA	BELLEZIA
TRICIA	F1: LETIZIA X MARTE
GRAZIA	F1: LETIZIA X MARTE
CAPTAIN	F1: LETIZIA X MARTE

3.3.3 Development of molecular markers

A first screening of *Diplotaxis* tenuifolia germplasm has been performed using 27 molecular markers already developed for cauliflower, exploiting the cross-transferability within Brassica

species (Tab.25). Moreover, 12 new primers pair have been drawn on specific gene sequences of *Diplotaxis tenuifolia* published on the NCBI database and tested on the same germplasm.

Once identified the sequence of interest, microsatellite finder tool has been used to detect the repeats of short nucleotide motifs. According to the motifs position, primer pairs have been successively designed with Primer3 online software. In the end, using the Oligocalc online tool (http://biotools.nubic.northwestern.edu/OligoCalc.html), the optimal parameters for each primer was verified.

The approach to molecular analysis involved two main steps: a preliminary test on specific crosses for the technical optimization and a second screening on the germplasm aimed at verifying the stability.

With the aim of determining the efficiency of crosses and technically testing the markers, specific crossings with specific combinations have been foreseen. All the single plants of the commercial varieties used for crosses were tested with different markers to obtain the corresponding genetic profile and to detect the presence of polymorphisms. After the identification of the polymorphisms, the plants were labeled and divided based on genotypes obtained from the markers, and then used to create F1 by manual crossing.

After crossings and harvest of the resultant seeds, F1 were also sampled and analyzed to evidence the real hybrid nature. The available molecular markers were used to confirm controlled crosses that were produced in the frame of the activity.

Table 25 Molecular markers tested in Diplotaxis tenuifolia in the current study with bibliography (pag.65-67).

GenBank (NCBI) accession /gene	Marker designation	Annealing temperature (°C) OligoCalc Salt adjusted	Origin	Bibliography
Rapa	Ra2E12_Rv	60,9	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Rapa	Ra2E12_Fw	60,5	SSR	
Oleracea	Ol10C05_Fw	60,9	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol10C05_Rv	60,1	SSR	
Oleracea	Ol12F08AF	56,4	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol12F08AR	54,3	SSR	
Napus	Na14H12_Rv	57,5	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Napus	Na14H12_Fw	58,4	SSR	
Oleracea	FITO498F	59,5	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	FITO498R	53,4	SSR	
Oleracea	FITO255F	53,8	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	FITO255R	52,3	SSR	
Oleracea	FITO361F	57,5	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	FITO361R	53	SSR	
Oleracea	pW143F	67,4	SSR	Tonu et al 2013
Oleracea	pW143R	63,2	SSR	
Oleracea	Ol10F11F	58,4	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol10F11R	60,5	SSR	
Oleracea	Ol12E03_Fw	60,5	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol12E03_Rv	62,5	SSR	
Napus	Na12D03_Rv	58,4	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Napus	Na12D03_Fw	58,4	SSR	
Oleracea	CB10139_F	55,4	SSR	pgdb doi: 10.1111/j.1439-0523.2011.01904.x ; doi: 10.1007/s00122-005-0080-6
Oleracea	CB10139_R	57,5	SSR	
Oleracea	PBCGSSRBo39_Fw	58,4	SSR	Branca et al 2017
Oleracea	PBCGSSRBo39_Rv	58,4	SSR	
AF241115	BoCALa	53,4	SSR	Tonguç and Griffiths 2004

		56,4	SSR	
Oleracea	Ol12F03_Rv	62,5	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol12F03_Fw	60,5	SSR	
Oleracea	KBrS003010N1_F	58,4	SSR	pgdb doi:10.1007/s11032-011-9665-8
Oleracea	KBrS003010N1_R	60,1	SSR	
Oleracea	V17_F	57,5	indel	pgdb doi: 10.1186/1471-2164-15-1094
Oleracea	V17_R	54,3	indel	
Oleracea	BnGMS301F	54,7	SSR	Afrin et al 2018
Oleracea	BnGMS301R	54,3	SSR	
Oleracea	BoGMS0971F	49,3	SSR	Afrin et al 2018
Oleracea	BoGMS0971R	53,8	SSR	
Oleracea	Ol10B06F	58,4	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol10B06R	62	SSR	
Oleracea	Ol10F11F	58,4	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol10F11R	60,5	SSR	
Napus	Na12B09_Fw	58,4	SSR	Astarini, 2008
Napus	Na12B09_Rv	58,4	SSR	
Oleracea	Bo_301522F1	60,9	SLAF	Zhao, 2016
Oleracea	Bo_301522R2	58,4	SLAF	
Oleracea	Ol10F07_Rv	58,4	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol10F07_Fw	57,5	SSR	
Oleracea	Ol12A04_Rv	61,5	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Oleracea	Ol12A04_Fw	61,2	SSR	
Oleracea	BoIAB94TF	57,5	gss	Louarn et al 2007
		56,3	gss	
Napus	Na10D03_Fw	54,3	SSR	BBSRC_ https://www.brassica.info/resource/markers/ssr-exchange.php
Napus	Na10D03_Rv	62,5	SSR	
Oleracea	A1_F	56,4	indel	pgdb doi: 10.1007/s11032-013-9925-x ; doi: 10.1186/1471-2164-15-1094
Oleracea	A1_R	54,3	indel	
Diplotaxis tenuifolia	Dt_FTSZ1-1_F	56,4	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FTSZ1-1_R	60,5	SSR	designed on NCBI sequences (Stockenhuber)

Diplotaxis tenuifolia	Dt_FTSZ1-1_F1	60,5	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FTSZ1-1_R1	58,4	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FTSZ1-1_F2	57,5	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FTSZ1-1_R2	62,1	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_XTH32_F	58,4	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_XTH32_R	59,5	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FERONIA_F	60,5	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FERONIA_R	60,5	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FERONIA_F1	58,4	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FERONIA_R1	58,4	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_SAHH1_F	58,4	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_SAHH1_R	60,5	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_SAHH1_F1	58,4	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_SAHH1_R1	58,4	SSR	designed on NCBI sequences (Stockenhuber)
Diplotaxis tenuifolia	Dt_FLC_F2	59	SSR	designed on sequences (Tylor 2015)
Diplotaxis tenuifolia	Dt_FLC_R2	59	SSR	designed on sequences (Tylor 2015)
Diplotaxis tenuifolia	Dt_FLC_F3	59	SSR	designed on sequences (Tylor 2015)
Diplotaxis tenuifolia	Dt_FLC_R3	59	SSR	designed on sequences (Tylor 2015)
Diplotaxis tenuifolia	Dt_GI_F	59	SSR	designed on sequences (Tylor 2015)
Diplotaxis tenuifolia	Dt_GI_R	59	SSR	designed on sequences (Tylor 2015)
Diplotaxis tenuifolia	Dt_TGD1_F	59	SSR	designed on sequences (Tylor 2015)
Diplotaxis tenuifolia	Dt_TGD1_R	59	SSR	designed on sequences (Tylor 2015)

3.3.4 Real-time PCR and HRM analysis

All the DNA analyses have been performed using High-Resolution Melting analysis (HRM), a post-polymerase chain reaction (PCR) method developed for the detection of genetic variants in nucleic acid sequences. The reaction mix for each sample is described in Tab.26. The mix was prepared and placed in the PCR Plate Spinner centrifuge for a few seconds and finally placed in the CFX Connect RealTime PCR thermal cycler Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The amplification conditions have been fine-tuned optimizing the number of cycles and the pairing temperature of primers. The amplification protocol had an initial cycle denaturation at 98 ° C for 3 minutes, which are followed 39-50 cycles in which two steps alternated:

- denaturation at 98 ° C for 5 seconds;

- pairing and amplification at 52-62°C (depending on the primer pairing temperature) for 30 seconds.

The amount of fluorescence for each sample, given from the incorporation of EvaGreen® into double-stranded DNA, was measured at the end of each amplification cycle and analyzed with the CFX-Manager Software v3.1 (Bio-Rad Laboratories, Inc.). The melting curve of the amplicons was obtained with temperatures between 55 ° C and 94 ° C. The data acquisition was performed every 0.2 ° C of temperature increase with one step 10 seconds. The samples were analyzed through the software Precision Melt Analysis ™ (Bio-Rad), which groups automatically samples based on their profile denaturation and assigns a confidence value to each sample.

Table 26 Reaction mix for single well

Reaction mix	**Vol. in each well**
SsoFastTM EvaGreen® Supermix (2x)	4,09 µl
Primer F (10 µM)	0,27 µl
Primer R (10 µM)	0,27 µl
Distilled water	3,37 µl
DNA	1 µl
Final volume	9 µl

3.4 RESULTS AND DISCUSSION

3.4.1 Manual cross method

The manual method of crossing developed and based on the literature in other related species, led to the formation of seed in 90% of performed crosses.

The problems occurred during the development of crossing technique were related to:

- the full isolation of plants with an insect net after pollination

- the moment of pollen collection and pollination of female part of flowers

In fact, crossings may not occur for different climatic variation or for execution errors.

It was less simple to obtain a F2 offspring because F1 plants do not self-pollinate due to the self-incompatibility mechanism. For this reason, multiple F1 sister plants were used to intercross (using pollinating insects) and obtain the F2 generation from sib-plants. This constitutes a further obstacle to obtain a commercial hybrid because the stabilization of lines is more complex due to allogamy.

3.4.2 Molecular markers analyses results

Molecular markers developed for cauliflower in ISI Sementi, did not produce clear and usable results in *Diplotaxis tenuifolia*. Out of 27 molecular markers from other *Brassica* species tested on rocket, 20 did not amplified on ISI Sementi accessions genetic backgrounds, so they were discarded; within, 7 were monomorphic among studied accessions.

A second screening have been done using SSR markers designed on specific *Diplotaxis tenuifolia* sequences and these shown readable output after HRM analysis. Out of 12 markers designed on *Diplotaxis tenuifolia* from NCBI accessions, only 3 gave distinct polymorphisms among analyzed accessions and they were used for this study. Moreover, Dt_FERONIA_F1/R1 markers have been discarded for technical reasons, Dt_FTSZ1.1 F1R1 was firstly used in the preliminary test for the determination of crosses efficiency and only in a second moment Dt_FLC_F2/R2 have been developed and used as an alternative marker for quality control. The results of the molecular markers analysis are summarized in Tab.27 below.

Table 27 Amplification and polymorphism obtained on rocket (Diplotaxis tenuifolia)

Marker name	Origin	Amplification		Polimorphic on rocket
		Diplotaxis tenuifolia	***Brassica oleracea***	
Ra2E12_F/R	*B.rapa*	No	Yes	No
Ol10C05_F/R	*B.oleracea*	No	Yes	No
Ol12F08AF/R	*B.oleracea*	No	Yes	No
Na14H12_F/R	*B.napus*	No	Yes	No
FITO498F/R	*B.oleracea*	No	Yes	No
FITO255F/R	*B.oleracea*	No	Yes	No
FITO361F/R	*B.oleracea*	No	Yes	No
pW143F/R	*B.oleracea*	No	Yes	No
Ol10F11F/R	*B.oleracea*	No	Yes	No
Ol12E03_F/R	*B.oleracea*	No	Yes	No
Na12D03_F/R	*B.napus*	No	Yes	No
CB10139_F/R	*B.oleracea*	No	Yes	No
PBCGSSRBo39_F/R	*B.oleracea*	No	Yes	No
BoCALa	*B.oleracea*	Yes	Yes	not readable
Ol12F03_F/R	*B.oleracea*	No	Yes	No
KBrS003010N1_F/R	*B.oleracea*	No	Yes	No
V17_F/R	*B.oleracea*	No	Yes	No
BnGMS301F/R	*B.oleracea*	No	Yes	No
BoGMS0971F/R	*B.oleracea*	No	Yes	No
Ol10B06F/R	*B.oleracea*	No	Yes	No
Ol10F11F/R	*B.oleracea*	No	Yes	No
Na12B09_F/R	*B.napus*	No	Yes	No
Bo_301522F1/R1	*B.oleracea*	Yes	Yes	not readable
Ol10F07_F/R	*B.oleracea*	Yes	Yes	No
Ol12A04_F/R	*B.oleracea*	Yes	yes	No
BoIAB94TF/R	*B.oleracea*	Yes	yes	not readable
Na10D03_F/R	*B.napus*	Yes	yes	not readable
A1_F/R	*B.oleracea*	Yes	yes	No
BoGMS1042_F/R	*B.oleracea*	Yes	yes	No
Dt_FTSZ1-1_F/R	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	No
Dt_FTSZ1-1_F1/R1	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	**Yes**
Dt_FTSZ1-1_F2/R2	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	No
Dt_XTH32_F/R	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	No
Dt_FERONIA_F/R	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	No
Dt_FERONIA_F1/R1	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	**yes, cluster not always clear**
Dt_SAHH1_F/R	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	No
Dt_SAHH1_F1/R1	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	No
Dt_FLC_F2/R2	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	**Yes**
Dt_FLC_F3 /R3	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	not readable
Dt_GI_F/R	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	not readable
Dt_TGD1_F/R	NCBI seq *Diplotaxis tenuifolia*	Yes	ND	not readable

3.4.3 Validation of crosses

The single parental plants used were selected on the basis of their genotypes for marker Dt_FTSZ1.1 F1R1 (AA, BB and the heterozygote genotype AB) to verify in the resultant F1 the hybridization. Melt curve with the distinct clusters is shown in Fig.12. Initially there were 100-200 plants for each line, to increase the probability of finding all the polymorphisms that the marker Dt_FTSZ1-1_R1 detected and to obtain different combinations and data. Based on these results, for each variety some single plants were selected to be used as parental plants for the subsequent crosses.

From the analysis with this marker, plants with different haplotypes were distinguished for each variety used for the crosses. In Tab.28 below parental combinations and distinct clusters are reported.

Figure 13 Melt curve with marker Dt_FSTZ1-1_F1R1 used for hybridization check

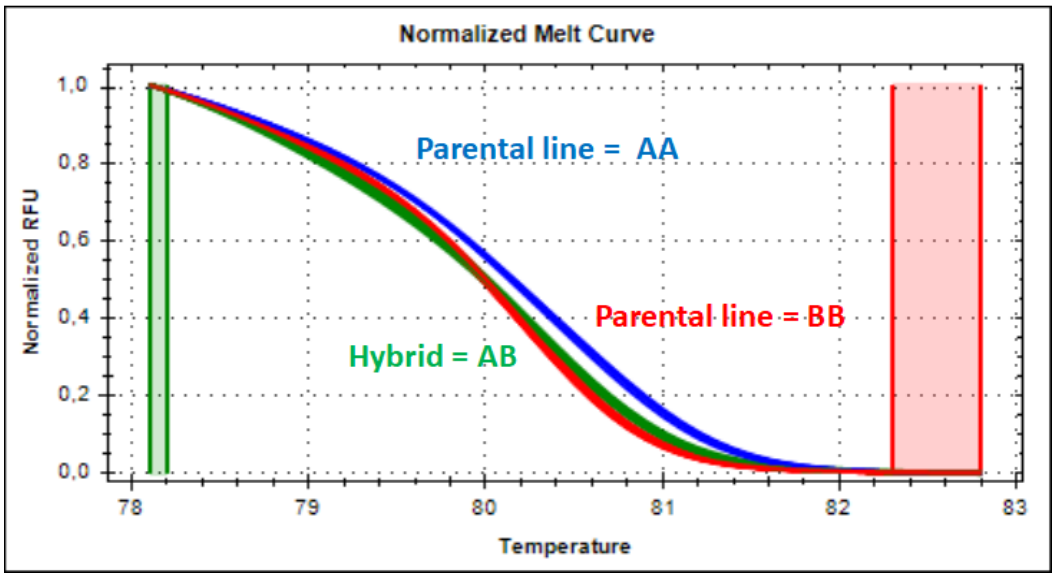


Table 28 List of the crossing combinations and relative genotypes obtained from the analysis with marker Dt_FSTZ1-1_F1R1

FEMALE	CLUSTER	MALE	CLUSTER
MARTE	AA	CAPTAIN	AA
MARTE	AA	CAPTAIN	AB
MARTE	AA	CAPTAIN	BB
MARTE	BB	CAPTAIN	AA
MARTE	BB	CAPTAIN	AB
MARTE	AA	CAPTAIN	BB
MARTE	BB	GRAZIA	AA
MARTE	BB	TRICIA	AA
MARTE	AA	EXTREMA	AA
MARTE	AA	ATLANTA	AA
MARTE	AA	DALLAS	AA
MARTE	AA	DALLAS	AB
MARTE	BB	DALLAS	AA
MARTE	BB	DALLAS	AB
CAPTAIN	AA	LETIZIA	AA
CAPTAIN	AA	LETIZIA	AB
CAPTAIN	AA	LETIZIA	BB
CAPTAIN	BB	LETIZIA	AA
CAPTAIN	BB	LETIZIA	AB
CAPTAIN	BB	LETIZIA	BB
GRAZIA	AA	LETIZIA	BB
TRICIA	AA	LETIZIA	BB
CAPTAIN	AA	BELLEZIA	AA
GRAZIA	AA	BELLEZIA	AA
TRICIA	AA	BELLEZIA	AA
TRICIA	AA	LETIZIA X MARTE F1	AB
GRAZIA	AA	LETIZIA X MARTE F1	AB
CAPTAIN	BB	LETIZIA X MARTE F1	AB

F1 were created with manual crosses between the selected parental plants, they were bagged and then sowed in trays. At the stage of second leaf, they were sampled and analyzed.

The marker allowed to discriminate the supposed F1: out of 37 crosses produced by manual pollination, 6 were excluded because resulted with clusters not present in their parental plants (probably there was contamination by pollen from other plants) and the remaining 31 validated (Tab.29)

Table 29 Cross harvested and analyzed with the molecular marker to check the success of the crossing

CROSS NAME	FEMALE	♀	MALE	♂	F1 expected (%)	F1 observed (%)
RK20004	Marte	BB	Grazia	AA	100 AB	100 AB
RK20008	Marte	AA	Atlanta	AA	100 AA	100 AA
RK20010	Marte	AA	Extrema	AA	100 AA	100 AA
RK20012	Marte	BB	Grazia	AA	100 AB	100 AB
RK20013	Marte	BB	Dallas	AA	100 AB	100 AB
RK20021	Marte	AA	Captain	BB	100 AB	90 AB, 10 AA
RK20023	Marte	AA	Captain	BB	100 AB	100 AB
RK20035	Marte	BB	Grazia	AA	100 AB	90 AB, 10 BB
RK20036	Marte	BB	Captain	AA	100 AB	100 AB
RK20038	Captain	AA	Letizia	BB	100 AB	100 AB
RK20041	Captain	AA	Letizia	AA	100 AA	100 AB
RK20042	Captain	AA	Letizia	BB	100 AB	100 AB
RK20046	Captain	AA	Letizia	BB	100 AB	100 AB
RK20048	Captain	BB	F1 (Letizia x Marte)	AB	50 AB, 50 BB	50 AB, 50 BB
RK20051	Captain	AA	Letizia	BB	100 AB	100 AB
RK20052	Captain	BB	Letizia	AA	100 AB	100 AB
RK20058	Captain	AA	Letizia	BB	100 AB	50 AB, 50 AA
RK20060	Captain	BB	Letizia	AA	100 AB	100 AB
RK20061	Grazia	AA	Letizia	BB	100 AB	100 AB
RK20063	Grazia	AA	Letizia	BB	100 AB	100 AB
RK20067	Grazia	AA	Bellezia	AA	100 AA	100 AA
RK20079	Grazia	AA	F1 (Letizia x Marte)	AB	50 AB, 50 AA	50 AB, 50 AA
RK20087	Tricia	AA	Bellezia	AA	100 AA	100 AA
RK20089	Captain	BB	F1 (Letizia x Marte)	AB	50 AB, 50 BB	50 AB, 50 BB
RK20090	Grazia	AA	F1 (Letizia x Marte)	AB	50 AB, 50 AA	50 AB, 50 AA
RK20099	Marte	BB	Dallas	AA	100 AB	100 AB
RK20100	Marte	BB	Dallas	AA	100 AB	100 AB
RK20107	Grazia	AA	Letizia	BB	100 AB	90 AB, 10 AA
RK20111	Captain	BB	Letizia	AA	100 AB	90 AB, 10 BB
RK20112	Captain	BB	Letizia	AA	100 AB	100 BB
RK20113	Captain	BB	Letizia	AA	100 AB	100 AB
RK20117	Tricia	AA	letizia	BB	100 AB	100 AB
RK20118	Tricia	AA	letizia	BB	100 AB	100 AB
RK20119	Tricia	AA	F1 (Letizia x Marte)	AB	50 AB, 50 AA	50 AB, 50 AA
RK20133	Marte	BB	Captain	AA	100 AB	90 AB, 10 BB
RK20135	Marte	AA	Captain	BB	100 AB	66.6 AB, 33.3 AA
RK20141	Marte	BB	Tricia	AA	100 AB	100 AB
RK20144	Marte	BB	Dallas	AA	100 AB	100 AB
RK20153	Marte	AA	Captain	BB	100 AB	58.3 AA, 41.6 AB
RK20154	Marte	AA	Captain	BB	100 AB	41.6 AA, 58.3 AB
RK20157	Marte	AA	Captain	AA	100 AA	90 AA, 10 AB
RK20161	Marte	BB	Captain	AA	100 AB	100 AB

3.5 Conclusions

Starting from a poorly supplied literature on the molecular biology of rocket and the few tools available in ISI to understand rocket genome, some progress has been made during the project.

The cauliflower molecular markers available did not produce measurable results .The publication of specific sequences of *Diplotaxis tenuifolia* constituted a turning point in the genetic study of the crop. The analysis carried out allowed to identify 3 molecular markers useful and applicable in breeding on rocket, e.g. to identify the successful pollination and the formation of F1.

In future it will be interesting to further explore the rocket markers associated with some traits useful for breeding, as plant pathologies or qualitative traits of the crop.

CHAPTER 4- SELF INCOMPATIBILITY AND MALE STERILITY INTROGRESSION

4.1. INTRODUCTION

For allogamous plants, methods for hybrids creation are different. The creation of pure lines is necessary for the constitution of classical hybrids, although it's not always easy or feasible in allogamous plants, because of their biology that naturally favored outcrossing.

In rocket, creating pure homozygous lines is present limits like the presence of self-incompatibility barriers, which have been identified in *Diplotaxis tenuifolia* and *Eruca sativa*. Besides, there are other *Diplotaxis* species (*Diplotaxis muralis*, *Diplotaxis erucoides*….) (Sharma et al., 2014; Vyas et al., 1995a) that self-fertilize and produce seeds. Self-incompatibility found in rocket is sporophytic, like in other species of the Brassicaceae family, and probably involves a high number of alleles.

For these reasons, is not possible to use pure self-fertilized lines in the constitution of hybrids. Aminul Islam et al. in Brassica breeding and biotechnology (2021) shows other ways to create hybrids, for example population-by-population, starting from local varieties or varieties that have undergone stages of genetic improvement. An advantage of this type of hybrid is the broad genetic basis, but with limited expression of heterosis . This hybrid does not correspond to the technical definition of a uniform F1 generation coming from the crossing of two lines in homozygosity.

In recent years, modern breeding explored different techniques for the constitution of hybrids, for example the self-incompatibility systems present in some species. Self-incompatibility is already present in a high number of cultivated species (and in their related wild types) to prevent self-pollination and the loss of genetic variability. In self-incompatible plants, for morphological or genetic reasons, pollen of the same plant is not recognized from the female part of the flower.

It is possible to create hybrids starting from two self-incompatible but inter-compatible parental varieties. In this case, hybrid seeds are collected by both parents. This technique can be used after careful research on self-incompatibility systems, which must be stable and uniform in the parental line..

Other systems broadly known are cytoplasmatic and nuclear male sterility. If present in the specie, plants are not able to produce fertile pollen (therefore they can only act as mother plants). This character can be naturally present in a species (as a result of a mutation) or introduced through

repeated crosses with a sterile wild relative in an originally fertile parental line. (Sharma et al., 2014; Vyas et al., 1995b)

4.1.1 Self-incompatibility: the background

During pollination, pollen grains reach the stigma, where they are recognized by pistil tissues. In here, they stick to the stigma papillae where they receive the hydration needed to germinate: this process leads to the development of the pollen tube. This one grows into the stile and the fertilization is permitted.

The self-incompatibility system (SI) prevents the fertilization with incompatible pollen, produced by the same plant or related plants, inhibiting the germination of pollen tube in the stile.

SI is controlled by genetic factors. The male and female floral organs are perfectly viable but not able to form the embryo, due to morphological and physiological obstacles.

This incompatibility system permits only the cross pollination and can act also with different genotypes that carry the same genetic SI information. The Self Incompatibility in the allogamous species is the base of genetic variability and adaptability.

Genetic bases of SI have been studied through appropriate cross combinations. Results led to the identification of a multiallelic locus (called locus S in *Brassica* species) responsible of self-incompatibility. It is believed that locus S has a size of 20 to 400 Kb, depending on the genotype and alleles involved at this locus. (Muñoz-Sanz et al., 2020a)

Pollen and pistil have the incompatibility reaction when they carry the same alleles in locus S. All the Self Incompatibility (SI) systems provide an auto-reaction, resulting in an unsuccessful fertilization and zygote formation. According to the mode of action, SI can be gametophytic or sporophytic. (Barcaccia, Genetica e Genomica)

In gametophytic systems, SI reaction is under the control of locus S genes, that can present themselves in numerous series without dominance or recessivity relationship.

Sporophytic systems can be distinguished in eteromorphic, if there are morphologic differences in different phenotypes, and homomorphic, if there are no morphologic differences and a monofactorial control with multiallelic series.

In homomorphic systems, SI is based on the mutual recognition of two glycoprotein molecules: one produced by the stigma and the other by pollen (gametophytic type) or by the pollinating

plant (sporophytic type) similarly to immune control in animals. The hypothesis is that after pollination, in case of self-incompatibility, the glycoproteins produced in the pollen tube can combine with those produced in the stylus: this process forms a dimeric protein that acts as a repressor that prevents the transcription of the DNA of the male gametophyte and therefore the action of RNA polymerase.

4.1.2 Male sterility and its use in breeding

Male sterility is the production of not functional male gametes or the missed production of male gametes. Sterility can be under nuclear the control of a single gene in the recessive status, as recently found in radish (Banga et al., 2003; Sharma et al., 2014) or under cytoplasmatic control. This second case, often found in *Brassica* species, is controlled by mitochondrial genes and therefore transmitted by the female parent in successive generations. In nature female sterility is also present, but it has not been found in Cruciferous family.

In species where nuclear or cytoplasmic male sterility is present, this trait can be exploited in the creation of hybrids. There are botanical families in which the presence of sterile male genotypes has been frequently found and exploited in breeding. In modern breeding male sterility has also been transferred from one species to another (fertile variety) through interspecific or intergeneric crosses. Interspecific and intergeneric crosses often present many pre and post zygotic fertility barriers: to overcome these barriers, a widely used laboratory method is the *embryo rescue* technique, that helps the growth of the embryos formed using specific medium. (Inomata, 2005a)

4.1.3 Embryo rescue technique

In modern breeding interspecific, and sometimes intergeneric, crossings are often used to insert specific characters between one species and another. The most classic example is the introgression of male sterility for the creation of parental lines for hybrids. If very different species are used, successive back crosses are necessary to approach the desired commercial phenotype.

Interspecific crossings are not always easy to implement: sometimes breeders need to use a bridge species, more similar to the commercial one but more compatible with the donor species of the characters of sterility or resistance, or to use *vitro* technique to save the formed embryo from the abortion and stimulate the growth of a normal seedling. (Agnihotri et al., 1990; Banga et al., 2003)

One of this technique is called *embryo rescue*, that is an in vitro-culture technique used to save weak, immature and hybrid or sometimes inviable embryos to prevents its degradation. The

procedure involves excising weak, immature plant embryos and culture them on specially devised culture medium. It plays an important role in plant breeding of important crop plants. In *Brassica*, the interspecific and intergeneric hybridization was done from very earlier breeding because the crop yield losses due to disease, biotic and abiotic stresses is very high. The earlier attempts were made in Chinese cabbage varieties i.e. *B. oleracea* and *B. campestris*. The hybrid embryos were carefully removed and cultured on the nutrient medium in vitro.

Interspecific, intergeneric and inter varietal hybrids have been generated in mango, banana, seedless grape, papaya and seedless citrus using embryo rescue.

Different *embryo rescue* technique *in vivo* are possible. In *Brassica* the best adopted methods for the embryo rescue are the direct ovule culture, siliqua culture and immature embryos culture.

In siliqua culture young fertilized pistils are excised 4 to 6 days after pollination and cultured on the MS medium. It absorbed the medium start to grow gradually but regular subculturing is required. The swollen pistil is again excised to dissect out developing ovule.

A second way is to culture ovules in a similar manner. The choose of the right day after pollination in which the ovules has to be cultured, may varies from plant to plant. To identify the fertilized pistil, pollinated pistil fixed in 70% ethanol 24 to 48 hour after pollination and stained in aniline blue, the pollen germination and pollen tube growth may be observed under the microscope.

For immature embryos culture, the siliqua formed is collected after 10, 15, 20, 25 or 30 days after pollination, depending on the species and the time needed for immature seeds development. In the embryo culture, the siliques are sterilized in ethanol and NaOH and ovules are dissected out from siliques. The young embryos are then cultured on MS medium to stimulate the formation of a plantlet.

Another possibility is ovary culture, also practiced in some *Brassica* species, can be done 4 to 14 days after pollination. The stalk of the ovary has to be cut from the base and cultured on the nutrient medium to induce development of the embryo.

All these methods of *embryo rescue* (Agnihotri et al., 1990; Inomata, 2005b; Vyas et al., 1995b) depend on the type and the condition of the hybrid embryos and the type of hybridization experiments.

4.1.4 Systems preventing self-pollination found in the Cruciferae and in rocket species

In Cruciferous family (which *Diplotaxis* spp. and *Eruca sativa* belong to) different genetic systems can be found to prevent the loss of genetic variability. (Sharma et al., 2014)

In this botanical family, there are generally high frequencies of allogamy (equal to 90%) and mainly entomophilic pollination. Moreover, in several species (*Brassica, Raphanus, Arabidospis*) (Barcaccia, Genetica e Genomica), breeders have found male sterility systems (citoplasmatic) and self-incompatibility barriers to self-fertilization.

The sporophytic homomorphic system has been observed in several *Brassica* species (*Brassica oleracea, Brassica campestris, Raphanus sativus* for example). Number of alleles can be very high and in certain botanical families, like Brassicaceae and Asteraceae, show dominance relations and are very rare, in order to maintain in populations a balance between Self-Incompatible genotypes and Self-Compatible ones. (Barcaccia, Genetica e Genomica)

In literature there are studies and examples of self-incompatibility in rocket. (Muñoz-Sanz et al., 2020b)studied a population of *Diplotaxis erucoides*, showing that inflorescences bagged in the field or greenhouse, generally set few or no seeds. Experimental hand pollination (Kunin 1991) verifies that these plants require cross-pollination to set seed. In another experiment, *Diplotaxis euroides* flowers were observed presence and absence of pollinating insects. Fully pollinated flowers pollinated by insect developed siliquae with up to 60 small seeds (average= 0.23 mg). Unpollinated flowers formed small, empty siliquae which are easily distinguished from aborted buds. These studies explained the importance of cross pollination in *Diplotaxis erucoides*.

A publication of 2017 by Williams et al. investigated the usefulness of floral characters as a potential indicator of breeding system in several species of Brassicaceae. The results of the pod set tests, clearly differentiated between self-compatible and self-incompatible species. For *Diplotaxis tenuifolia* and *Diplotaxis tenuisiliqua* the breeding classification was Self Incompatible (SI), for *Diplotaxis muralis* was Self Compatible (SC) Also results of a second experiment for pollen tube growth, confirmed the results of the bagging experiment. In *Diplotaxis tenuifolia* self-pollinated plants, pollen grains germination was retarded or with small number of germinating pollen grains. In cross pollinated plants, both pollen grain, germination and pollen tube growth was normal, with a large number of fertilising ovules and germinating pollen grains.

4.2 OBJECTIVES OF THE EXPERIMENT

Given that in literature there are several studies that indicate the presence of self-incompatibility in rocket, this has been exploited during the phD project in an experiment carried out using accessions available at ISI Sementi, with isolation and bagging of plants.

Self-incompatibility, as mentioned before, is a complicated character to be used in breeding and constitution of hybrid lines. For this reason, another aim of the project was to try to introgress male sterility from other related species of the Cruciferae family through interspecific crosses and *embryo rescue* technique to save the embryo formed.

All these attempts were done in order to find a usable way to develop hybrids in rocket, and this was one general goal of the breeding project.

To sum up, the goals of the two experiments were:

- To verify the presence of Self-Incompatibility on *Diplotaxis tenuifolia* accessions;
- To cross *Eruca sativa* and *Diplotaxis tenuifolia* with a male-sterile *Brassica oleracea* in order to introgress cytoplasmatic male sterile character for subsequent breeding use, through *embryo rescue* technique *in vitro* to facilitate the formation of the interspecific seedling.

4.3 . MATERIALS AND METHODS

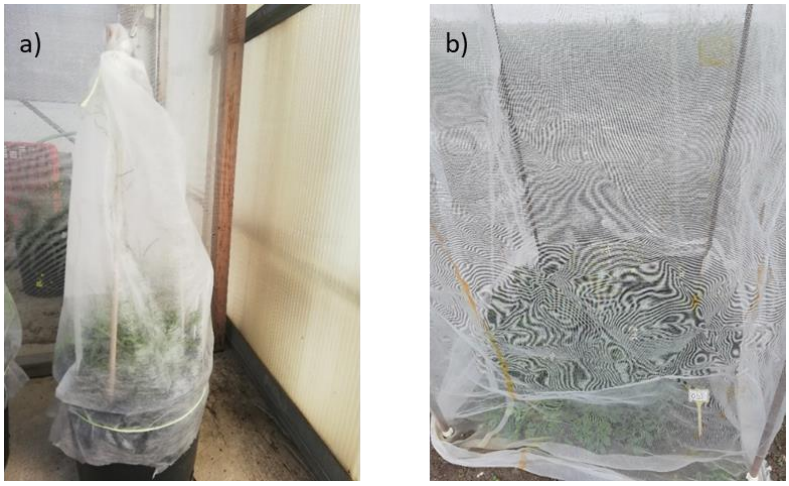
4.3.1 Plant material used for self-incompatibility study

The conditions for the experiment are summarized in Tab.30 below. Insect pollination has been performed with subsequent launches of pollinating insects (flies produced by the company *Bioplanet*). Plants were put under cages protected with tightly meshed insect net to avoid unwanted pollinators and maintaining flowers isolated for all the duration of flowering (Fig.13). Four launches (one per week) were made during flowering time in the cages at the larval stage, and in 24-48 hours the pupae turned into adults being able to pollinate flowers.

Table 30 Plant material and conditions for the self-incompatibility study in rocket

Plant material tested	Conditions studied
ISI 1 sib, ISI 2 sib, ISI 3 sib	Insect pollination, isolation with net
Single plant ISI 1, ISI 2, ISI 3	Insect pollination, isolation with net
Clones of single genotype (ISI 1, ISI 2, ISI 3)	Insect pollination, isolation with net

Figure 14 a)single plant in anti-insect net for self-incompatibility validation; b)cage with different plants used in field or in greenhouse for isolation of sister plants.



The first experiment was set up with sister plants of the same variety isolated under the same cage protected with anti-insect net; the second one was conducted on single plants isolated singularly with anti-insect net with very small mesh; the third one provided clones of the same genotype isolated together under the same net cage. Clones of different genotypes were made cutting the plant at the base of auxillary buds, making them root in high humidity and transferring them after 2 weeks in pots.

At the end of the experiment, after a month of flowering plant isolation, both cages and isolation net have been removed and a check of the seeds formed has been carried out to confirm the hypothesis of Self Incompatibility presence.

The seeds formed were analyzed with the molecular marker used for the previous experiment of hybridization check (FTSZ1-1 FR1).

4.3.2 Plant material used for crosses for CMS introgression:

Crosses were made manually between *Diplotaxis tenuifolia* and *Eruca sativa* accessions and a CMS line of *Brassica oleracea* C301, the material used is reported in Tab.31.

The species used for interspecific crosses have different flowering time; for this reason, gradual sowings have been made to favor the synchronization of flowering.

Table 31 Plant material used for crossing and introgression of male sterility

Plant material	Start flowering after sowing day	Full flowering after sowing day
Male sterile *Brassica* accession C301	85-90 days	140-155 days
Eruca sativa commercial genotype A	30-40 days	50-60 days
Diplotaxis tenuifolia commercial genotype B	30-40 days	50-60 ays

Figure 15 a)Brassica male sterile flower , b) Eruca sativa flower used as male parent, c)immature silique harvested 20 days after crossing and sterilized for embryo rescue culture

4.3.3 Protocol for embryo rescue

Immature siliques were collected 4 to 6 weeks after pollination, before the invitro culture, siliques were sterilized in 3% NaClO for 15 minutes and then washed in sterile water for 1 minute.

Immature seeds were collected and placed on MS Medium (ph 5.9-6.1) in a growth chamber with a photoperiod 16/8 h 20-25° C day / night, following the published Nothnagel protocol. The medium used for sustaining the development of the sampled embryo is reported in Tab.32.

In parallel, some Petri with siliques were placed in the dark for the duration of the experiment, to evaluate if there were differences or improvements in the development of the embryo.

Table 32 Recipe of Murashige and Skoog medium (MS)

Chemical	Formula	Concentration
Macronutrients (10 X)		100 mL/L
Ammonium nitrate	NH_4NO_3	16.5
Potassium nitrate	KNO_3	19.0
Calcium chloride	$CaCl_2.2H_2O$	4.4
Magnesium sulfate	$MgSO_4.7H_2O$	3.7
Potassium dihydrogen orthophosphate	KH_2PO_4	1.7
Micronutrients (100 X)		10 mL/L
Manganese sulphate	$MnSO_4.4H_2O$	2.23
Zinc sulphate	$ZnSO_4.7H_20$	0.86
Potassium iodide	KI	0.086
Cupric sulphate	$CuSO_4.5H_2O$	0.0026
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.025
Cobalt (ous) chloride	$CoCl_2.6H_2O$	0.0026
Boric acid	H_3BO_3	0.62
Vitamin source (100 X)		10 mL/L
Nicotinic acid	$C_6H_5NO_2$	0.05
Thiamine hydrochloride	$C_{12}H_{17}ClN_4OS.HCl$	0.01
Pyridoxine hydrochloride	$C_8H_{12}N_2O_2.2HCl$	0.05
Glycine	$C_6H_{12}O_6$	0.2
Iron source (100 X)		10 mL/L
Sodium EDTA	$C_{10}H_{14}N_2O_8Na_2H_2O$	2.78
Ferrous sulphate	$FeSO_4.7H_2O$	3.72
Myo-inositol		0.1 g (freshly add)
Sucrose	$C_2H_5NO_3$	30 g
Phytagel		2 g

Petri were stored in the growth chamber for 60-80 days to see the formation of calluses and, eventually, plantlets. After the first 30-40 days of culture, embryos were checked and the embryos that seemed to have developed the most, were removed from the MS medium and moved to a new medium with plant hormones to stimulate the root growth.

4.3.4 Use of molecular markers for the experiment

In a first step of the research for hybrids creation with the use of male sterility and self-incompatibility, a screening of the rocket plant material in our possession was done. To verify the presence of male cytoplasmic sterility in rocket germplasm in possession of ISI Sementi, the marker of the *ogura* gene used for other *Brassica* species (ORF138YT_FR, Yamagishi H., Terachi T. 1996) was tested.

In a second step, in the experiment of interspecific crosses and subsequent *embryo rescue*, to verify the interspecific hybrid we tested the seedlings obtained by the embryos with the molecukar marker BoIAB94TF (Louarn et al 2007), see Tab.33. The choice of this marker depended on the from optimal amplification on all three species used in the experiment (BoIAB94TF resulted polymorphic on cauliflower (*Brassica oleracea*) and on *Eruca sativa,* monomorphic on *Diplotaxis*

tenuifolia), defining very different patterns. The fact that the patterns are so distinct allows to identify the identification of F1 clusters in an interspecific hybrid.

Table 33 Markers used in male sterility introgression study

gene		Marker designation	Annealing temperature (°C) OligoCalc Salt adjusted	Bibliography
oleracea	h_F	BoIAB94TF	57,5	Louarn et al 2007
oleracea	h_R		56,3	
oleracea	h_F	ORF138YT_FR	52,7	Yamagishi H., Terachi T. 1996
oleracea	h_R		58,4	

4.4 RESULTS AND DISCUSSION

4.4.1 Self incompatibility on accessions:

Table 34 Plant material for self-incompatibility validation on rocket accessions

Plant material	Experiment condition	Seed formation
Sister plants ISI 1	Insects under cage	Yes
Sister plants ISI 1	Isolation with net	No
Sister plants ISI 2	Insects under cage	Yes
Sister plants ISI 2	Isolation with net	No
Sister plants ISI 3	Insects under cage	Yes
Sister plants ISI 3	Isolation with net	No
Single plant ISI 1	Insects under cage	No
Single plant ISI 1	Isolation with net	No
Single plant ISI 2	Insects under cage	No
Single plant ISI 2	Isolation with net	No
Single plant ISI 3	Insects under cage	No
Single plant ISI 3	Isolation with net	No
Clones genotype 1	Insects under cage	No
Clones genotype 2	Insects under cage	No
Clones genotype 3	Insects under cage	No

As it can be seen from Tab.34, different results were obtained according if under the cage there were more plants or just a single one: seed formation, in fact, occurred only in the case of the presence of sister plants of the same line (for both ISI1, ISI2 and ISI3) under cage. In fact, sister

plants in rocket are not stable, a rocket variety is not a pure line but each variety, while phenotypically homogeneous, is open pollinated being a multigenotypic variety made by a pool of highly heterozygotic genetically different plants. Therefore the plant recognizes the sister as genetically different and fertilization is possible. The absence of seed formation in absence of insects again support the need of entomophilic pollination in rocket. Genetic recognition probably takes place and prevent the self-pollination.

The results of the various tests carried out to verify the self-incompatibility of *Diplotaxis*, shows that the species confirms the presence of this typical characteristic of the Brassicaceae family. In fact, single plants of all genotypes do not form seeds in any of the cases. Instead, sister plants of the same variety (referred to as ISI1, ISI 2 and ISI 3) normally form seeds, as long as pollinating insects are present. The plants of the same variety are not, in fact, homogeneous from the genetic point of view, but tend to preserve a certain variability as a consequence of the free crossing to which the species tends.

The test conducted on cuttings of the same plant, gave results consistent with the hypothesis of the auto incompatibility of the species. The test also highlighted the predisposition of *Diplotaxis tenuifolia* to propagation through cuttings. In fact, from the same plant it was possible to obtain 5 or 6 cuttings that formed roots within 21-28 days with a success rate greater than 70%.

From the results of the tests, it can be assumed that there is a genetic mechanism underlying the self-incompatibility of this species. It will be a goal for the future to try to better understand the biological and genetic mechanisms that cause this phenomenon and if this happens also in *Eruca sativa*, that has not considered in the present study.

The experiment was also verified using the molecular marker FTSZ1-1 FR1 previously used for hybridization check (see Chapter 3). The marker has been used only in case of seed formation, to confirm that the genotype was the same as the sister plants isolated under the same cage.

4.4.2 Interspecific crosses and *embryo rescue* technique

In order to introduce the male-sterility of cauliflower in rocket, inter-generic crosses were made between accessions of *Brassica oleracea* (used a sterile male with cytoplasmic sterility as female part of the cross) and plants of *Eruca sativa* and *Diplotaxis tenuifolia* used as male parentals. The number of crossings that has been made was high: this was necessary, since percentage of fruit

set is generally very low (especially between such different species). In fact, out of 250 crosses made, 70% of them broke off from the mother plant without fruit set, after 10 days.

Interspecific crosses among *Eruca* and *Diplotaxis* are already very complicated to carry out, due to the presence of physical barriers such as the conformation of the flower pistil or the size of the pollen grains. In the specific case of crosses made between *Brassica oleracea* C301 and *Diplotaxis tenuifolia*, in the majority of cases, floral abortion occurred. When abortions were not present, the siliqua with the immature seed inside was collected but the seeds were not viable. On the contrary, in crosses made between *Brassica oleracea* C301 and *Eruca sativa* a higher number of immature siliques has been harvested and cultured. 55 immature siliques formed on *Brassica* were collected and several attempts at embryo rescue were made using two different growth substrates, as described in the section "Materials and Methods".

In most cases, the embryos have not developed. In 18 cases the formation of a green callus was obtained, but this was the result of a vegetative propagation of the somatic organs of the cauliflower, not of an hybrid. In 5 case the formation of a small potentially hybrid seedling was obtained.

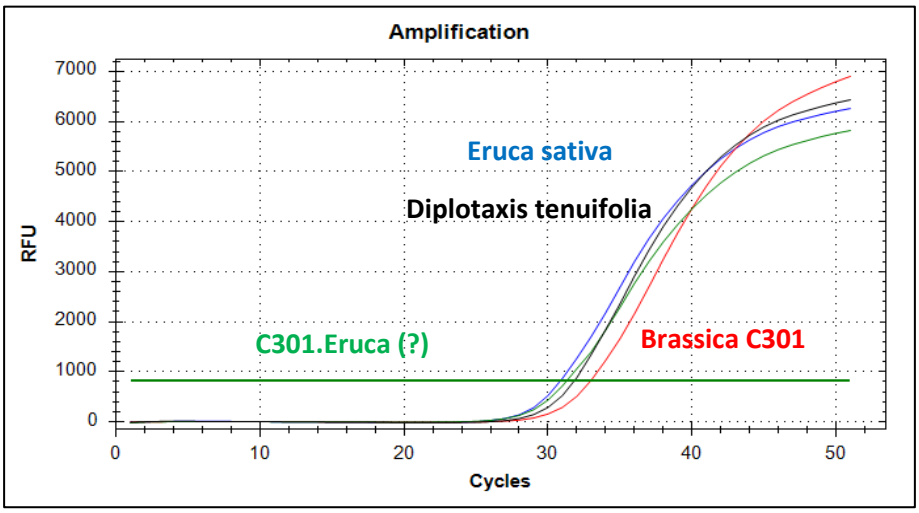
The hypotheses that can be drawn from the low compatibility between the species *B. oleracea* and *D. tenuifolia* is the presence of a too big genetic distance that prevent the possibility to overcome the post-zygotic barriers. The problem could be solved by using a bridge species or making crosses in different stages of maturation of the female and male organs, to understand if it can be a problem of floral maturity.

4.4.3 Molecular analysis

A first step in the research for male sterility for rocket was made by screening germplasm with the molecular marker *ogura* (*Brassica oleracea*). The aim was to find sterile male accessions that could become parental varieties in the creation of a hybrid of *Diplotaxis tenuifolia*. The marker *ogura* did not amplified among rocket accessions.

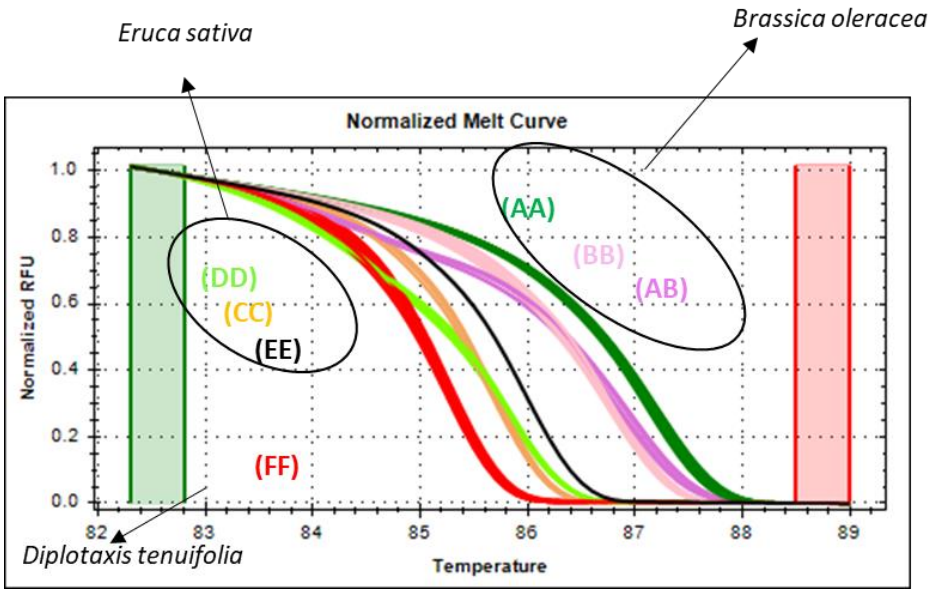
The second step was to use a male sterile cauliflower accession (C301) to test interspecific crosses. The five seedlings developed from these interspecific crosses grown in vitro using *embryo rescue* substrates, were sampled and tested with the marker BoIAB94TF**.** This marker amplified correctly on the *Diplotaxis tenuifolia* samples and *Eruca sativa* samples compared to the cauliflower control (C301), see Fig.

Figure 16 Amplification curve of marker tested on Brassica oleracea, rocket accessions and the interspecific crosses



From the HRM analysis, the patterns of the different species *Diplotaxis tenuifolia*, *Eruca sativa* and *Brassica oleracea* can be identified. The marker BoIAB94TF identified in cauliflower 3 distinct genotypes in the analysis (genotype 1 AA, genotype 2 BB and genotype 3 AB); in *Eruca sativa* the 3 genotypes were DD, CC, EE and in *Diplotaxis tenuifolia* only one genotype has been identified with this molecular marker (FF in the graphic, the marker is monomorphic for this species). Genotypes distinction is better explained in Fig.

Figura 17 Genotypes identified by the marker BoIAB94TF



The molecular analysis showed that none of these 5 seedlings were actually an interspecific hybrid, but they were all genetically identical to the parental plant (*Brassica oleracea*).

No plantlets formed with embryo rescue and then analyzed with the molecular markers showed the presence of alleles of both parental lines, but only alleles from *Brassica oleracea*. All the callus and then the plantlets formed were then considered as vegetative propagation of the female parents.

4.5 Conclusion

Research aimed at understanding Self Incompatibility and the introgression of male cytoplasmic sterility are a starting point for the creation of a hybrid, the main objective of the research project. In the next years, it will be necessary to repeat cycles of interspecific crossbreeding to re-test the *embryo rescue* protocol and use it in a more successful way. If an interspecific hybrid could be obtained, repeated back-crosses must be carried out to introduce CMS into rocket, thus losing the phenotypic characteristics of cauliflower. This method is difficult and laborious to implement, therefore alternative ways are being explored to obtain hybrids, such as the use of micropropagation (through cuttings) or the fusion of protoplasts. The insertion of male sterility in rocket (specifically in *Eruca sativa*) is possible and has been described in a patent, reason why attempts have been made.

Nevertheless, no results were obtained, probably for the unsuccessful crossings or the *embryo rescue* method that has to be changed or implemented.

Various factors can affect obtaining hybrid plantlets with *embryo rescue*. It depends on the age of the embryo, intactness of the suspensor, excision procedure, sterilization, culture medium supplementation, temperature and light requirements etc. Highly immature embryo rescue is very difficult and requires special medium requirements. The aim should be rescue for primary embryos secondary embryogenesis should be avoided.

CHAPTER 5 : PHYTOPATHOLOGY FOR ROCKET BREEDING

5.1 INTRODUCTION ON ROCKET PATHOLOGIES

Considering the nowadays intensive and multi-seedling cycle cultivation involving leafy vegetables and in particular the baby leaf market, seed market must offer varieties resistant to the main pathogens that cause problems during cultivation. A new variety must therefore possess these resistances and maintain a high quality level in the post-harvest.

The most common pathogens for the ready-to-use salads are the soil born fungal pathogens, like *Fusarium oxysporum* spp. (Synder and Hansen), *Sclerotinia sclerotiorum* (partially diffused in Italy) and downy mildew on leaves (*Phytoptora parasitica*/*Hyaloperonospora parasitica*). (Pane et al., 2017)

There are also other minor pathologies for rocket crop, for example the bacterial disease caused by *Xanthomonas* spp., that cause spots and blights on leaves and stem. The leaf spot disease on wild rocket was reported in 2005 and the pathogen has been identified as *Xanthomonas campestris* pv. *raphani* Pammel (Pernezny, 2007). Another fungal pathology is caused by *Alternaria japonica*, especially during the final stages of rocket cultivation during seed production. This fungus can reduce the production and the germinability of the seeds (Gilardi et al., n.d.)

Among the pest insects, *Phyllotreta nemorum* (a Coleopter known with the common name Altica) can cause very dangerous infestations during summer, when temperatures arise at 25-30°C causing losses of product both in cultivation and in seed production. The insect eats the leaf lamina, making the product unsaleable and slowing down the vegetative and reproductive cycle of the plant. These infestations are generally controlled by repeated chemical treatments when the number of individuals exceeds the accepted threshold.

The management of soil-borne diseases is instead difficult, therefore finding resistant cultivars is crucial for a more sustainable management. The exploration of rocket germplasm to find sources of disease resistance started a few years ago (Gilardi et al., n.d.) by research groups interested in this crop. Further study to extend knowledge on response to biotic stress of rocket genetic sources are still necessary, particularly for those conserved in germplasm bank which will soon represent the reservoir of traits to exploit in view of increased food demand by a growing world population and climate change. (Pane et al., 2017)

From the very beginning of this PhD project, different partnerships have been established with private analysis laboratories and public research centres, concerning the development of phytopathological tests for the selection of genotypes resistant to the various pathogens of rocket. The aim of these collaborations was the development of protocols of infection on rocket seedlings/plants with *Xanthomonas campestris* (CREA di Pontecagnano), *Hyaloperonospora parasitica (*project RemiRucola INIAV Portugal, 2019) and *Fusarium oxysporum f.sp. raphani* (Ceersa di Albenga phytopathological analysis laboratory). Accessions available at ISI Sementi were sent to the various research units to verify the presence of sources of resistance.

Information collected have been useful to organize the breeding programme and to create new internal phytopathological protocols in ISI Sementi. In the company there was already present a laboratory performing resistance test to fungal disease in other species (lettuce, tomato, onion) and it was a starting point for rocket breeding.

5.2 *FUSARIUM OXYSPORUM*:

Fusarium wilt is caused by the soil-born fungus *Fusarium oxysporum*, which has a wide host range that includes hundreds of crop species. Fusarium disease has been detected on several species of Brassicaceae family, including rocket (both *Diplotaxis* species and *Eruca* species) with a highly host specialization and several formae specialis.

5.2.1 Classification of *Fusarium oxysporum* in Cruciferae and in rocket:

Of the *F. oxysporum* isolates, *F. oxysporum* f. sp. *raphani* (*For*) has been identified as a pathogen that infects radish plants or seedlings (*Raphanus sativus* L.), which is one of the major limiting factors for radish production (Baik et al. 2011). This fungal pathogen was originally classified from one of the five *F. oxysporum* f. sp. *conglutinans* races that cause Fusarium wilt on cruciferous crops (Armstrong and Armstrong 1981; Ramirez-Villupadua et al. 1985).

In radish (Lievens et al 2008) two QTLs were identified (one on chromosome 5 and one on chromosome 11) as responsible for resistance in varieties (Michelmore & Kesseli, 1991) “QTL gene responsible of FW resistance in radish”.

According to the pathotypic variation in *F. oxysporum* on crucifers, there were defined 5 races of this fungus. Subsequently, *F. oxysporum* f. sp. *conglutinans* race 1 and 5 were categorized as *F.*

oxysporum f. sp. *conglutinans* race 1 and 2, respectively, with host specificity to *Brassica oleracea* L. (Kistler et al. 1987). Race 3 and 4 were also changed to *F. oxysporum* f. sp. *matthioli* race 1 and 2, respectively, with host specificity to Matthiola incana (L.) R. Br. (Kistler et al. 1987). Race 2 was finally classified as *F. oxysporum* f.sp. *raphani* based on its ability to infect radish plants (Brayford 1992).

Fusarium oxysporum was also reported in Europe since 2003 on both *Eruca vesicaria* (cultivated rocket) and on *Diplotaxis* spp. (Gilardi et al., n.d.). Three formae speciales were isolated on these plant species and identified through DNA intergenic spacer region sequencing and named *F. oxysporum* ff. spp. *raphani, conglutinians* and *matthioli* (Srinivasan et al. 2012). However, the forma specialis *raphani* is distinguished for its higher virulence on wild rocket (Srinivasan et al. 2012).

Fusarium oxysporum was reported on *Eruca vesicaria* (cultivated rocket) and on *Diplotaxis* spp., in Europe since 2001 and reported in Northern Italy since 2002 (Garibaldi et al. 2003). During the summer 2001 and spring 2002, rocket plants (*Eruca vesicaria* and *Diplotaxis tenuifolia*) cultivated in a protected environment in Bergamo showed typical symptoms of this disease; then a new epidemic happened in 2003-04, expanding from Lombardy to all of northern Italy in different cultivation areas. In the following years, epidemics of this pathogen also occurred in the Campania cultivation area, which is the first production site in Italy (De Martino).

5.2.2 Symptoms

Symptoms induced by this pathogen includes wilting and the development of necrotic, chlorotic lesions on the vessels, yellowed leaves, wilting and finally death of the whole plant. *F.oxysporum* spores can survive over long period in soil (in the form of chlamydospores) even in the absence of suitable hosts (Dinolfo et al.2017).

Wilting disease symptoms are typical, appearing firstly as leaf chlorosis, plant stunting and browning of vascular tissues. Then, in progress, the disease causes death of the plant. The fungus has been reported as a seed-borne and seed-transmissible pathogen (Gilardi et al., n.d.)

5.2.3 Objectives of the study

Given the great importance of this pathogen during the cultivation of rocket, the project immediately focused on the search for resistant genotypes. Given the unavailability of a specific test for rocket, we started from the development of the protocol, and then applied it for our breeding purposes. The objectives therefore were:

- Classification and taxonomy of different strains of *Fusarium oxysporum* formae speciales collected in cultivation areas in Italy, with the application of the sextet code useful to define the forma specialis
- Developing an infection protocol and symptom rating scale for the pathogen *Fusarium oxysporum* f.sp. *raphani* on rocket accessions and populations (*Diplotaxis tenuifolia* and *Eruca sativa* accessions)
- Studying the genetics of resistance to *Fusarium oxysporum* in rocket populations F2 to define whether the basis of genetics is qualitative (Mendelian hypothesis) or quantitative (QTL gene)
- Screening of plant material for breeding aims to obtain a resistant OP variety competitive in the market and to find resistance sources.

5.2.4 Materials and methods *Fusarium oxysporum f.sp.raphani* phytopathological assay:

For the infection with *Fusarium oxysporum* f.sp. *raphani*, the starting point was an article published by (Pane et al., 2017): the protocol was then adapted to the conditions of the ISI Sementi laboratory.

Fungal strains used for the protocol development were:

- *Fusarium oxysporum* f.sp. *conglutinans*, isolated from field radish by CeRSAA (Albenga, SV) and used for the first tests;
- *Fusarium oxysporum* f.sp. *raphani*, two strains isolated from CREA Battipaglia, labelled as 09413 and 10223;

Plant material tested:

- Accessions, varieties, germplasm present in ISI Sementi seed bank (Tab.7 in Chapter 2)
- 18 segregant populations (up to 200 plants inoculated for each population in F2,F3 or F4) for breeding purpose (the list of genetics selected for confidentiality of the research by the breeding company are not specified),
- A specific F2 population deriving from a F1 cross between a resistant parent and a susceptible one to study the distribution of resistance classes in the F2 generation.

Protocol for infection:

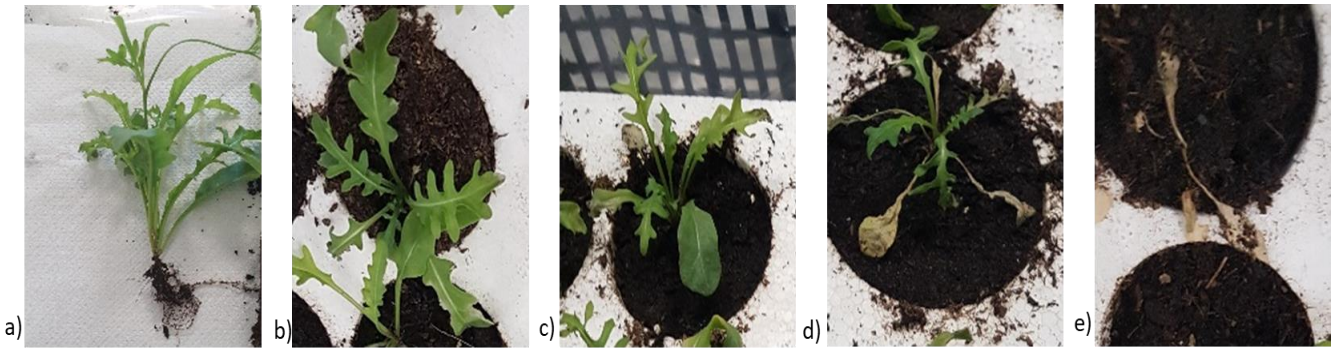
For the fungal growth, Potato Dextrose Agar solidified (21 grams/500 ml) was used for fungal vegetative propagation in Petri dishes. Casein hydrolysate (16 grams/500 ml) was used as liquid medium for spore induction (it took 10-14 days to obtain an optimal number of spores for the test).

Plants for Fusarium wilt test were sowed in plateaux and placed in greenhouse with controlled temperature and humidity. The infection was performed on 21 days old plants. Seedlings were removed from cultivation trays, cleaned and the roots were cut and dipped into the fungal solution (prepared and diluted to a concentration of 10^6 spores/ml) for 10 minutes. Infected plants were transplanted in plateaux containing 60 plants and placed in a growth chamber at 23-25°C 12/12 day/night for the duration of the test (21 days).

Evaluation phase:

The evaluation takes place after 21-28 days from the infection, when the susceptible control show disease symptoms. A scale of disease susceptibility was used (Fig.17).

Figure 18 a) High resistant plant (value 1), with developed roots and leaves, no yellowish or symptoms on cotyledons; b) Resistant plant (value 2), with yellowish only on cotyledons, normal growth; c) Intermediate plant (value 3) with yellowish of cotyledons and leaves, small size, reduced growth and small roots; d) Susceptible plant (value 4), with very reduced development and death of leaf tissue; e) High susceptible plant (value 5) dead or very injured



5.2.5 Results and discussion

5.2.5.1 Protocol development

As mentioned before, three formae specialis have been detected on rocket (*Eruca vesicaria* and *Diplotaxis tenuifolia*): *conglutinans*, *raphani* and *matthioli.*

ISI germplasm and populations created during 2019 were analyzed for *Fusarium oxysporum* f.sp. *conglutinans* by an external laboratory during summer 2019. The laboratory had previously isolated the fungus from radish (*Raphanus sativus*) and then the infection was done on plants 21-30 days old. The resistant plants obtained from the F2 population were selected and multiplied under cages in open field (with insect controlled impollination) to obtain successive generations.

However, the test did not always give repeatable results and it was strongly depending on the finding and isolation of the pathogen in the field. For this reason, and to further investigate this pathogen, it was decided to try to internalize the Fusarium phytopathological assay in ISI Sementi laboratory.

The pathogen was isolated from roots of susceptible plants on PDA medium (Potato Dextrose Agar) in ISI Sementi. The fungus was grown vegetatively on PDA and two different liquid culture media were tested for the production of spores: PDB (Potato Dextrose Broth) and Hydrolyzed Casein were used according to the protocols in literature (Pane, 2017).

The pathogen was then tested in vivo in 2019 and 2020 on commercial varieties, which were used as controls. The test done by CeRSAA showed that forma specialis *conglutinans* was not very aggressive on *Diplotaxis tenuifolia* and on *Eruca sativa*, suggesting that this strain/forma specialis is not very specific for rocket.

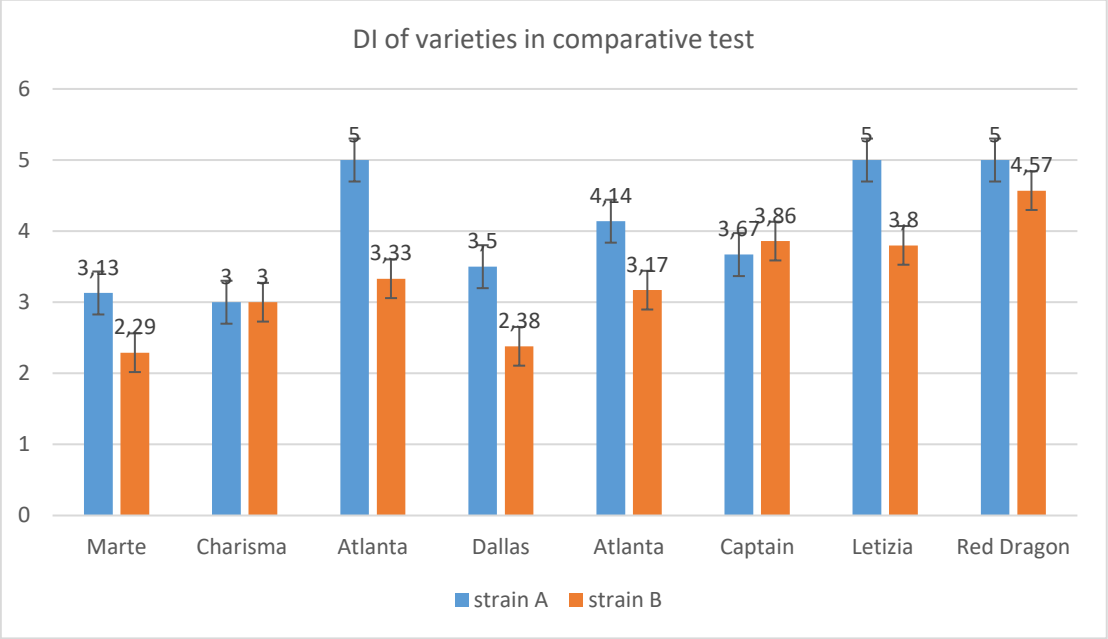
Therefore in 2020, with the collaboration of CREA of Battipaglia, ISI Sementi obtained and tested other two strains of *Fusarium oxysporum* f.sp. *raphani*, in order to define a protocol valid for *Diplotaxis tenuifolia*. The forma specialis was defined by CREA after a sextet infection test using different standard varieties of cauliflower (*Brassica oleracea* L. var. *botrytis*) variety Snowball, radish (*Raphanus sativus* L.) variety Champion and on wild rocket variety Tricia. The host specificity was assessed based on the disease index (D.I.) as reported in Tab. 35 and in Fig.18 (De Martino, Sigillo, 2016).

The two strains (A and B) were both multiplied in vitro using PDA medium for fungal growth. A comparative test done on commercial varieties with both strains permitted to identify strain A as the most selective one, to be used for breeding purposes on screening material.

Table 35 Different DI of a sextet of varieties with strain A and strain B of Fusarium oxysporum f.sp.raphani

Variety	strain A	strain B
Marte	3,13	2,29
Charisma	3,00	3,00
Atlanta	5,00	3,33
Dallas	3,50	2,38
Atlanta	4,14	3,17
Captain	3,67	3,86
Letizia	5,00	3,80
Red Dragon	5,00	4,57

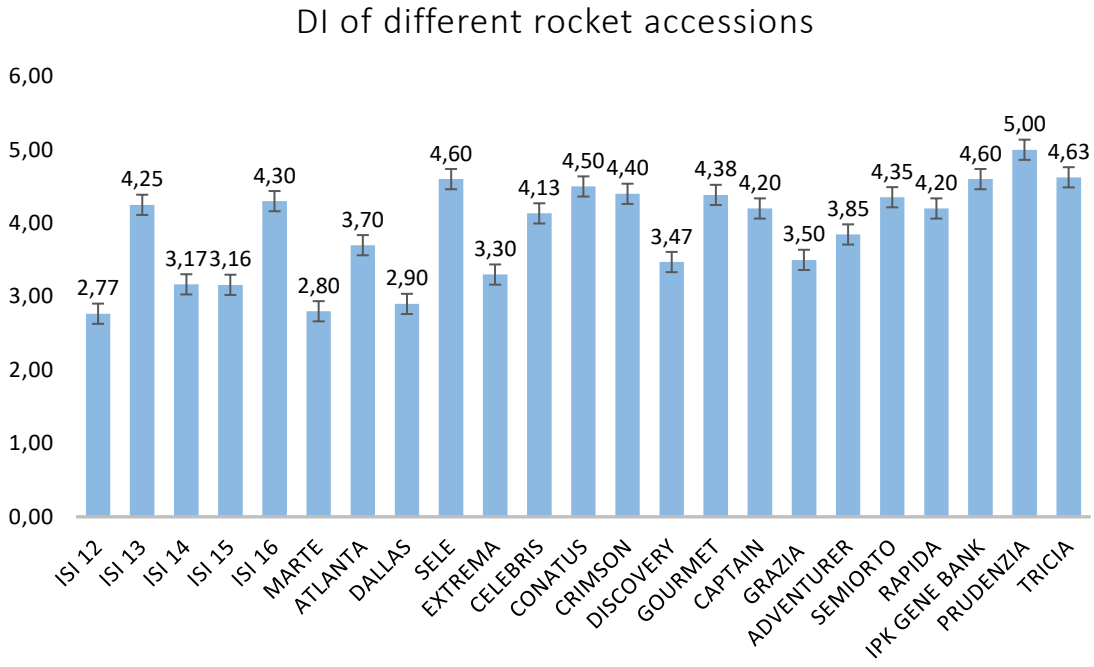
Figure 19 Graphic of DI differences with the two strains (A,B) tested



After the protocol development and improvement, plant material of different origin was tested. Screening of commercial varieties and accessions (Disease Index in Fig.19) led to the collection of information on genetic material possible sources of resistance, useful to plan future crosses for breeding.

Tests were performed also on 18 segregant populations and permitted to obtain resistant genotypes and subsequently improved rocket populations resistant to *Fusarium oxysporum f.sp.raphani*.

Figure 20 Disease index in rocket accessions tested with Fusarium oxysporum f.sp.raphani



Test on accessions provided information on tolerance or resistance of the varieties, showing an overall low level of resistance, except for ISI 12, Marte and Dallas: these have an average disease score lower than 3,00 and can be considered as intermediate. Around intermediate value (just over 3,00) there are also ISI 14, ISI 15, Extrema, Discovery and Grazia.

The tests gave fairly homogeneous results within the same variety, however there are genotypes of the same variety that behave differently from the average of the others, probably giving the remaining percentage of heterozygosity in the populations, typical of a species like rocket in which the populations are strongly allogamous.

5.2.5.2 Study on genetics of Fusarium oxysporum disease

In 2020 manual crosses described in Chapter 2 led to the creation of several F1. These F1 were multiplied under cage to obtain F2 populations and to create a new germplasm for selection and breeding activities.

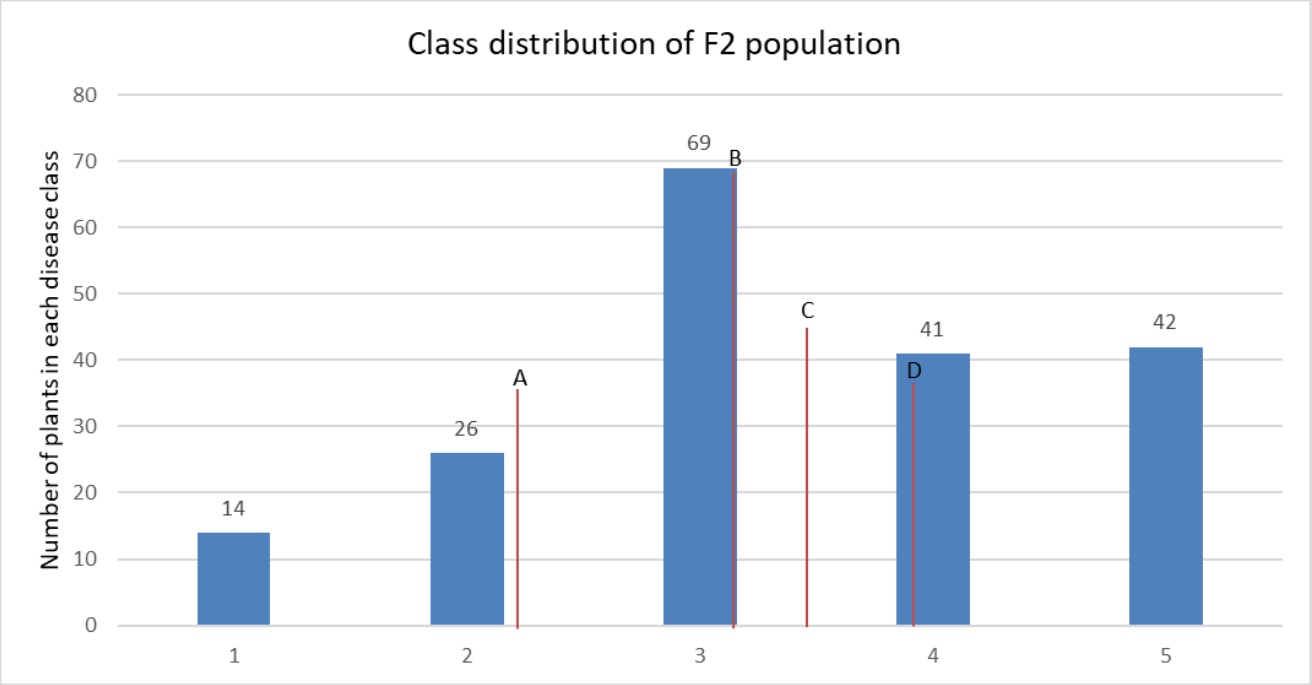
A selected F1 (cross between tolerant parent and susceptible parent), its F2 generation (192 plants) and the respective parental varieties were tested with *Fusarium oxysporum* f.sp*. raphani* to study the distribution of classes in F2 and to better understand the genetics of resistance against this pathogen.

F2 distribution (reported in Tab.36 and in the graphic in Fig.20) suggests QTL genes involved in the genetic of resistance against *Fusarium oxysporum* f.sp. *raphani.*

Table 36 Distribution of disease classes in a F2 population, relative F1 and parentals

Plant material	Tot.infected plants	1.HR	2.R	3.IR	4.S	5.HS
F2	192	14	26	69	41	42
F1 (RxS)	4			2	2	
P1	16	5	4	4	2	1
P2	11			0	11	

Figure 21 Class distribution of F2 population (A is the average value of the resistant parent P1, B is the average value of the F2 population tested, C is the average value of F1 P1xP2 and D is the average value of the susceptible parent P2)



To discard the hypothesis of single dominant or recessive gene involved in resistance against *Fusarium oxysporum*, and to demonstrate that resistance against Fusarium wilt has a complex genetic basis, chi square test has been performed and values are reported in the table below (Tab. 37, Tab.38, Tab.39).

Table 37 chi square value with hypothesis of dominant gene that explain the resistance (classes 1,2,3 are considered resistant and class 4 and 5 as susceptible)

	Hypothesis Dominant gene			
	Expected	Real		X^2
R	144	109		34,02
S	48	83		

Table 38 chi square value with hypothesis of three classes (plants in intermediate class 3 are heterozigous)

	Hypothesis heterozigosity			
	Expected	Real		X^2
R	48	40		34,44
IR	69	69		
R	48	83		

Table 39 chi square value with the hypothesis that a recessive gene explain resistance against Fusarium wilt (intermediate class 3 has been unified with susceptible classes)

	Hypothesis Recessive gene			
	Expected	Real	χ^2	Error %
R	48	40	1,77	10%
S	144	152		

Although in the case of the hypothesis with recessive gene involved in *Fusarium* resistance there is a statistically more acceptable chi square value, error percentage is high (10%), therefore all the three hypothesis must be discarded.

Analyzing the distribution of the disease classes in the assay, it is possible to say that QTL genes are involved for *Fusarium oxysporum* resistance in rocket (also found in bibliography in other Cruciferous species, like radish). QTL genes have a complex genetic base. Sometimes one single locus influences a quantitative character (pleiotropic effect, one gene is involved in more characters) or more genes are associated on the same chromosome, and therefore inherited together.

Observing the graphic with the distribution of F2 plants in classes, the hypothesis is that at least two allelic pairs are involved. The additive model is based on the absence of dominance, so more genes are responsible in the same way for the resistance. Dominance or superdominance relationship between genes can instead explain the presence of a major QTL gene, responsible in a greater percentage for resistance.

Moreover, the environmental effect cannot be underestimated. The more the environment is able to change the phenotypic expression of a genotype, the more different phenotypes will show and the distribution curve will be continuous (normal). The environment can influence the expression of quantitative characters with the number of genes involved.

An allelic test should be performed by testing different resistant parental lines crossed and F1 and F2 tested to better know how many genes are responsible for *Fusarium* resistance in rocket.

The complementation or allelic test in genetics is an useful test for determining whether two mutations associated with a specific phenotype represent two different forms of the same gene (alleles) or are variations of two different genes. The complementation test is relevant for

recessive traits (traits normally not present in the phenotype due to masking by a dominant allele). In instances when two parent organisms each carry two mutant genes in a homozygous recessive state, causing the recessive trait to be expressed, the complementation test can determine whether the recessive trait will be expressed in the next generation.

5.3 *HYALOPERONOSPORA PARASITICA*: DOWNY MILDEW DISEASE

Downy mildew in horticultural plants is a disease with specific characteristics caused by parasitic Oomycetes from different genus: *Hyaloperonospora*, *Peronospora, Phytoptora*. Infected plants generally show symptoms on leaves and stem, like chlorosis and subsequent necrosis of leaf tissues. Frequently, there is also sporulation on infected leaves, more often on the underside of the leaves. The losses are more severe at the seedlings stage than on mature healthy plants (Hladilova, n.d.). Cool and moist conditions are favourable for the disease development (Smith et al., 1988).

5.3.1. Classification of the pathogen in rocket

Hyaloperonospora parasitica (Pers.) Constant. is an Oomycete from the family Peronosporaceae, also found in literature with the name Peronospora brassicae Gäum. or Peronospora parasitica (Pers.) de Bary. It has been considered for a long time to cause severe symptoms of downy mildew in a variety of species within the Brassicaceae. The former name for *H. parasitica* was *Peronospora parasitica* until it was reclassified and put in the genus *Hyaloperonospora*. It is most famous for being a model pathogen of *Arabidopsis thaliana* which is a model organism used for experimental purposes (Hladilova, n.d.)

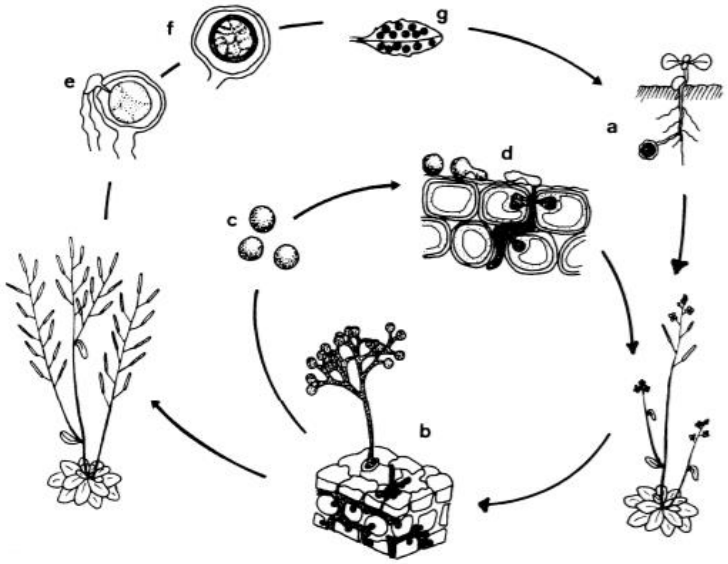
Downy mildew is a pathology wich strikes more during the seasons with high humidity and temperature range between night and day. In rocket crop, downy mildew is caused by *Hyaloperonospora* (Peronospora) spp.: in particular *Hyaloperonospora parasitica* is indicated in literature as the most host-specific species found in wild rocket. Other indications in literature indicate as pathogenic for *Diplotaxis tenuifolia* the species *Peronospora diplotaxidis* and the species *Peronospora erucastri* for the causal agent of downy mildew on *Eruca sativa*. The species are different and they can also be very numerous, it is very common in Oomycetes where there is a very high level of host specialization (Coelho et al., 2017)

5.3.2 Pathogen cycle and symptoms

Hyaloperonospora parasitica and *Peronospora* spp. are both polycyclic pathogen with a sexual and an asexual stage which thrives in cool moist environments. Over winter oospores survive and wait for spring in the soil. In warmer conditions, when the temperature is about 9-12°C, oospores germinate and produce an appressorium or form a short germ tube. The mycelium grows intracellularly and haustoria penetrate through the host cells when the temperature is around 15°C. After about 1–2 weeks when the temperature is between 15-17°C, conidiophores (the asexual stage) form out of the plant stomata bearing conidia. Sporulation occurs at night and the conidia disseminate in high humidity mornings (10°C). Meanwhile, during the sexual stage, the antheridia (the male sex organ) fertilizes the oogonium (the female sex organ) with a fertilization tube causing the development of an oospore. Sporulation on rocket leaves is showed in Fig.21, while the pathogen cycle is summarized in Fig.22 below.

Figure 22 Sporulation on rocket leaves caused by downy mildew

Figura 23 Cycle of Hyaloperonospora parasitica, different stages: a) oospores germinate in soil (starting infection on plantlets); b)colonization of the plant by growing mycelium; c) conidiophores out of stomata, after 1-2 weeks of growth, d) conidia starting new rounds of infection; e)-f)-g) oospores are formed concurrently with asexual spores.



5.3.3 Objectives of the study:

The damage caused on rocket by *Hyaloperonospora parasitica* can cause major economic losses if not managed properly. The recommendation for management is generally weekly fungicide application. The weakness of the management for this pathogen regard the very low or absent level of phytochemical residuals accepted in GDO market on the fresh cut material which limits the possibility of frequent chemical interventions. On the other hand, the pathogen has a tendency to change rapidly with always new favourable mutations, giving the fast life cycle and to develop resistance towards the treatment. For these reasons, it would be a great value to create a rocket variety resistant to this disease, which would avoid the need for chemical treatments on the crop.

The interest in this resistance regards the main European areas where the largest volumes of baby leaves are grown for the fresh-cut market, mainly the South of Italy and the Iberian peninsula, where rocket seeds are commercialized and cultivated also in autumn and winter cycles.

It is useful to specify that for this crop, for which registration of variety is not needed in the majority of countries, there is still a lot of confusion in the classification of the resistance. Some varieties are sold as tolerant, but there is no international resistance testing association like in lettuce and other species, as rocket is still considered a wild species in most European and non-European countries.

With this premises, during the research the objectives pursued were:

- To collect local strains of downy mildew (*Hyaloperonospora parasitica*) in cultivation areas of interest for ISI Sementi, in order to develop a protocol of infection and maintenance of the pathogen in ISI Sementi lab;
- To screen accessions and populations to find sources of resistance in rocket germplasm in possession of ISI Sementi;
- To create a sextet of accessions or commercial varieties for pathogen identification, as done with other plant species and their related pathogens.

5.3.4 Materials and method for *Hyaloperonospora parasitica* phytopathology test:

- Fungal material:

- a *Hyaloperonospora parasitica* strain collected in a field Portugal within the partnership of Remirucola project;

-a local Italian strain collected in Piana del Sele on ISI Sementi varieties during autumn 2021 trials and then multiplied *in vivo* in laboratory;

-strain collected in Portugal during summer 2022 in a cultivation area for baby leaf and rocket multiplied *in vivo* in laboratory.

- Plant material used for test:

-ISI Sementi variety Atlanta (susceptible) used as maintainer *in vivo* for pathogen reproduction;

-Commercial varieties and accessions present in ISI Sementi germplasm tested for resistance sources research (Tab.7 in Chapter 2)

- Plant maintenance for the test:

Varieties were sowed in plateaux (30 plants per variety were generally sown) and placed in greenhouse. The infection was performed after 10-14 days after sowing.

Given the biotrophic nature of the pathogen, the sowing, infection and evaluation operations were repeated cyclically every week to keep the pathogen always alive and virulent.

- Inoculum preparation:

For the inoculum preparation, infected leaves were rinsed in 15 ml of tap water, put in the vortex for 1 min and then filtered with a gauze to eliminate residues. The filtered material was observed under microscope to count the spores and used for infection. The optimum spores concentration is around 10^6 spores/ml.

- Infection:

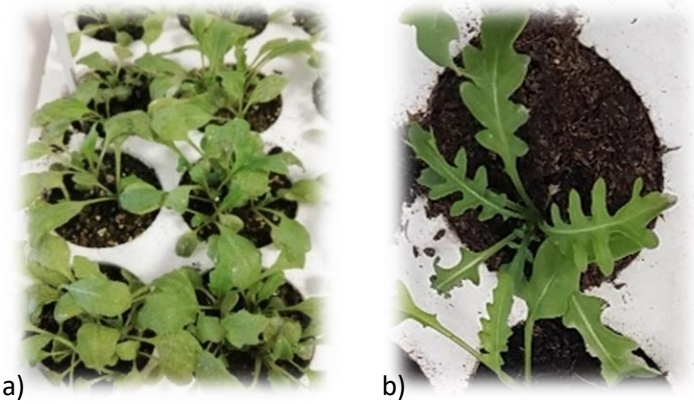
The suspension with spores was directly sprayed with a micro vaporizer/nebulizer on 10-14 days old rocket plants to maintain the pathogen *in vivo*. After the infection, plants were stored at 14°C in a plastic box to ensure the 90% of U.R. and placed in a growth chamber at 13°C (photoperiod of 14/10 hours light/night) to induce sporulation. Sporulation occurred after 7-10 days, making it possible to repeat the infection on fresh leaf material.

- Evaluation:

Plants are evaluated after 7-10 days from the infection, around 21 days after sowing. For the evaluation the protocol referred to the *Bremia lactucae* one (CPVO protocol *lactuca sativa*).

Plants with yellowing and sporulation on the leaf surface are indicated as susceptible, and plants with no symptoms on the leaf or that show necrosis with absence of sporulation (hypersensitivity reaction) are indicated as resistant (Fig.29). Spores are observed both on cotyledons and first leaf surface.

Figure 24 Downy mildew symptoms on rocket leaves: a) Yellowish of the surface with abundant sporulation (Susceptible plant) and b) no yellowish of the leaves, no evident sporulation, necrosis if hypersensitivity reaction occurs (Resistant plant)


a) b)

5.3.5 *Hyaloperonospora parasitica* - Results and discussion

5.3.5.1 Results within Remirucula project

In 2019, at the beginning of the doctoral project, a collaboration with INIAV (Instituto Nacional de Investigacao Agraria e Veterinaria, Portugal) began regarding a project that involves the screening of different accessions of rocket (many of which sent by different seed companies) in order to find resistance sources to *Hyaloperonospora parasitica*. To perform these tests, a strain isolated from a field in Portugal has been used. This experiment provided information for the accession resistances present in the ISI germplasm (reported in Tab.40)

Table 40 Results for varieties and accessions within the Remirucola project (legend Disease Index (DI) response: R=Resistant (DI≤2.5), PR=Partially Resistant (2.5<DI≤4.0), S=Susceptible (4.0<DI≤5.0), and HS=Highly Susceptible (DI>5.0))

Project code	Variety/Accession	Phenotipic category: cotyledons/leaves	DI (Disease Index) cotyledons/leaves
ISI 1	Captain	S/PR	4.8/3.5
ISI 3	Captiva	HS/S	5.3/4.1
ISI 4	Charisma	HS/S	5.2/4.3
ISI 5	Celebris	HS/S	5.8/4.9
ISI 6	Conatus	HS/S	5.1/4.4
ISI 7	Crimson	HS/HS	6/5.8
ISI 16	Virtus (*Eruca sativa*)	R/R	1.2/1.1
ISI 17	*Diplotaxis* accession	HS/S	5.6/5
ISI 19	*Eruca sativa* accession	R/R	1.3/1.0
ISI 20	Extrema	HS/HS	5.9/5.3
ISI 21	Atlanta ISI	HS/S	5.5/5.0
ISI 22	Gourmet	HS/HS	5.8/5.7
ISI 27	Bellezia	HS/S	5.2/4.2
ISI 28	Prudenzia F1	S/S	5.0/4.4
ISI 29	Grazia	S/PR	4.9/3.9
ISI 30	Letizia	S/S	5.0/4.5
ISI 31	Tricia	S/PR	4.8/3.7
ISI 32	Marte	S/PR	4.8/3.0

The presence of sporulation at the cotyledons and first two leaves was evaluated on 21-day-old plants (Fig.30). Both *Eruca sativa* accessions (ISI 16 and ISI 19) were resistant at the cotyledons and leaves to the *Hyaloperonospora* spp. isolate used for the project, collected on wild rocket plants in the field. Generally, wild rocket accessions were more susceptible in the cotyledons than in the

first and secondary leaves. The most frequent response in cotyledons/leaves was the HS/S combination , followed by the S/PR combination (ISI 1, ISI 29, ISI 31 and ISI 32). No accessions were observed to be highly susceptible in cotyledons and resistant in leaves. Fig.24 is an overview of varieties tested in tray at the evaluation day, after sporulation occurred on leaves.

Figure 25 Plants at 21 days after sowing (evaluation stage)

This Remirucola collaboration constituted a first approach to the knowledge of this pathogen, of the protocol for the *in vivo* maintenance for *Hyaloperonospora parasitica* and of the timing for infection. The test showed that there were very few resistant accessions in our germplasm, except for accession of *Eruca sativa*, probably because the strain was not host-specific.

The strain used in the project was characterized and identified as *Hyaloperonospora parasitica*, local strain collected in cultivated field in Portugal.

5.3.5.2Protocol development with local strain

In 2021 in Piana del Sele, a local *Hyaloperonospora parasitica* strain has been collected from infected plants in cultivation. Leaves with spores were sent to ISI Sementi phytopathology lab and used for infection *in vivo*. The susceptible variety Atlanta from ISI Sementi catalogue has been used as maintainer for propagation.

In order to maintain the pathogen for subsequent infection, every week the susceptible variety was sowed and infected after 7-10 days from sowing with fresh infected material. This method is

necessary for the biotrophic obligate pathogens, in fact freezing of the infected material can easily lead to the loss of vitality by the spores.

In the first attempt to decide the best conditions for the protocol, two different temperatures were tested: after infection plants were placed both at 23°C (as indicated in the Remirucula project for the Portuguese strain) in a growth chamber and at 14°C in a second growth chamber, normally used for the propagation of *Bremia lactucae* in ISI Sementi laboratory.

Sporulation occurred both at 14 °C and 23°C, but at 23°C it was faster: in 5 days spores appeared on the leaf surface and in two days rots developed on the leaf. Therefore 14°C chamber has been chosen for the next propagations, because it was more repeatable and did not occur in leaf rots and death of the plant.

Once the protocol was defined and repeated for several cycles of propagation of the pathogen, various accessions present in our seed bank were screened.

All the varieties tested so far confirm the susceptibility to this strain of *Hyaloperonospora parasitica* as well. The goal is to test as many genetics as possible (deriving also from European seed bank) to be able to find sources of resistance and be able to proceed with selection or crossing.

5.3.5.3 Reproduction of Portuguese strain collected in the field

In summer 2022, infected leaves were sampled from a field under cultivation in Portugal, where there were ISI Sementi varieties in cultivation.

The strain was added to the pathogen collection of the phytopathology laboratory in ISI Sementi, reproduced on Atlanta ISI variety and propagated in the same way of the strain sampled in Piana del Sele in 2021. Currently, strains are being screened with this additional strain to see if there are any differences.

Even with this second strain obtained in the ISI Sementi phytopathology lab, no resistant varieties were found among those present in our germplasm. Screening tests on other commercial varieties and from seed banks will continue in the next year, with the aim of defining a variety sextet that can facilitate the identification of different strains.

5.4 PHYTOPATHOLOGY CONCLUSIONS

As part of the phD project, among the pathologies studied, greater importance and urgency was given to resistance to *Fusarium oxysporum* in rocket. This is because, as explained above, the pathogen is widespread in the soil in the areas where rocket is grown in intensive agriculture and it is difficult to control with crop protection products.

The research of the strains and subsequently the obtaining of the protocol for the infection with *Fusarium oxysporum f.sp.raphani*, have given satisfactory results and have made it possible to select the ISI Sementi varieties with an improved level of accuracy.

The phytopathology test allowed to screen rocket germplasm and can now be used as a selection tool for breeding purposes. Selection and reproduction of resistant plants are already leading to the current state to the creation of improved OP varieties.

with the *Fusarium oxysporum f.sp.raphani* test were, at the beginning of the research, in finding the specific strain to carry out the infection and to create a standard evaluation form, not present in literature of the species and based on repeated observations and test.

Regarding the second pathogen used for our research purposes, *Hyaloperonospora parasitica*, it was difficult to find information and to obtain a classification of the different strains.

The *Hyaloperonospora* test protocol was therefore difficult to develop, because lacks on scientific literature on this pathology and for the absence of a official protocol of infection and classification of the pathogen. In other species, like lettuce, for the test *Bremia lactucae,* there are organizations that define new strains, resistance genes and sextet of varieties used to classify races. .

It was however a positive result to obtain two different strains from the cultivated varieties affected by this pathogen, store and reproduce them in the pathology laboratory and start using them for resistance tests and germplasm screening.

For what concerns *Fusarium oxysporum* f.sp.*raphani*, a sextet has been defined in a previous publication (De Martino, Sigillo 2016) and there are more information available on the pathogen.

The goal is to obtain new populations with increasingly stable sources of resistance, basing the selection and crossing patterns precisely on this resistance desired by the market.

CHAPTER 6 - CONCLUSIONS

Before the start of the PhD project, ISI Sementi proposed two varieties of rocket in its commercial range. The initial aim was the creation of a breeding program for this species, developing new selection criteria and breeding for modern varieties.

New traits were defined by ISI Sementi marketing team, to propose a plant ideotype useful to overwhelm competitor's varieties. The greatest urgency was certainly to deepen the knowledge on the pathogens (mainly *Fusarium oxysporum* f.sp.*raphani* and *Hyaloperonospora parasitica*) that attack the rocket in intensive cultivations, widely spread both in the North and in the South of Italy.

The tools that have been developed to meet the main market needs are summarized in Tab.41.

Table 41 Summary of objectives and solution for breeding

PROBLEM	SOLUTION	DEPARTMENT OF RESEARCH
Fusarium wilt fungal disease	Developing phytopathology assay protocol for resistance	Phytopathology and breeding
Taste	Component analysis	Quality
Agronomical traits (leaf shape, jaggedness, color of the leaves)	setting of new agronomic selection criteria	Breeding
Mildew disease	Developing phytopathology assay protocol for resistance	Phytopathology and breeding
Abiotic stress resistance	Water content and dry matter measurement, resistance to salt stress	Quality

The first aim during this PhD project has been to set up a breeding program for a crop of which very little was known, both in the company and for the short history of breeding for this species.

The first objective was to obtain tools to make breeding more specific and detailed, and not based on mass selection of populations on agronomic basis. The most useful tools was the setting of a crossing method and its verification by means of a hybridization marker, the tools for agronomic selection and phytopathology protocols.

The phytopathology protocols developed during the PhD are currently used in the selection programs, which made it possible to obtain material tolerant to *Fusarium oxysporum* f.sp.*raphani.* Some of the plant selected in the early stage of breeding were already advanced in the subsequent stages of screening and evaluation. Besides, other most segregating materials will be selected starting from this phytopathological assay.

Regarding the phytopathological test for resistance to *Hyaloperonospora parasitica*, the results have not yet led to the achievement of populations resistant to this pathogen. This is due to the lack of resistant genotypes in the screened germplasm, all of which were found to be susceptible to downy mildew. Therefore, the first problem to be solved is to obtain new genotypes, including wild ones, from which resistance genes can be found. It is also necessary to clarify the nature of the strains used and the creation of a reference sextet useful for strains classification, exploiting *Bremia lactucae* sextet as a model.

Three new rocket varieties (Fig.31) will be soon added in the ISI Sementi commercial range, that have been selected for improved agronomical traits, like more jagged leaves, better cutting angle, fast growth and tolerance to fusarium wilt.

- RB0013 is a wild rocket OP variety suitable for autumn-winter cultivation with good leaf shape and jaggedness, intermediate tolerant to Fusarium wilt.
- RB0014 is a wild rocket OP variety suitable for spring-summer cultivation with optimal leaf jaggedness, fast growth. Low resistance to Fusarium wilt compared with RB0015.
- RB0015 is a wild rocket OP variety suitable for spring-summer cultivation, susceptible to low temperatures, fast growth in summer and good tolerance to Fusarium wilt.

Figure 26 RB0013 (3), RB0014 (4) and RB0015 (5) in cultivation in ISI field

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