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In Vitro Propagation by Axillary Shoot Culture and Somatic Embryogenesis of *Daucus carota* L. subsp. *sativus*, 'Polignano' Landrace, for Biodiversity Conservation Purposes

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Abstract: Carrots are a multi-purpose vegetable; hence, they have become a very popular consumers' choice, also thanks to their nutritional value with health benefits. Several varieties and landraces exist of carrot (*Daucus carota* L. ssp. *sativus*). In Apulia (Italy), for example, a multicolored carrot landrace is present, the Polignano carrot (PC), threatened by the risk of genetic erosion. With the purpose of verifying the possibility of using in vitro culture for ex situ conservation purposes of PC, a rapid and reliable protocol for micropropagation and a highly efficient somatic embryogenesis protocol have been set up. For micropropagation studies, axillary buds from taproot were used as initial explants. Supplementation on basal medium (BM, a Murashige–Skoog modified medium) with low cytokinine (6-benzyladenine, BA, 0.22 μM) dosage was enough to obtain a good multiplication rate, also depending by the PC color. Rooting of proliferated shoots occurred easily in hormone-free medium and acclimatization was successfully reached in 6 weeks. Somatic embryos were formed from embryogenic callus induced by taproot sections placed on 2,4-dichlorophenoxyacetic acid (2,4-D)-added medium. After in vitro conversion in hormone-free medium, somatic embryos were also successfully acclimatized. Both microplants and somatic embryos produced true-to-type taproots (multicolored carrots), demonstrating the suitability of in vitro techniques for biodiversity conservation purposes.

Keywords: carrot; in vitro culture; somatic embryos; endangered species; ex situ conservation

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1. Introduction

Carrot (*Daucus carota* L. ssp. *sativus*) is a biennial herbaceous species, belonging to the Apiaceae family and one of the most cultivated vegetables in the world [1]. Carrots are a multi-purpose vegetable; hence, they have become a very popular consumers' choice, also thanks to their nutritional value with health benefits. In fact, carrots are one of the richest source of carotenes, providing a significant amount of dietary vitamin A, other vitamins (ascorbic acid, thiamine, riboflavin and niacin) and minerals [2]. In addition to these nutrients, black carrots contain anthocyanins, conferring an increased nutraceutical value [3].

The black or purple carrot (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) is considered the first domesticated carrot, with the primary domestication center in Central Asia (Afghanistan) [4–6]. By genetic mutation of the purple carrot, a yellow carrot originated and there was distribution of both towards the Mediterranean area, as early as the 10th century [4]. Since the 17th century, the orange carrot, a mutation from the yellow type, has taken the place of the purple and yellow types in Europe, through human preference and selection [4,5,7].

By the end of 13th century, carrot cultivation was documented in Italy too, probably thanks to the trades Venice undertook with Middle Eastern countries [7]. Moreover, it can be hypothesized that through Venetians, carrots (purple and yellow) came to the

Apulia region (South Italy), where their cultivation has been documented since the 18th century [8]. Nowadays, only a few local landraces of multicolored carrot are present in Apulia, threatened by the risk of genetic erosion [9]. The yellow–purple carrot of Polignano is a multicolored carrot which grows in the municipality of Polignano, near Bari (Apulia region). This carrot landrace is a mixed population of *Daucus carota* L. ssp. *sativus*, with variable pigmentation, from yellow to orange and to purple, combined differently in the cortex and in the core [3].

The need to preserve the local plant genetic biodiversity has pushed the Apulia Region Administration to launch and fund, years ago, specific projects (under the 2007–2013 Rural Development Program), in which also the Polignano carrot (from here onward named PC) was included [9,10].

The conservation strategy of endangered species is usually accomplished by in situ collection. However, out of the suitable area, ex situ conservation can be considered a complementary conservation strategy to support and integrate the programs for the conservation of plant biodiversity, with the aim to reintroduce the plants in their natural habitats [11]. Among the different approaches for ex situ plant conservation, in vitro culture techniques are of great interest for the collection, multiplication and storage of plant germplasm [11–13]. Biotechnological methods, such as micropropagation and in vitro regeneration, are valuable means for conservation of endangered species [14]. Propagation (including micropropagation) of endangered species and landraces is an important tool, not only for conservation purposes, but also as a means to maintain the genetic diversity which can be useful in genetic improvement programs [15].

Micropropagation is the in vitro propagation of a plant which is cloned in a high number of individuals, using synthetic growth media and an artificial environment [16]. This technique has been applied to the propagation of both plant crops and endangered species [13,17]. The advantage of this technique is the large-scale propagation, and the reduced space requirements necessary to grow a large number of plants, in a sterile environment, without need for pest control [16].

To obtain a large-scale propagation of individuals, the plantlet can derive from a pre-existing bud which is induced to grow and multiply, or through an adventitious regeneration process (organogenesis or somatic embryogenesis) [18]. Micropropagation through axillary bud is a two step-process: the first is to obtain the axillary shoot proliferation, and the second one is for rooting and obtaining a complete plant [19]. The same process is used for adventitious bud regeneration through organogenesis. Somatic embryogenesis, on the other hand, allows the regeneration of a complete individual, provided by both the shoot meristem and the root meristem at the same time. Both micropropagation and somatic embryogenesis can be considered clonal propagation, unless genetic modifications (somaclonal variation) occur [20].

The aim of the present work was to verify the possibility of using in vitro culture for ex situ conservation purposes of PC. To this aim, (i) a rapid and reliable protocol for in vitro propagation by axillary shoots, and (ii) a high-efficiency somatic embryogenesis protocol, have been set up.

2. Materials and Methods

2.1. Plant Material

Taproot of carrots (a mixed population of *Daucus carota* L. ssp. *sativus*), belonging to the 'Polignano' carrot (PC) landrace were provided by smallholder farmers of the area near Polignano (Bari, Italy).

2.2. Culture Establishment and Micropropagation by Axillary Shoots

For each color type (yellow, orange and purple), three to five taproots were considered, and the apical part (3 cm long, approximately) was cut and placed in a container filled with tap water, leaving the collar out of the water. After the buds at the collar developed (nearly 15 days), the developing shoots were then sterilized with 1.4% sodium hypochlorite (NaClO)

for 15 min, following extensive washing with sterile water. The explants (developing shoots) were transferred (a total of 12/each color) to a basal medium (BM, a Murashige–Skoog modified medium, see Supplementary Table S1), supplemented with 6-benzyladenine (BA), at 0.22 and 0.44 μM; this is referred to as multiplication medium (MM).

Three subsequent subcultures (lasting three weeks) were performed on the same MM, and several parameters (number of newly axillary formed shoots, length and number of leaves per each shoots) were recorded for each subculture.

After three subcultures, around 40 microcuttings of each color were transferred to a rooting medium (RM), composed by BM added with different doses of α-naphthaleneacetic acid (NAA) (0, 2.68 μM, 5.37 μM). The percentage of rooted microplants, number and length of the roots were measured after four weeks on rooting medium.

2.3. Regeneration via Somatic Embryogenesis

The median part of the taproot (each color) was used to obtain transversal slices (2 cm thick), which were sterilized with NaClO (1.4%) for 30 min, following extensive washing with sterile water. Afterwards, from each taproot slice, triangles (around 1.5 mm long, 16 mm^2) were obtained, all containing phloem, meristematic tissue and xylem, and placed on embryogenic media (EM) composed by BM or Murashige and Skoog medium (MS) [21] added with different 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations (0, 2.26, 4.52 μM).

After 30 days of incubation, the developed callus was transferred to the same BM or MS medium lacking plant growth regulators (PGRs). The percentage of callus induction as well embryogenic callus induction were recorded after 30 days. After transferring of the embryogenic callus on PGRs-free medium, the somatic embryos (SEs) induction time was considered, as the first-time SEs induction occurrence (days).

2.4. Culture Conditions

The MM and RM contained 2% sucrose, while EM contained 3% sucrose.

All the media had the pH adjusted to 5.7 with 1M KOH before adding 0.7% (*w*/*v*) agar (Oxoid), and then they were autoclaved at 121 °C for 20 min. All in vitro cultures were maintained in a growth chamber at 22 ± 1 °C, under a light intensity of 50 mmol m^{-2} s^{-1} provided by Philips TLD/83 white fluorescent tubes, positioned 40 cm above the level of the cultures, with a 16 h photoperiod. Subcultures were performed every three weeks.

2.5. Acclimatization and Transfer of Micropropagated and Regenerated Plants to the Field

Four week-old rooted microplants were removed from in vitro containers, gently rinsing the radical apparatus in distilled water, then transferred to plastic pots containing peat and vermiculite (3:1 *v*/*v*), and covered with transparent plastic. Plantlets were grown in greenhouse under a natural light environment at 25 ± 1 °C (day) and 18 ± 1 °C (night). Relative humidity varied from 85–90% until 50–60% over 20 days.

All acclimatized plantlets (both micropropagated and regenerated) were then transferred to field conditions, at the Department of Soil, Plant and Food Science of the University 'A. Moro', Bari (Italy), to monitor the production and phenotyping characteristics.

2.6. Statistical Analysis

For each carrot color and in a triplicate experiment for each stage, the following number of explants have been considered. At the culture establishment stage, 12 developing buds were considered. At the multiplication stage, around 40 microcuttings were transferred to rooting medium. Twenty microplants were grown in a greenhouse for acclimatization, and at least 10 plants were transferred to the field. For somatic embryos, around four carrots (each color) were considered, obtaining slices and triangles to be put in culture (96 triangles/each color).

Data were subjected to analysis of variance (ANOVA), using the CoStat software Version 6.40.

The Student Newman Keuls (SNK) test ($p \leq 0.001$) was used to compare the means of the different treatments. Before ANOVA analysis, percentage data were subjected to angular transformation.

3. Results

3.1. Micropropagation by Axillary Bud Development

3.1.1. Culture Establishment and Shoot Proliferation

For each carrot color (yellow, orange and purple), after two weeks, the already existing buds at the collar developed enough in size (nearly two cm) to be used as an initial explant for in vitro establishment (Figure 1A). After sterilization, the developing shoots were placed on MM (Figure 1B).

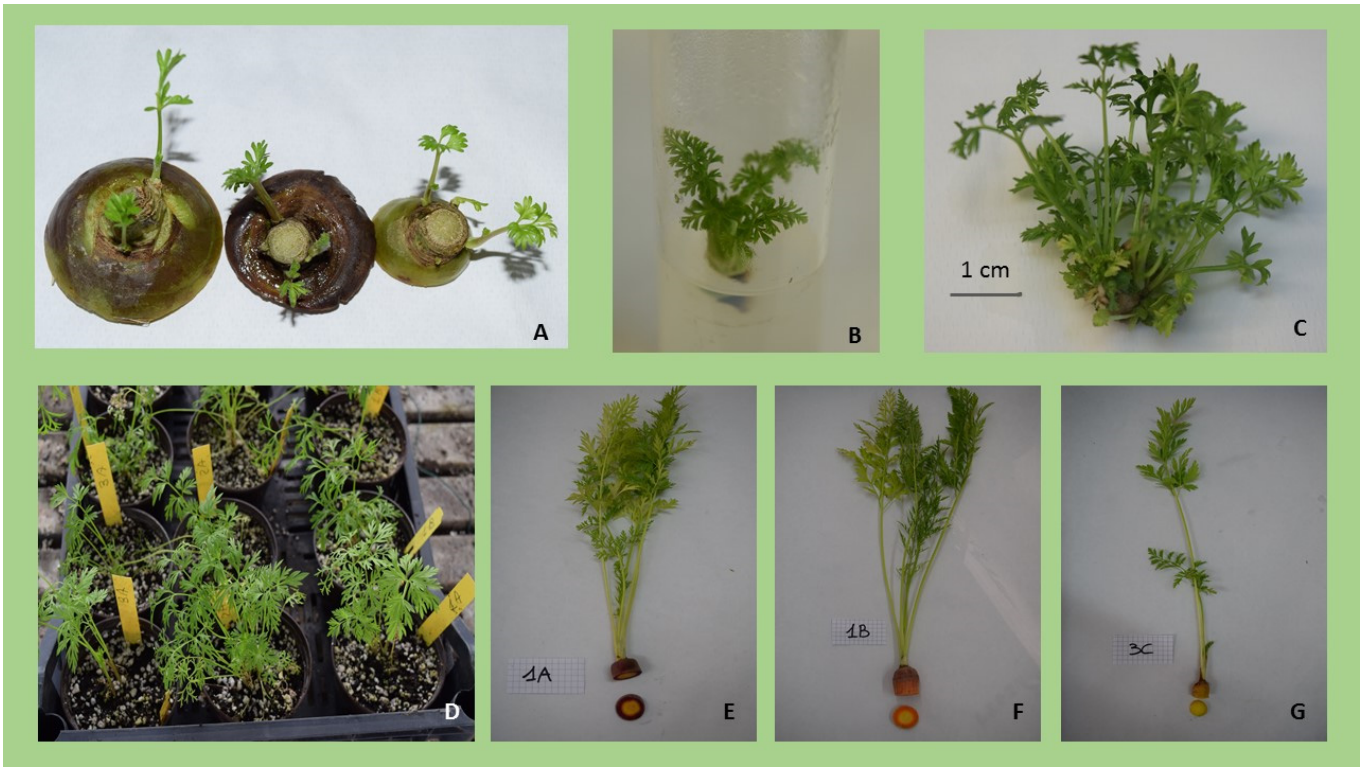

Figure 1. Micropropagation steps in *Daucus carota* L. ssp. *sativus*, Polignano landrace (PC). (**A**) Developing buds at the collar. (**B**) First step of shoot proliferation. (**C**) Shoot cluster developing on MM. (**D**) Acclimatized plants in pots. (**E**–**G**) Taproots of the three colors produced by field grown-micropropagated plants.

The best results for carrot axillary shoot development were obtained with the lowest BA concentration, that is 0.22 µM (Figure 1C), in respect to the highest dose (0.44 µM), which reduced significantly the proliferation index by nearly a half (from 50 to 60 %) in all the three colored carrots (Table 1).

Table 1. Effect of 6-benzyladenine (BA) concentration on proliferation and leaves features in *Daucus carota* L. ssp. *sativus*, Polignano landrace (PC) microcuttings.

Color	BA (µM)	MMI * (n)	Leaves Number	Leaves Length (cm)
Orange	0.22	4.27 ab	9.3 a	4.3 a
	0.44	2.30 c	7.7 a	4.1 a
Yellow	0.22	6.08 a	7.0 a	4.6 a
	0.44	3.09 c	7.0 a	4.5 a
Purple	0.22	4.38 ab	8.7 a	4.2 a
	0.44	2.65 c	8.3 a	4.3 a

MMI * = Mean multiplication index was calculated as the mean number of microcuttings obtained during one subculture. Data were a mean of three subcultures. Different letters for each column indicate significant differences (SNK test at $p \leq 0.001$). No significant differences were observed in the leaves' number and length.

3.1.2. Rooting and Acclimatization

Proliferated shoots were isolated from the cluster and transferred to a rooting medium containing NAA at different concentrations. After four weeks, roots were well formed and the average rooting percentage was 79% for orange, 89% for yellow and 66% for purple carrot, with no significant difference among the different NAA concentrations (Table 2). In addition, the number and length of roots were independent from the hormonal treatment.

Table 2. Effect of α-naphthaleneacetic acid (NAA) concentration on rooting and roots features in *Daucus carota* L. ssp. *sativus*, Polignano landrace (PC) microcuttings.

Color	NAA (µM)	Rooting (%)	Root Number	Root Length (cm)
Orange	0	78 b	4 a	3 a
	2.68	80 b	3.9 a	3 a
	5.37	80 b	4.2 a	2.9 a
Yellow	0	89 a	4.2 a	2.8 a
	2.68	88 a	4 a	3.1 a
	5.37	90 a	4.3 a	3 a
Purple	0	65 c	3.9 a	3.1 a
	2.68	66 c	3.8 a	3.2 a
	5.37	69 c	4.1 a	2.9 a

Different letters for each column indicate significant differences (SNK test at $p \leq 0.001$). No significant differences were observed in the root number and length.

After four weeks in rooting medium in vitro, all plants were transferred to plastic pots in a controlled greenhouse. After six weeks, the acclimatization process was considered complete (Figure 1D) and three parameters were calculated, as reported in Table 3. The micropropagated plants had a true-to-type phenotype, as the newly formed taproot was the same color of the donor plant (Figure 1E–G).

3.2. Somatic Embryogenesis Regeneration

3.2.1. Embryogenic Callus Induction and Somatic Embryos Regeneration

The sterile culture of taproot carrot, of different colors, was successfully established (Figure 2C).

Table 3. Growth parameters of acclimatized plants of *Daucus carota* L. ssp. *sativus*, Polignano landrace (PC), from microplants and somatic embryos, after six weeks acclimatization.

Color		Survival Rate (%)	Leaves Number	Leaves Length (cm)
Orange	Microplants	93 a	14.3 a	6.0 a
	Somatic embryos	91 a	13.9 a	6.6 a
Yellow	Microplants	91 a	13.3 a	5.9 a
	Somatic embryos	91 a	13.7 a	6.2 a
Purple	Microplants	90 a	14.0 a	6.4 a
	Somatic embryos	92 a	14.2 a	6.3 a

Different letters for each column indicate significant differences (SNK test at $p \leq 0.001$).

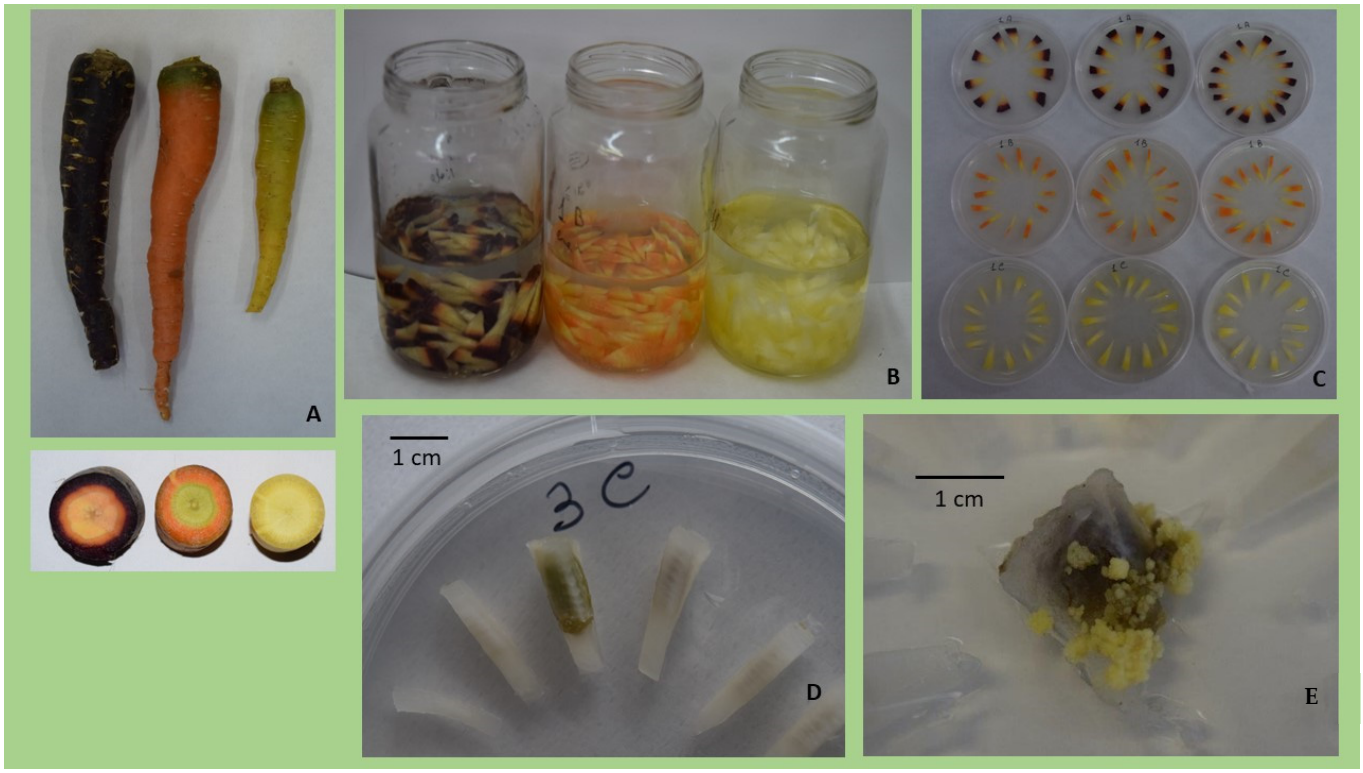

Figure 2. Somatic embryogenesis process in *Daucus carota* L. ssp. *sativus*, Polignano landrace (PC), initial steps. (**A**) PC taproot features, three colors. (**B**,**C**) PC explants before and after in vitro set up. (**D**) Starting callus proliferation. (**E**) Embryogenic callus proliferation.

After 30 days incubation, the explants started to show callus proliferation (Figure 2D), differently on BM or MS, and at a different level depending on the 2,4-D concentration (Table 4). Callus proliferation was much higher in MS- than BM-based medium, in all the carrot colors. The highest callus proliferation was obtained at the highest 2,4-D concentration (4.52 µM), reaching 90% in the orange carrot (Table 4). Not only callus proliferation was considered but also the embryogenetic capability of the newly formed callus. Only MS-based medium produced embryogenic callus (callus which was able to develop embryos when transferred on the suitable medium) (Figure 2E). Only the highest 2,4-D concentration gave a high % embryogenic callus induction (Table 4).

Table 4. Callus proliferation from *Daucus carota* L. ssp. *sativus*, Polignano landrace (PC) taproot explants after 30 days incubation.

Color	Medium	2,4-D (µM)	Callus Induction (%)	Embryogenic Callus Induction (%)
Orange	BM	0	0 f	0 d
		2.26	10 e	0 d
		4.52	12 e	0 d
	MS	0	0 f	0 d
		2.26	40 c	5 b
		4.52	90 a	85 a
Yellow	BM	0	0 f	0 d
		2.26	10 e	0 d
		4.52	18 de	0 d
		0	0 f	0 d
		2.26	60 b	8 b
		4.52	88 a	81 a
Purple	BM	0	0 f	0 d
		2.26	13 e	0 d
		4.52	20 de	0 d
		0	0 f	0 d
		2.26	25 d	2 c
		4.52	76 ab	73 ab

Different letters for each column indicate statistically significant differences (SNK test at $p \leq 0.001$). BM: basal medium, as reported in Table 1; MS: Murashige and Skoog [21] medium.

After transferring of the embryogenic callus on the PGRs-free medium (expressing medium, EM), somatic embryos (SEs) developed (Figure 3A,B) and the time of first day SEs appearance was recorded (Table 5). This stage was observed between 20 and 30 days for all the colors, with the highest percentage SEs in the yellow carrot explants (95%) and 34 embryos produced *per* explant (Table 5). Only the best developed and coetaneous embryos were counted and their growth was followed after three weeks of culture in the same EM (Figure 3C). Again, yellow carrot produced the better performing SEs, in terms of leaves and roots development (Table 5).

Table 5. Somatic embryogenesis process features and somatic embryos (SEs) characteristics in *Daucus carota* L. ssp. *sativus*, Polignano landrace (PC).

Color	SEs Induction (Day)	SEs (%)	SEs/Explant	Leaves Number	Leaves Length (cm)	Root Number	Root Length (cm)
Orange	22	83 ab	28 a	5.4 a	4.7 a	3.1 a	2.2 a
Yellow	24	95 a	34 a	6.3 a	4.9 a	3.3 a	2.4 a
Purple	28	72 b	21 b	5.9 a	4.4 a	3.1 a	2.1 a

Different letters for each column indicate statistically significant differences (SNK test at $p \leq 0.001$).

3.2.2. Acclimatization

After four weeks in EM in vitro, the embryo-derived plantlets were ready to transfer to plastic pots in a controlled greenhouse. As in the case of the microplants, after six weeks the embryo-derived plantlets were fully acclimatized (Figure 3D) and some parameters were considered, as reported above in Table 3. Furthermore, the somatic embryos produced a true-to-type phenotype, as the newly formed taproots showed the same color of the donor plant (Figure 3E–G).

Figure 3. Somatic embryogenesis process in *Daucus carota* L. ssp. *sativus*, Polignano landrace (PC), later steps. (**A**) Somatic embryos converting into plantlets. (**B**) Somatic embryo-derived plantlet. (**C**) Somatic embryo-derived fully developed plantlets. (**D**) Somatic embryos-derived plants acclimatized in pots. (**E**–**G**) Taproots of the three colors produced by field grown-plants derived from somatic embryos.

4. Discussion

In vitro culture of carrots has been studied since the first reports of Steward (1958) [22] and Reinert (1959) [23], and the carrot later became a model system for somatic embryogenesis [24]. In addition to representing a model system for studying the plant cell totipotency and the mechanisms for regeneration of somatic embryos, in vitro carrot culture can be used in conservation programs of rare or endemic genotypes, as in other crop plants [25,26]. In this meaning, in vitro culture can be applied to the 'Polignano' carrot (PC) landrace studied here.

The experiments on axillary shooting, starting from the already existing shoot buds at the collar, allowed us to define the optimal conditions for the first stage of micropropagation. In all the three color of PC, the best results for axillary shoot development were obtained with the lowest BA concentration (0.22 µM), while the highest BA dose (0.44 µM) reduced significantly the proliferation index by nearly a half (from 50 to 60%), in all the three carrot colors. This behavior is different from the results on *D. carota* L. subsp. *halophilus* shoot proliferation from in vitro seedlings [26]. In that study, increasing BA concentration (from 0.44 µM and higher) resulted in a multiplication index increase, with values similar to the values obtained in our experiments at the lowest BA concentration (0.22 µM). This could be due to the different endogenous hormonal features in the two species. We hypothesized that PC carrot does not need high cytokinins dosage, as it probably has an endogenous high cytokinins level.

Rooting of carrot shoots occurred easily on MS with or without PGRs. Additionally, in *D. carota* L. subsp. *halophilus*, increased auxin concentration (in that case IBA) did not gave higher rooting percentage [26]. Likewise, rooting in medium lacking PGRs has already been reported [26,27].

Acclimatization of microplants was successful, and the following year, a taproot with the same color of the donor plant was produced, demonstrating a true-to-type phenotype. This feature is particularly important within micropropagation protocol, when the goal is to propagate an elite genotype, as in the case of PC landrace. Clonal stability of micropropagated plants, in fact, is a prerequisite to maintaining the advantages of desired elite genotypes [28].

Our results on the induction of somatic embryogenesis indicate that PC is also able to differentiate somatic embryos, as a normal *D. carota* cell line. Somatic embryogenesis has been described in the carrot since the early studies of Reinert (1959) [23] and many others (see [29] and reference therein), as the developmental process through which a somatic cell gives origin to a complete embryo, capable of developing into a whole plant. Two patterns can be expressed in vitro: direct embryogenesis, where embryos are directly formed from the explant, and indirect embryogenesis, where callus proliferation precedes the somatic embryo development. This latter is the case of PC. Callus proliferation is clearly observed after few weeks of incubation of PC taproot explants on media added with 2,4-D (Figure 2D).

The synthetic auxin 2,4-D is a pre-requisite hormonal treatment for the induction of somatic embryogenesis [30]. The indirect somatic embryogenesis process described here is based on one month treatment with 2,4-D (induction phase of the callus), followed by a development stage of the somatic embryos on a medium lacking PGRs. Some authors prefer to follow the direct somatic embryogenesis procedure, with somatic embryos directly arising from the original explant tissue, after a short exposure to high dosage of 2,4-D, followed by a development step [31]. This is because the long exposure of donor tissue to the synthetic auxin can cause genetic variation in the newly-formed callus cells [32]. However, recent studies contribute to better define the regeneration processes, including somatic embryogenesis [18,33]. In our experiments, we did not find variation, at least at the phenotypic level; in fact, the color of the regenerated taproots arising from somatic embryos was the same as the donor plants.

5. Conclusions

The biotechnological manipulation applied to the PC landrace has demonstrated the possibility of using in vitro culture for ex situ conservation purposes of PC. In fact, both microplants and somatic embryos have produced true-to-type taproots (multicolored carrots), demonstrating the suitability of in vitro techniques for biodiversity conservation purposes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8121150/s1; Table S1: Basal Medium (BM) composition.

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