horticulturae

MDPI

Review

Somatic Embryogenesis in Spinach—A Review

Snežana Zdravković-Korać *, Maja Belić, Dušica Ćalić and Jelena Milojević

Department of Plant Physiology, Institute for Biological Research "Siniša Stanković"—National Institute of the Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia; mmilic0905@gmail.com (M.B.); calic@ibiss.bg.ac.rs (D.Ć.); jelena.milojevic@ibiss.bg.ac.rs (J.M.)
* Correspondence: szk@ibiss.bg.ac.rs

Abstract: A spinach-supplemented diet exerts numerous health benefits, but high levels of oxalic acid and nitrate can cause medical problems, so their levels should be reduced, while the levels of vitamins and phytochemicals could be further increased by breeding. Conventional spinach breeding is limited by the very complex sex determination. However, these limitations could be circumvented in synergy with a biotechnological approach. Accordingly, tissue culture techniques allow rapid and efficient clonal propagation of selected valuable genotypes, and somatic embryogenesis has been recognized as a superior process for clonal propagation because somatic embryos resemble zygotic embryos and therefore can spontaneously develop into complete plants. Since spinach has been considered recalcitrant to in vitro regeneration for decades, a deeper insight into the mechanisms underlying somatic embryogenesis is important for a better understanding and further improvement of the efficiency of this process. In this review, a comprehensive overview of the major factors affecting somatic embryogenesis in spinach is presented and discussed, with particular emphasis on the synergistic effects of α-naphthaleneacetic acid, gibberellic acid, light, and the intrinsic predisposition of individual seedlings to somatic embryogenesis, as well as the expression of genes encoding key enzymes involved in the maintenance of gibberellin homeostasis and the levels of endogenous gibberellins.

Keywords: Amaranthaceae; gene expression; gibberellins; somatic embryogenesis; *Spinacia oleracea* L.; tissue culture

Citation: Zdravković-Korać, S.; Belić, M.; Ćalić, D.; Milojević, J. Somatic Embryogenesis in Spinach—A Review. *Horticulturae* **2023**, *9*, 1048. https://doi.org/10.3390/horticulturae9091048

Academic Editor: Barbara Ruffoni

Received: 9 August 2023
Revised: 10 September 2023
Accepted: 14 September 2023
Published: 19 September 2023

1. Introduction

Spinach (*Spinacia oleracea* L.) is an economically important green leafy vegetable grown worldwide. It is considered a functional food [1] because spinach leaves are rich in vitamins, minerals, phytochemicals, and dietary fiber [1–6]. Due to its increasing popularity, spinach production and consumption have increased significantly in recent decades, especially baby spinach, which is consumed mainly as an unprocessed food [7]. In addition, powdered spinach leaves are often used in the production of cheese and bread to improve their nutritional value, antioxidant properties, color, and taste [8–11].

Spinach leaves are a rich source of chlorophylls and carotenoids [5], and aqueous extracts from the leaves exhibit exceptionally potent antioxidant activity, surpassing that of green tea, lettuce, cabbage, onions, and even pure vitamin E [12,13]. A spinach-supplemented diet therefore has numerous health-promoting effects [1], such as anti-cancer and anti-angiogenic effects [14], the alleviation of inflammatory vascular diseases [15], the retardation of age-related decline in cognitive and motor functions of the nervous system [13,16], and the prevention of diabetic complications [17,18]. In addition, the consumption of raw spinach has been shown to promote the diversification of the gut microbiota, contributing to the maintenance of intestinal homeostasis and the prevention of chronic diseases [19–24].

However, the consumption of spinach also has some negative aspects. Spinach plants contain high levels of oxalic acid [25], which can lead to the formation of kidney stones [26],

and nitrate [27], which can cause methemoglobinemia [28]. High variability in spinach germplasm has also been observed in terms of the nutritional and chemical composition, such as ascorbic acid [6], folate [3], total phenols and flavonoids [29], oxalate [25], and carotenoids and chlorophylls [5]. All of these traits may vary depending on the growing and storage conditions, but all have a significant genetic background. Therefore, the most important goals in spinach breeding programs, in addition to disease resistance, abiotic stress tolerance, and delayed bolting, are to improve the nutritional quality [6,7,30–32].

Numerous accessions of the genus *Spinacia* are maintained in collections worldwide [7,30,33,34], including cultivars and landraces of cultivated spinach (*S. oleracea*) and wild populations of *S. turkestanica* and *S. tetrandra*, sexually compatible relatives of cultivated spinach [30,34]. These wild species are of paramount importance for breeding purposes because they are a valuable source of genes that confer disease resistance and agronomically important traits [7,30]. This is particularly important because intraspecific genetic variation in cultivated spinach is low [7].

Recently, a high-quality genome assembly of two spinach inbred lines, Sp75 [35] and Monoe-Virofly [36], was generated, and SpinachBase (available at http://spinachbase.org/ accessed on 27 June 2023) was established [37]. In addition, numerous markers for marker-assisted selection have been developed [38–43], providing an excellent basis for facilitating spinach breeding and enabling gene expression profiling [36].

However, conventional spinach breeding is limited because of the very complex sex determination and plasticity of sex expression in plants [2]. Spinach is a dioecious plant species, but monoecious plants bearing both pistillate and staminate flowers as well as hermaphrodite flowers bearing both pistils and stamens also occur in spinach populations [2,44–47]. Dioecious plants often show so-called "leaky" sex expression, i.e., they occasionally produce some flowers of the opposite sex, which is enhanced by external stimuli [48], or in the absence of specimens of the opposite sex [49]. This is also found in spinach plants [2], as a variety of external factors can alter the ratio of male to female plants or trigger plant sexual instability [44,45,50], including temperature [46,47], light conditions [51], plant growth regulators [50], ion particles [52], and in vitro culture [53–55]. Sex determination in spinach has not been fully elucidated, although numerous molecular markers associated with sex-determining genes have been found [56–59], and a high-density linkage map covering these loci has been constructed [60]. A male-specific candidate gene (*NRT1/PTR 6.4*) has been proposed to drive stamen initiation/carpel repression [61], while *GIBBERELLIC ACID INSENSITIVE*, a member of the DELLA transcriptional repressor family, has been shown to be necessary for female organ development by repressing genes required for stamen development [62]. These findings may explain the sexual plasticity previously observed in spinach under ex vitro and in vitro cultivation.

In addition to the complex sex determination in spinach, conventional breeding is a time- and labor-intensive process, but these limitations could be circumvented in synergy with biotechnological approaches [63]. Accordingly, tissue culture techniques allow the rapid and efficient clonal propagation of selected valuable genotypes and the creation of pure lines in only a few steps and in much shorter time compared to conventional breeding [64]. Several processes enable the in vitro regeneration of plants, including de novo shoot organogenesis, somatic embryogenesis, and embryogenesis from immature male and female gametic cells (androgenesis and gynogenesis, respectively) [65]. Somatic embryogenesis is recognized as a superior process of clonal propagation because somatic embryos (SEs) resemble zygotic embryos and therefore can spontaneously develop into complete plants [66].

Spinach has been considered recalcitrant to in vitro regeneration for decades [67], and the protocols established to date are not generally applicable to all genotypes [68–71]. The first report of a tissue culture response in spinach was obtained by de novo shoot organogenesis from seedling shoot tips, but with very low efficiency [72]. It took almost twenty years to achieve the next success, when a plethora of reports appeared. Briefly, the system of micropropagation from shoot primordia was established [73], while de

novo shoot organogenesis was induced from hypocotyls [74–80], cotyledons [78,80–82], roots [78,83,84], leaves [68,85,86], mature dry seeds [87], and leaf protoplasts [88–91]. In addition, spinach calli are suitable for the establishment and maintenance of cell suspension cultures [92]. Androgenesis from spinach anther cultures and gynogenesis after pollination with irradiated pollen have also been reported [93]. These initial achievements in spinach tissue culture responses were described in several excellent reviews [67,94,95]. However, later research on the intrinsic predisposition of individuals to somatic embryogenesis, the synergistic interaction of α-naphthaleneacetic acid (NAA), gibberellic acid (GA_3), and light, and altered gibberellin homeostasis during SE induction provided deeper insights into the mechanisms underlying this process in spinach. Therefore, this review presents these new findings.

2. Somatic Embryogenesis

Somatic embryogenesis in spinach was achieved in parallel in two laboratories in 1993, one in France [96] and the other in the authors' laboratory in Serbia [97]. In the following decades, numerous reports have been published on this subject, and the mechanism of SE induction and the importance of the factors affecting this process have become better understood. However, the established protocols are still genotype-dependent and require further improvement, as a robust and generally applicable protocol for efficient and reliable SE induction is needed for further application in biotechnological crop improvement.

In all studies, somatic embryogenesis was induced indirectly via an intermediate callus phase [69,70,84,96–102]. Rarely was the direct formation of SEs from the leaves of SE-derived plants observed [98]. In some regeneration systems, somatic embryogenesis and de novo shoot organogenesis proceeded in parallel [84,96–99,101], while in the majority of studies only SEs were observed [69–71,100,102–105]. In some cases, shoot organogenesis preceded the formation of SEs [96–99], while Nguyen et al. [84] were able to alternately induce SEs or shoot buds from the same explant type by controlling the combination and concentration of plant growth regulators (PGRs). Interestingly, the same regeneration procedure using root explants resulted in the regeneration of shoot buds in some cultivars [83], while in others only SEs were formed [71,102,105]. It is also worth noting that explants of the same cultivar produced exclusively SEs in one procedure [71,102,105] and both SEs and shoot buds in another [97–99].

2.1. Factors Affecting Somatic Embryogenesis

Numerous factors significantly influence somatic embryogenesis in spinach. The most important effect on this process is the genotype, explant type, media composition (particularly the PGR combination, concentration, and treatment duration), and environmental factors such as light conditions and temperature. The effects of these factors on the initiation of SEs are presented on the following pages and summarized in Table 1.

To facilitate the understanding the text, the following terminology is used: "embryogenic capacity" is used as a general descriptive measure of the ability of explants to regenerate SEs, whereas "embryogenic response" indicates that explants are able to regenerate SEs in response to SE induction treatment. "Regeneration frequency" (defined as the proportion of explants regenerating SEs out of the total number of explants subjected to SE induction treatment) and "mean SE number" per explant are used to quantitatively describe SE regeneration.

2.1.1. Genotype

Genotype has been recognized as one of the most important factors that strongly influences not only somatic embryogenesis [69–71,102] but also callus induction [68] and the regeneration of shoot buds from calli or protoplasts [68,90]. The frequency of SE regeneration from seedling root segments varied widely among the cultivars tested [69,101]. A single study tested the frequency of SE regeneration in eight spinach cultivars: Jiromaru, Nihon, Hoyo, Minsterland, Virofly, King of Denmark, Nobel, and Ujo [69]. The frequency of

SE regeneration varied from 17.1% (in the cultivar Ujo) to 78% (in the cultivar Jiromaru) [69]. However, results obtained by the same research group in different experiments under the same experimental conditions showed high variability in the embryogenic response of seedling root segments within a highly responsive cultivar (Jiromaru), ranging from 36% to 78% [69,100,104]. Overall, these results suggest large differences in individual (seedling) response between and within seed lots. Indeed, a large variation in the embryogenic response of root sections isolated from 30 randomly selected seedlings of the cultivar Nippon was later found following the same regeneration protocol in the same laboratory [70]. Of 30 seedlings tested, explants from 10 seedlings had low, 13 had medium, and seven had high embryogenic capacity, with 20%, 20–70%, and 80% embryogenic explants per seedling, respectively [70].

A rather similar embryogenic response was observed for root tips of the cultivar Matador, previously considered extremely recalcitrant to in vitro regeneration [72]. Out of 30 randomly tested seedlings at the same developmental stage (4–5 leaves), root apices of 26 seedlings responded with SE regeneration frequencies ranging from 0.3% to 100%, while roots of four seedlings did not respond at all [102]. The mean SE number per explant in the responsive lines ranged from 0.001 to 9.96, and 227–347 SEs were obtained from 30 root explants of each of the four most responsive lines over 12 weeks [102]. The obtained SEs developed into plants, flowered, and set seed in vitro, so the embryogenic response of the resulting 69 seedlings was tested [102]. The progeny of the poorly responding parental lines always responded in a similar manner, with SE regeneration frequencies below 12% and up to 0.47 SEs per explant, while the lines with moderate embryogenic capacity (with SE regeneration frequencies of 20–70%) produced progeny with highly variable SE regeneration frequencies (0.86–98.5%) and mean SE numbers per explant (0.001–10.41). However, one line that showed moderate embryogenic response (35.3% and 8.74 SEs per explant, 115 SEs from 30 explants) resulted in progeny with high embryogenic capacity after 3–4 cycles of self-fertilization [102]. The root apices of these lines regenerated SEs at a frequency of 96.4–100%, with 40.6–68.3 SEs per explant, and produced 1547–2181 SEs from 30 explants over 12 weeks [102]. This was 13.4–19.0 times more than the mother plant and 4.5–6.3 times more than the explants of the best performing seedling randomly selected from the seed lot tested in the same study [102]. In fact, in later experiments, we tested the embryogenic capacity of almost one thousand randomly selected seedlings in different studies, and none of them showed even close embryogenic capacity compared to the four elite lines. Moreover, the above-mentioned highly responsive lines regenerated SEs much faster, starting from the fourth week of culture, while lines with low embryogenic capacity regenerated SEs most frequently only after 6–8 weeks of culture initiation [102]. Roots isolated from SE-derived plants behaved similarly to the roots of the corresponding seedlings, suggesting the temporal stability of this trait within the line [102]. The four elite lines obtained in this study were maintained through cycles of SE-induction from the roots of SE-derived plants, which retained a high embryogenic capacity for 5–7 years. In agreement with this, Ishizaki et al. [106] maintained root cultures of highly responsive lines on PGR-free medium and found that their embryogenic capacity was high and stable for at least 12 months.

Deeper insights into the intrinsic variability of the embryogenic capacity in spinach were obtained by studying embryogenic capacity at both population and individual (single seedling) levels in the cultivar Matador [71]. Large differences were found among seeds of the Matador cultivar obtained from nine European seed companies from Slovenia (Sl), Poland (P), Serbia (Sr), England (E), Germany (G), Lithuania (L), Ukraine (U), Russia (R), and Italy (I). The frequency of seedlings with embryogenic capacity (i.e., seedlings whose root apices regenerated at least one SE) was highest in population Sl (100%), followed by P (98%), Sr and E (88% each), G (60%), L (58%), U (34%), R, and I (0% each) during a 12-week cultivation period [71]. The mean SE number per root explant, calculated at the population level, was also highest in the Sl population (14.4), followed by the P, Sr, and E (2.6–4.1), G, L, and U (0.3–0.6), and R and I (0 each) populations. The speed of regeneration of SEs

from root explants followed the same order as the frequency and mean SE number, from the fastest to the slowest: Sl, P, Sr, G, E, L, U, R, and I [71]. The first SEs were observed on Sl explants in the fourth week of culture initiation in 10.3% of Sl seedlings, and a 100% regeneration response was achieved in the seventh week of culture initiation.

At the seedling level, the Sl population was found to be composed of highly responsive seedlings, as 82% of Sl seedlings had 80–100% regenerating explants, whereas in the intermediate responsive populations (Sr, P, and E), 21–28% of seedlings were non-responsive and 40% of seedlings had 20–60% regenerating explants [71]. As expected, in the least responsive populations (G, L, and U), explants from 52–85% of seedlings were non-responsive, and explants from only up to 1.3% of seedlings regenerated SEs at a frequency greater than 60%. Accordingly, explants from Sl seedlings regenerated the highest number of SEs: 27.6% of seedlings regenerated more than 20 SEs per explant, 43.5% regenerated 10–20 SEs, and only 19.6% regenerated 1–10 SEs per explant [71]. In contrast, 73.8%, 59.7%, and 36.1% of P, E, and G seedlings, respectively, regenerated only 1–10 SEs per explant.

The observed large differences in the embryogenic capacity of seedlings may be due to genotypic effects, the physiological age of the seed at harvest, and postharvest storage conditions [107]. Because the seeds used in the above study were produced under different climatic conditions, the physiological age of the seeds at harvest is unknown, and the seeds were stored in different storage locations until shipment. Selected seed populations (Sl, Sr, and U) of the same seed lots used in the study by Belić et al. [71] were sown and grown under the same environmental conditions, and the embryogenic capacities of the root apices of the obtained seedlings were compared. Seeds from each seed population were sown in a separate planter, and each planter was placed in an isolated cage to prevent cross-pollination between plants from the different seed populations, as suggested by Morelock and Correll [2]. The root apices of the obtained seedlings showed a similar embryogenic response as that in the study by Belić et al. [71]: Sl seedling explants had the highest embryogenic capacity, Sr medium, and U very low (Figure 1) (Zdravković-Korać et al., unpublished results), indicating a strong influence of genotype on the embryogenic response [71]. Therefore, due to the significant variation between lots, it is strongly recommended to test the plant material (seed lot) prior to any research on somatic embryogenesis [71].

2.1.2. Explant Type

Numerous explant types have been used to induce somatic embryogenesis in spinach (Table 1). In most studies, seedling organs have been used as explants, not only because young organs generally respond more efficiently to induction treatments than older ones, but also because seeds are more robust than other plant organs and allow the use of more stringent sterilization procedures to successfully eliminate microorganisms [98,101].

Comparative studies have examined the frequency of SE induction from hypocotyls, cotyledons, and roots [69,101]. Seedlings were typically 8–10 days old, and sections were 5–8 mm long [69] or 400–500 µm thick transverse thin cell layer (TCL) sections [101] (Table 1). In addition, 2–3 mm long hypocotyl segments [96], the hypocotyl and middle part of cotyledons (5–10 mm), and leaf discs (5 mm) isolated from 1–2 month-old plants were also examined [69,98,99].

Of all explants, root sections responded most efficiently to the SE induction treatments [69,100,101,104]. In the cultivar Jiromaru, 75% of the root sections formed embryogenic callus, followed by the hypocotyl segments and basal cotyledon segments (28% and 16%, respectively), while the distal cotyledon segments formed hardly any calli and showed no embryogenic capacity [69]. In the cultivar Carpo, only root sections responded with a frequency of up to 25% in a series of treatments [101]. Interestingly, in the same study, in cultivar RZ1, only a few (0.79%) hypocotyl TCLs responded, while root and cotyledon TCLs did not [101]. Similarly, in the cultivars Matador and Virofly, hypocotyl-derived calli were more frequently embryogenic (3.7% and 5.7%, respectively) than cotyledon-derived calli (1.07% and 0%, respectively) [98]. However, in the cultivar Matador, leaf discs derived from

2-month-old greenhouse plants were the most responsive explants (83%) [98], whereas leaf segments isolated from 30-day-old in vitro-cultured plants of the cultivar Jiromaru showed no embryogenic capacity [69].

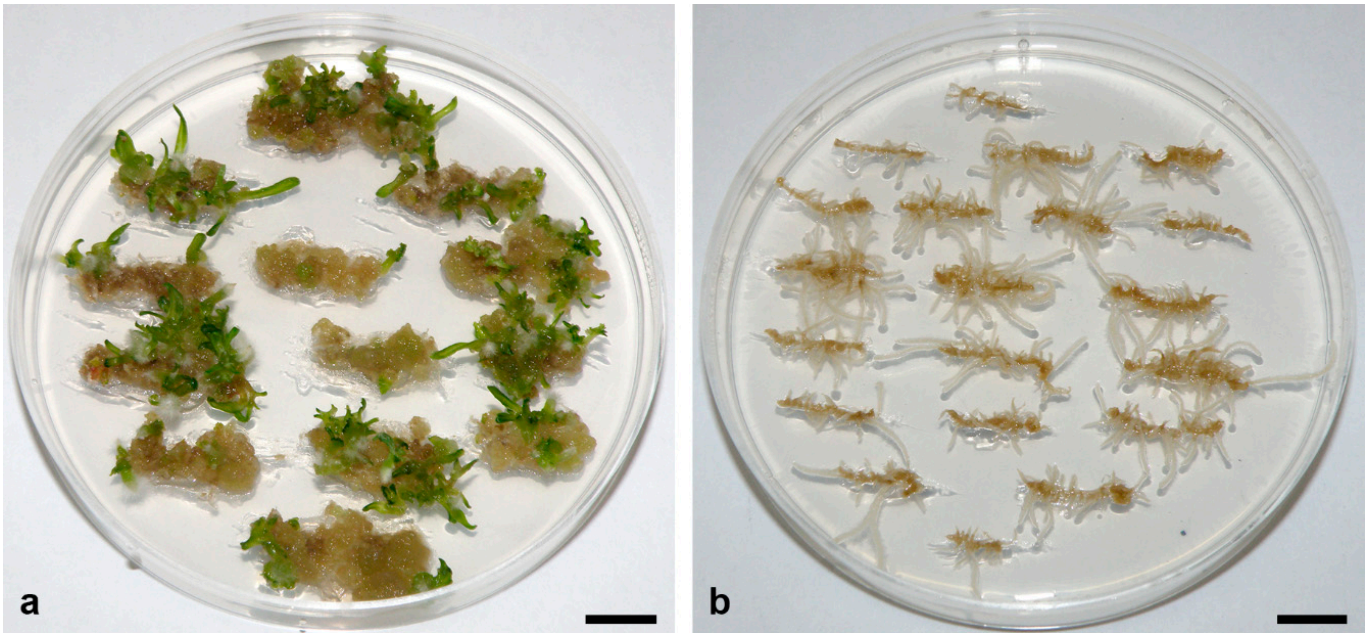

Figure 1. Regeneration of somatic embryos (SEs) from root apices of randomly selected seedlings from (**a**) Slovenian (Sl) and (**b**) Ukrainian (U) populations of the spinach cultivar Matador. Root apices were cultivated for eight weeks on MS medium supplemented with 20 μM NAA + 5 μM GA_3 under a long-day photoperiod (16 h of light) and a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1}. Regeneration of the SEs from Sl explants began from the fifth week of culture. Scale bar: 1 cm. Unpublished from the authors' laboratory.

Because root segments had the highest embryogenic capacity in most studies, they were the preferred explants in subsequent studies of somatic embryogenesis in spinach [70,71,84,102,105,108–110]. Moreover, some studies found that the regeneration response differed along the roots—the apical and middle fragments of the roots responded more strongly than the basal ones [83], which is why some authors used only the root apices [71,102,105,108–110]. However, Ishizaki et al. [70] used root sections (5 mm) from previously established root cultures grown on PGR-free medium, and Nguyen et al. [84] used 5–10 mm long main and lateral root fragments without root tips to induce somatic embryogenesis.

As mentioned above, organs from young seedlings 1–2 weeks old were most commonly used for culture initiation. However, to our knowledge, the effect of donor plant age on the SE regeneration capacity has never been studied in spinach, although it has been shown that cotyledons from five-day-old seedlings showed the best de novo shoot regeneration response compared to younger and especially older cotyledons that lacked the ability to regenerate shoots [81].

Somatic embryogenesis was also induced from protoplasts obtained from the cotyledons, roots, and hypocotyls of 10-day-old seedlings and from the leaves of 30-day-old plants of the cultivar Jiromaru [91].

Table 1. Summary of literature data on the induction of somatic embryogenesis from different types of explants isolated from different sources in spinach cultivars. The content of plant growth regulators in the media used for the induction of embryogenic callus and somatic embryo regeneration is shown in the table.

Cultivar	Explant Source	Explant Types Tested	Callus Induction	SE * Regeneration	Reference
Carpo	1-week-old seedlings	Hypocotyl segments (2–3 mm)	MS + 85.7 µM IAA + 100 µM GA_3 + 2% sucrose	MS + 11.4 µM IAA + 10 µM GA_3 + 2% sucrose	[96]
Carpo, RZ1	8-day-old seedlings	Cotyledon, hypocotyl, root (TCL: 400–500 µm)	MS + 100 µM NAA + 1 µM BA + 10 µM GA_3 + 5% sucrose	MS, PGR-free	[101]
Gyeowoonae	2-week-old seedlings	Root segments (5–10 mm)	1/2 MS + 10 µM NAA + 0.3 µM GA_3 + 2% sucrose	10µM NAA + 0.1 µM GA_3 + 2% sucrose	[84]
Jiromaru, Nihon, Hoyo, Minsterland, Viroflay, King of Denmark, Nobel, Ujo	10-day-old seedlings 30-day-old plants	Cotyledon, hypocotyl, root segments (5 mm each), leaf fragments (5 mm^2)	MS/2 + 10 µM NAA + 0.1 µM GA_3 + 1% sucrose	MS, PGR-free + 2% sucrose	[69]
Jiromaru	10-day-old seedlings	Root segments (8 mm)	MS/2 + 30 µM NAA + 100 µM GA_3 + 1% sucrose	MS, PGR-free + 2% sucrose	[100]
Jiromaru	10-day-old seedlings	Root segments (8 mm)	MS/2 + 30 µM NAA + 100 µM GA_3 + 29 mM fructose	MS, PGR-free + 59 mM sucrose	[104]
Matador	2-month-old plants	Leaf discs (5 mm)	MS + 4.4 µM 2,4-D + 4.6 µM Kin + 2% sucrose	MS + 4.6 µM Kin + 2% sucrose	[97,99]
Matador, Virofly	2-month-old plants, 10-day-old seedlings	Leaf discs (5 mm), hypocotyl segments (5–10 mm), middle part of cotyledons	MS + 4.4 µM 2,4-D + 4.6 µM Kin + 2% sucrose	MS + 4.6 µM Kin + 2% sucrose	[98]
Nippon	in vitro root culture	Root segments (5 mm)	1/2 MS + 10 µM NAA + 0.1 µM GA_3 + 5.4 g/L fructose	1/2 MS, PGR-free + 2% sucrose	[70]

* SE—somatic embryo; 2,4-D—2,4-dichlorophenoxyacetic acid; Kin—kinetin; IAA—indole-3-acetic acid; GA_3—gibberellic acid; NAA—α-naphthaleneacetic acid; BA—benzyladenine.

2.1.3. Media Composition—Mineral Elements

In most studies, a full or half-strength MS mineral solution was used for embryogenic callus induction in spinach (Table 1) [96–99]. However, Nitsch's and 1/2 MS solutions were found to induce the highest frequency of embryogenic response in root sections of the Jiromaru cultivar (51.1% and 37.8%, respectively) among the six mineral solutions tested: White's [111], MS [112], half-strength MS (1/2 MS), B5 [113], Nitsch's [114], and SH [115], whereas explants cultured on medium supplemented with White's mineral solution showed no embryogenic response [69]. The ratio of nitrate and ammonium in a mineral solution also had a significant effect on embryogenic response, and the highest frequency of embryogenic explants (62.2%) was obtained in the explants cultured at a nitrate:ammonium ratio of 2:1 [69]. The total nitrogen concentration (nitrate + ammonium) was optimal at a nitrate:ammonium ratio of 2:1 at 10–30 mM [69], which is why these authors used 1/2 MS mineral solution for embryogenic callus induction in later studies [69,70]. However, Leguillon et al. [101] compared the effects of MS and MW mineral solution, the latter containing White's macroelements, Nitch's microelements, and MS vitamins, and obtained better results with MW, although the regeneration response was generally low. For SE regeneration, the MS mineral solution was used in all studies (Table 1).

2.1.4. Plant Growth Regulators

The most common approach to induce somatic embryogenesis in plants is to administer auxin for a short period of time and then withdraw it to allow SEs to develop [116]. However, this approach failed in spinach. Komai et al. [100] tested several of the most commonly used auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), NAA, and 2,4-dichlorophenoxyacetic acid (2,4-D) at a wide range of concentrations (0.1–100 µM) and found that none of them was sufficient to induce somatic embryogenesis from root sections of the cultivar Jiromaru. Moreover, combinations of these auxins with a number of cytokinins: *trans*-zeatin [6-(4-hydroxy-3-methylbut-*trans*-2-enylamino) purine] (ZEA), 2-isopentenyl-adenine (2-iP), N^6-furfuryladenine (Kinetin, Kin), N^6-benzyladenine (BA), and N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) at 0.01–10 µM, did not yield satisfactory results, as only several combinations of NAA or 2,4-D with ZEA or 2-iP elicited embryogenic callus formation and SE regeneration, but with very low frequency (up to 16%). Other research has also tried numerous combinations of Kin with 2,4-D, IAA, or NAA and failed to induce a response in several explant types of the Carpo and RZ1 cultivars [96,101].

Calli derived from leaf discs and hypocotyls of the cultivar Matador on medium supplemented with 4.4 µM 2,4-D + 4.6 µM Kin for 8 weeks and then transferred to medium supplemented with 4.6 µM Kin, however, gradually transformed into a new callus type—a deep red, compact callus within the next 8–12 weeks [97–99]. The greenish and red callus types survived side by side in the same cultures, but SEs were observed only on the red parts of the calli. Thus, SEs developed only secondarily on organogenic calli grown on Kin-supplemented medium, in 33.3% of callus clones derived from leaves and 36.4% of callus clones derived from hypocotyls. Calli continuously cultured on callus induction medium containing 4.4 µM 2,4-D + 4.6 µM Kin were organogenic and retained their bud regeneration capacity for up to 10 months, whereas those cultured on Kin-supplemented medium were embryogenic and retained their embryogenic capacity for more than 7 years [98,99]. Repeated rounds of cultivation of red callus on callus induction and regeneration media always followed the same pattern: the formation of green undifferentiated callus on callus induction medium (4.4 µM 2,4-D + 4.6 µM Kin), which gradually became organogenic, and the subsequent formation of red callus, which regenerated SEs after transfer to medium supplemented with 4.6 µM Kin [98].

Moreover, an inverse relationship between callus growth and SE induction has been observed [98,99]. Similarly, an inverse correlation between callus growth and shoot bud regeneration has been observed in several spinach cultivars [74,85]. A good example of this observation is the differential effect of two structurally similar cytokinins, BA and Kin, on these processes. Compared with Kin, BA significantly promoted callus growth but suppressed SE differentiation and vice versa [98]. Consistent with this, 4 and 10 µM abscisic acid (ABA) in combination with BA or Kin decreased callus weight to 20–50% of controls but contributed to a further increase in SE number when combined with Kin. The highest SE number per 1 g of callus (678 SEs/g) was achieved with 4 µM ABA + 5 µM Kin and was almost twice as high as with 5 µM Kin alone (325 SEs/g) [99]. The effect of ABA suggests that the enhancement of somatic embryogenesis in its presence was probably a stress response of the cells, as the cells were prevented from dividing [117]. Conversely, the inhibition of somatic embryogenesis was observed when red embryogenic callus was cultured on BA- or Kin-supplemented media in combination with 0.3, 1, 3 or 10 µM GA_3, whereas GA_3 significantly promoted callus growth, especially in combination with BA [98,99].

However, in other regeneration systems, GA_3 has been found to be essential for SE induction (Table 1) [96,100,101]. Xiao and Branchard [96] applied IAA at high concentrations (85.7 or 48.6 µM) in combination with GA_3 (10 or 100 µM) to induce somatic embryogenesis from hypocotyl discs (2–3 mm) of one-week-old seedlings of the cultivar Carpo (Table 1). Embryo-like structures were observed on calli after only three weeks of cultivation on media supplemented with 85.7 µM IAA + 10 or 100 µM GA_3, but SEs were obtained from explants cultured on all of the above IAA/GA_3 combinations in callus induction medium

after an additional four-week period of cultivation. The highest frequency of embryo-forming calli was obtained in explants cultured on medium supplemented with 85.7 µM IAA + 100 µM GA_3 for seven weeks and then subcultured on medium containing 11.4 µM IAA + 10 µM GA_3 for four weeks (Table 1). In addition, subculturing the calli in liquid medium supplemented with 2.86 µM IAA + 10 µM GA_3 was beneficial for the release of SEs from the calli, but with a higher frequency (10%) of SE malformations [96].

Consistent with this, auxins, which were not sufficient to induce embryogenic callus formation from root segments of the cultivar Jiromaru, either alone or in combination with cytokinins, were able to induce somatic embryogenesis when combined with GA_3 [100], confirming the requirement of GA_3 for SE induction in spinach postulated by Xiao and Branchard [96]. However, GA_3 alone was not sufficient to induce cell proliferation or to initiate SEs (Figure 2a). The optimal amount of auxins and GA_3 varied among cultivars. In contrast to the cultivar Carpo, high concentrations of IAA (100 µM) and GA_3 (1–100 µM) were not effective for SE induction from root explants of the cultivar Jiromaru, with only 4–12% of explants responding with 9.0 SEs per explant [100]. In combination with GA_3, IBA and 2,4-D caused a rather low frequency (up to 20%) of embryogenic callus formation, while 10 and 30 µM NAA proved to be much more effective, causing embryogenic callus formation in 16–72% of explants, with 30 µM NAA + 100 µM GA_3 being the best combination (Table 1) [100].

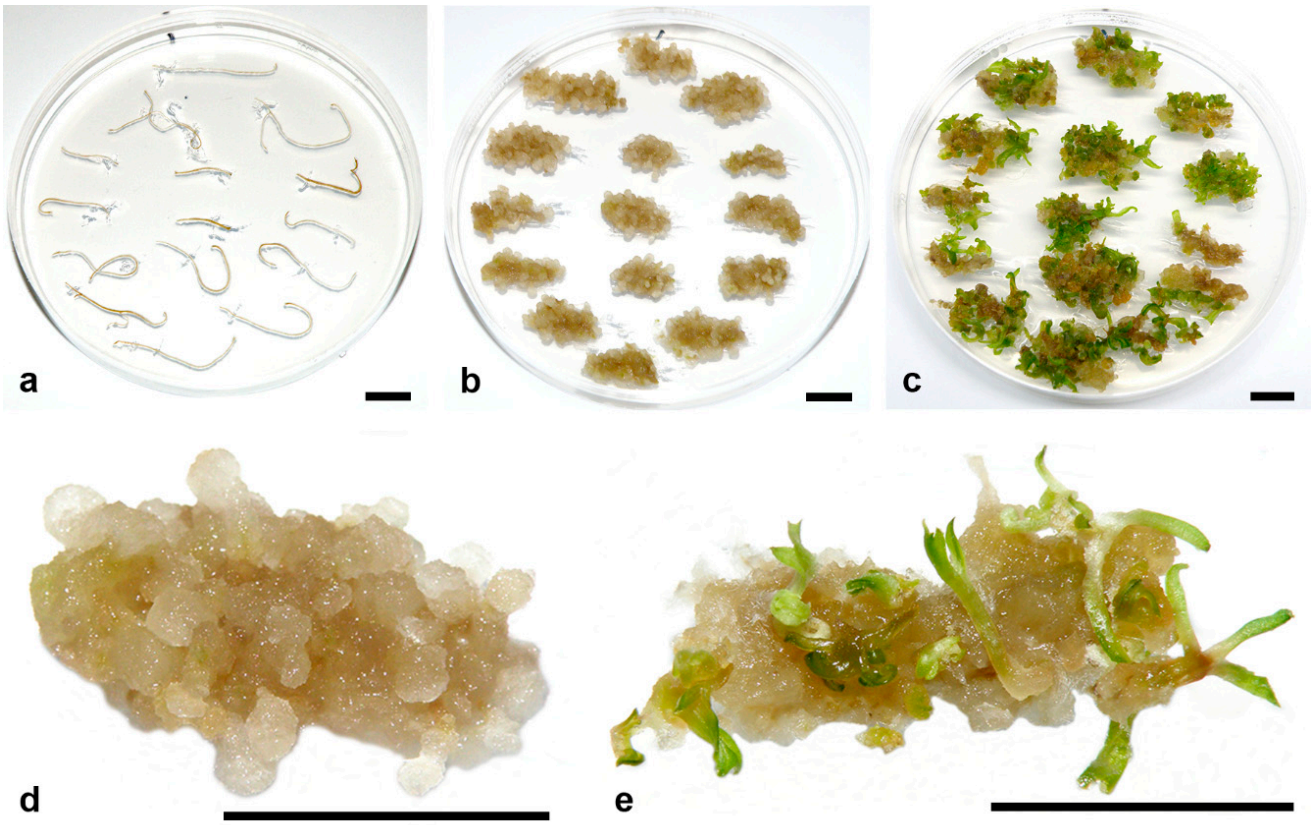

Figure 2. Proliferation and SE regeneration from spinach root apices cultured for eight weeks on medium supplemented with (**a**) 5 µM GA_3, where root apices only elongated but did not proliferate or regenerate SEs; (**b**,**d**) 20 µM NAA, where abundant proliferation occurred, but SE formation was extremely rare; (**c**,**e**) 20 µM NAA + 5 µM GA_3, where explants proliferated and regenerated SEs. Three repetitions with 15 explants in each repetition were used per treatment (n = 45). Scale bars: 1 cm. Unpublished from the authors' laboratory.

Later, the same group of authors demonstrated that GA_3 is not required for the induction of SEs from the hairy roots of four spinach cultivars [118]. This phenomenon has

not been clearly elucidated, although hairy roots are known to exhibit altered hormone homeostasis [119] and sensitivity to growth regulators [120,121], but no study has addressed this issue.

In another study, 20 μM NAA + 5 μM GA_3 proved to be the most effective PGR combination for inducing shoot buds from root sections of the cultivars RS no. E and Longstanding Round [83]. However, the same treatment induced somatic embryogenesis from root sections of the cultivar Matador, while shoot regeneration was never observed under these conditions [71,102,105,108,109].

Ethylene also plays an important role in the induction of somatic embryogenesis from spinach roots, in a complex manner—it is promotive for the induction of embryogenic callus but inhibitory for SE differentiation [122]. Ethephon (1–100 μM), an ethylene-releasing compound, increased the frequency of embryogenic callus formation, but only in combination with 0.1 μM GA_3. Conversely, 2-aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, and silver ions, which inhibit ethylene signaling, suppressed embryogenic callus formation at 10 μM and 1 μM, respectively, but significantly increased callus proliferation. The inhibitory effect of 1 μM AVG was abolished by the application of 10 μM ethephon. However, when ethephon was applied after the callus induction phase, i.e., added to the PGR-free medium through the SE proliferation phase, it strongly inhibited SE differentiation. Conversely, silver ions promoted SE differentiation at 1–100 μM, with the effect being strongest at 10 μM [122].

Not only PGR concentration but also treatment duration affected embryogenic efficiency. Komai et al. [69] applied callus induction treatment for only 4 weeks and then subcultured the explants on PGR-free medium. However, many reports showed that longer periods of callus induction were more effective [84,96,98,99]. In the cultivar Gyeowoonae, a 6-week callus induction was much more effective than shorter ones (2 and 4 weeks) for the acquisition of embryogenic competence [84].

2.1.5. Other Media Components

Carbohydrates are also a mandatory component of the medium. For the induction of embryogenic callus, 2% sucrose has been used most frequently (Table 1) [96–99]. However, Komai et al. [104] showed that sucrose is not the best choice for efficient SE induction from root explants of the Jiromaru cultivar. The highest frequency of embryogenic callus formation was obtained with fructose (72.5%), raffinose (64.4%), and maltose (52.0%), while glucose (38.6%) and sucrose (36.0%) showed a more modest effect, and mannose and sorbose completely inhibited cell proliferation and callus formation, so these sugars, together with galactose and lactose, were not suitable for cell proliferation. The effect of these hexoses and oligosaccharides was tested at 29, 87, and 145 mM, and all sugars showed the best effect at the lowest concentration of 29 mM [104]. Moreover, the weekly measurement of the residual sugar content in a liquid medium showed that root cells isolated from seedlings of the Jiromaru cultivar preferentially utilized fructose [104]. Therefore, these authors used 1% fructose (29.21 mM) in further studies (Table 1) [70]. However, in TCL explants of the Carpo and RZ1 cultivars, sucrose at 2% favored regeneration, while fructose impaired TCL development, although SE regeneration was observed [101].

Vitamins are also obligatory components of the medium. For the induction of somatic embryogenesis in spinach, some studies used an MS formulation of vitamins [96,101], while other studies enriched media with a range of B vitamins: 0.4–2 mg/l thiamine (B_1), 0.5–2 mg/L pyridoxine (B_6), and 0.5–5 mg/L nicotinic acid (B_3) [69,97–99]. Xiao and Branchard [96] supplied 0.01 mg/L biotin (B_7). Other adjuvants such as glutamine [96] or coconut water [53] were rarely used.

In addition to the standard media constituents, several other compounds have been shown to have a significant effect on the embryogenic response of spinach root apices. These compounds include hygromycin B (Hyg), trichostatin A (TSA), and dimethyl sulfoxide (DMSO). Hyg, an antibiotic commonly used to select genetically transformed cells, significantly promoted somatic embryogenesis from root apices and secondary somatic em-

bryogenesis when administered at low doses of 0.5–1.0 mg/L [108]. As mentioned earlier, spinach roots are the preferred explant type for efficient SE induction. However, spinach root apices are fragile and tend to become necrotic during the selection of transformed cells (Milojević et al., unpublished results). Therefore, stepwise selection offers the possibility of improving the probability of the recovery of transformed SEs [108]. However, similar enhancement of the embryogenic response by Hyg was not observed in the leaf explants of in vitro spinach plants, presumably because only a high concentration of 20 mg/L was used for the selection of transformed cells [123].

In a preliminary study, promoting effects of TSA, an inhibitor of histone deacetylases, and DMSO, a widely used solvent, on SE initiation were observed [124]. DMSO at a concentration of 0.05% significantly enhanced SE induction compared with the control, probably by enhancing the uptake of NAA and GA_3 into plant cells, whereas the enhancement of the embryogenic response obtained with 0.1–0.5 μM TSA suggests a significant role of histone acetylation in the epigenetic regulation of SE induction.

2.1.6. Culture Conditions

The effects of culture conditions have been studied only to a limited extent in spinach. In most studies, cultures have been exposed to diffuse light provided by cool white fluorescent tubes under a long-day photoperiod (LD, 10–16 h of light) and a photosynthetic photon flux density (PPFD) of 20–250 μmol m^{-2} s^{-1} from the beginning of culture [69,84,97–100,104]. Only Xiao and Branchard [96] and Leguillon et al. [101] kept cultures in the dark for the first 1 and 2 weeks, respectively, after culture initiation.

However, when root apices of the same seedling were divided into two groups, one of which was exposed to LD conditions (16 h photoperiod) and the other to short-day (SD) (8 h photoperiod) conditions, the LD condition was always favorable for the induction of SEs [109]. Explants from 7 of the 40 lines tested were able to regenerate SEs only under LD conditions. Moreover, regeneration under SD conditions was delayed by four weeks in all lines compared to LD conditions. Moreover, the embryogenic response of root apices of SE-derived plants was most efficient at a PPFD of 100 μmol m^{-2} s^{-1} [109]. In an opposite study, SD was found to be more effective in promoting shoot bud regeneration from spinach cotyledons [82]. The discrepancies between the two studies may be due to the use of different explant types and/or genotypes, as Geekiyanage et al. [82] used a different cultivar and did not examine the response of individual seedlings under either light regime.

Ambient temperature has a strong influence on the de novo shoot regeneration of spinach leaves [123]. Regeneration efficiency was 3–4 times higher in leaf explants cultivated at 14 °C than at 20 °C and 25 °C among different cultivars. To our knowledge, the effect of temperature on SE induction has never been extensively studied in spinach.

3. Auxin, GA_3, and Light Synergistically Promote SE Induction in Spinach

From all of the above information, it is clear that several factors act synergistically to allow the full expression of the embryogenic potential of spinach root apices. These include NAA, GA_3, and light. The absence of any single factor results in a drastic reduction or even abolition of SE initiation, although the presence of all factors does not guarantee the success of SE induction. An intrinsic predisposition to somatic embryogenesis is mandatory for a high SE response, making genotype an indispensable factor for SE induction.

Since the first report on spinach response to tissue culture [72] and most subsequent studies on SE induction [70,83,86,96,100,105], GA_3 has been considered indispensable for SE induction in spinach. However, GA_3 alone is unable to induce root cell proliferation and embryogenic callus formation (Figure 2a) [125], so it must be combined with auxin [100]. GA_3 had a differential effect on individual lines (derived from a single seed) of the cultivar Nippon [70]. In general, the highly responsive lines required a lower GA_3 concentration (0.1 μM) to achieve a high frequency of embryogenic explants (80%), and further increasing the GA_3 concentration to 10 μM did not result in a statistically significant increase in the embryogenic response. Moreover, explants from a highly responsive line did not require

GA_3 for SE regeneration and responded to 10 µM NAA alone, albeit with an SE regeneration frequency of only about 20% [70]. Conversely, GA_3 was indispensable in the lines with the lowest embryogenic capacity, and in a least responsive line, only 10 µM GA_3 was sufficient to induce a modest embryogenic response in about 30% of explants [70], suggesting that different lines require different GA_3 concentrations for SE initiation. However, GA_3 at higher concentrations (in combination with 10 µM NAA) was not sufficient to trigger a high embryogenic response (>50%) in lines with low intrinsic embryogenic capacity [70]. Interestingly, the SE regeneration frequencies of hairy root clones were proportional to the corresponding non-transformed clones, suggesting a high genotype dependence even in hairy root clones with altered hormone homeostasis [113]. Thus, these observations indicate that multiple factors are involved in the acquisition of embryogenic competence in spinach and that genotype at the individual level is a very important factor that should not be overlooked.

Consistent with this, in the Matador cultivar, SE induction without exogenous GA_3 was possible only in two previously selected highly responsive lines [105]. These lines were maintained through cycles of SE initiation from the root apices of SE-derived plants. Under optimal conditions, in the presence of NAA, GA_3 or GA_1, and fluorescent light (16 h photoperiod at a PPFD of 100 µmol m^2 s^{-1}), 95–100% of explants of these lines regenerated 16.7–40.7 SEs per explant. NAA alone induced the proliferation of root explants (Figure 2b,d), but the induction of SEs was extremely rare, 0.42 ± 0.42% of root apices derived from randomly selected seedlings of the cultivar Matador regenerated 0.003 ± 0.001 SEs per explant [125]. In the preselected highly responsive lines and under an LD photoperiod, NAA induced the regeneration of SEs at a fairly high frequency, in 19–89% of explants, but with only a few SEs per explant [105,126]. In darkness, only explants of a superior lineage were embryogenic, but at an extremely low frequency of 0.95% and only 0.14 SEs per explant [105]. Root apices derived from randomly selected seedlings of the cultivar Matador responded at the frequency of 82.48 ± 1.14% and regenerated 8.78 ± 0.05 SEs per explant (Figure 2c,e). Apparently, the interplay of NAA, GA_3, and light is crucial for efficient SE induction (Figure 2c,e). Selected spinach lines exhibited different requirements for GA_3 for efficient embryogenic response: 2.5 µM GA_3 was optimal for a line with higher embryogenic capacity, while 5 µM GA_3 was required for lines with lower embryogenic capacity [105]. Moreover, GA_3 was more effective than GA_1 for SE induction in all lines tested [105].

It has already been suggested that GA_3 is not required for the induction of regeneration, but only to stimulate the development of shoot primordia from calli competent for regeneration [78]. However, in the cultivar Matador, only a synergistic action of NAA and GA_3 from the beginning of explant cultivation resulted in successful SE induction (Zdravković-Korać et al. unpublished results).

Given the importance of GA_3 in the induction of SEs, it was expected that paclobutrazol (PAC), an inhibitor of gibberellin biosynthesis, would inhibit the induction of SEs. In contrast, PAC enhanced this process when combined with 20 µM NAA [127]. The combination of 5–10 µM PAC + 20 µM NAA was as efficient as 5 µM GA_3 + 20 µM NAA, whereas the combination of 20 µM NAA + 5 µM GA_3 + 2.5 µM PAC resulted in the highest mean SE number per root explant [127]. As PAC interferes with the terpenoid pathway, it affects abscisic acid and chlorophyll synthesis [128,129]. In addition, PAC affects ethylene and cytokinin levels and increases the activity of the antioxidant system, thereby increasing stress tolerance [130]. This is important because in vitro culture is considered stressful for the plants and their organs.

What is the basis of the interaction between GA_3 and light? Light is known to increase the biosynthesis of bioactive gibberellins (GAs) [131,132] through the upregulation of the *GA20-oxidase* (*GA20-ox*) gene [133–135], which is the checkpoint for maintaining GA homeostasis [136,137]. GA20-ox catalyzes the successive oxidation of GA_{53} to GA_{20} or GA_9 [133], which are further converted to bioactive GA_1 or GA_4, respectively, in a reaction catalyzed by GA3-oxidase (GA3-ox) [131,138], whereas GA2-oxidase (GA2-ox) inactivates

bioactive GAs. Therefore, GA20-ox, GA3-ox, and GA2-ox are the key enzymes regulating the levels of bioactive GAs [136,137].

Isolated root apices of Matador cultivar seedlings grown on PGR-free medium actively expressed GA-ox genes. The expression levels of *SoGA20-ox1*, *SoGA3-ox1*, *SoGA2-ox1*, *SoGA2-ox2*, and *SoGA2-ox3* were increased 0.93-, 2.39-, 2.55-, 3.32-, and 3.94-fold, respectively, in the roots after four weeks of cultivation under LD conditions compared with cultivation in darkness [105]. However, under LD conditions, the expression levels of *SoGA20-ox1* and *SoGA3-ox1* were lower by 8.56- and 7.41-fold respectively, whereas the expression levels of *SoGA2-ox1*, *-ox2*, and -ox3 were 0.48-, 3.69-, and 1.23-fold higher, respectively, in explants cultured on medium supplemented with 20 μM NAA + 5 μM GA_3 than in those cultured on PGR-free medium [105].

Moreover, the levels of immediate precursors and bioactive GAs were significantly higher in embryogenic explants cultured on medium supplemented with 20 μM NAA + 5 μM GA_3 than in nonembryogenic explants cultured on medium supplemented with 20 μM NAA, under LD conditions [126]. In nonembryogenic explants, only a transient increase in the content of endogenous GA_3 was observed after 24 h of cultivation, whereas no significant difference was observed in the levels of other bioactive GAs compared with the control root apices frozen immediately after isolation from LD-grown spinach seedlings [126]. However, in embryogenic explants, a significant increase in the content of all bioactive GAs was detected after 24 h of cultivation, which remained steady after seven days of cultivation, demonstrating the involvement of enhanced GA metabolism in the acquisition of the embryogenic competence of spinach root explants [126].

4. Genetic Background of the Induction of Somatic Embryogenesis

It is still not known what exactly constitutes the genetic background for the predisposition to somatic embryogenesis and de novo shoot regeneration, but it is becoming increasingly clear that this trait is controlled by multiple genes [139,140]. A genome-wide association study (GWAS) of 190 natural *Arabidopsis* accessions revealed that variation in shoot regeneration efficiency from root apices is related to a number of genes encoding transcription factors, hormone signaling, chromatin remodeling proteins, miRNAs, cell wall-modifying enzymes, among others [139]. Consistent with this, a single nucleotide polymorphism (SNP) detected in some of these genes, which affects protein conformation and thus protein function, has been shown to be different in responsive and non-responsive lineages or natural accessions [141]. This is the case for receptor-like protein kinase 1 (RPK1), which is essential for shoot regeneration from *Arabidopsis* root tips and is required for the epidermal cell shape and PIN1-mediated polarity of *Arabidopsis* embryos and cotyledon primordia formation [142]. Based on GWAS, it has been proposed that de novo shoot organogenesis is controlled by several universal master regulators (such as WUSCHEL) and numerous conditional fine-tuning factors that depend on explant type, media composition, and culture conditions [139,140]. This is also likely true for somatic embryogenesis, as the same master regulators play a central role in meristem formation in both shoot primordia and SEs [143]. Moreover, SNP variations found in the promoter regions of genes involved in regeneration induction, together with natural epigenomic and transcriptomic variations, suggest that subtle transcriptional changes may also contribute to the efficiency of regeneration [139,140]. Consistent with this, low embryogenic capacity in *M. truncatula* has been shown to be genetically determined [144]. However, SEs of these lines with low responsiveness gave rise to three lines with high embryogenic capacity after several cycles of selection and SE induction in independent studies [144]. As no genetic differences were found between the wild-type line Jemalong and a highly embryogenic line 2HA, it has been proposed that 2HA is an epigenetic variant of the wild-type Jemalong [144]. In addition to the above examples, a variety of proteins have been found to be involved in SE and de novo shoot induction, including histone modifying enzymes [145], DELLA proteins [146], ferredoxin-nitrite reductase [147], thioredoxin [148], and superoxide dismutase [149]. The different functions of these proteins and the possible involvement of environmental factors

in regulating their expression also suggest a complex nature of the initiation of SEs and the de novo regeneration of shoot buds.

5. A Molecular Marker for Embryogenic Capacity Assessment

To our knowledge, there is no molecular marker developed in spinach to determine the intrinsic predisposition to the high embryogenic capacity of individual plants. However, a 31-kDa basic protein has been found to accumulate to significantly higher levels in embryogenic compared with nonembryogenic calli or seedling tissues [106]. This protein was characterized as a type 1 ribosome inactivating protein (RIP) and designated SoRIP1, whereas another RIP protein was cloned and designated SoRIP2 [150].

The expression of *SoRIP1* increased markedly during embryogenic callus formation from root segments cultured on medium containing 10 µM NAA + 0.1 µM GA_3, whereas it remained very low in nonembryogenic calli cultured on medium containing only 10 µM NAA [150]. SoRIP1 accumulated preferentially in the proembryos and meristem of SEs [150]. In contrast, the expression of *SoRIP2* was low, and its level was similar in embryogenic and nonembryogenic calli during the callus induction phase but increased markedly in embryogenic calli during SE regeneration and remained steady in isolated SEs. SoRIP2 was found in the epidermis of SEs [150]. Thus, the expression profiles of both genes suggest developmental regulation [150,151].

Although these RIP proteins are not themselves involved in the transition from the vegetative to the embryonic stage, their expression coincides with the acquisition of embryogenic competence and the early stage of embryo development, and they have been used as molecular markers to quantify embryogenic capacity in subsequent studies [71,110]. Indeed, the expression of *SoRIP2* proved to be very useful and reliable for the rapid and early assessment of the embryogenic capacity of root explants of the cultivar Matador. The expression of *SoRIP2* was very low in seedling roots under noninductive conditions (PGR-free medium) and in roots cultured on SE-induction medium prior to the initiation of SEs but increased sharply (285-fold) in SEs at the globular stage of development, then decreased at later embryogenic stages and reached control levels at the late cotyledonary and early somatic seedling stages [71,110]. Thus, this method allowed much faster and easier quantification of embryogenic capacity compared to conventional SE quantification using a stereomicroscope. SEs at the earliest stages of development are difficult to delineate because they are embedded in root tissue proliferations, making them difficult to observe through a stereomicroscope, such that differences between low and high embryogenic capacity lines are visually detectable only after 8–10 weeks of cultivation [110], whereas the expression of *SoRIP2* could be used to distinguish between low and high embryogenic capacity lines after 4–6 weeks of cultivation [71]. Therefore, the expression of *SoRIP2* in explants after 4–6 weeks of cultivation showed a high positive correlation with classical SE quantification after 12 weeks of cultivation [110].

6. The Origin of SEs, SE Development, and Conversion to Plants

As mentioned earlier, SEs developed from proliferating root tissue. In some cases, SEs developed from massive calli arising from root sections cultured on medium supplemented with NAA + GA_3. Histological examination revealed that SEs originated from the epidermal layer of root tissue [103] or from the pericycle and parenchyma associated with root vascular tissue [105].

The PGR balance in the media significantly influenced the development of SEs [99]. The percentage ratio of globular (GE), bipolar (heart-shaped + torpedo-shaped, HTE), and cotyledonary (CE) SEs (GE:HTE:CE) was 61:17:22 and remained similar in explants cultured on Kin- and BA-supplemented media. However, the addition of ABA, GA_3, or IAA to Kin- or BA-supplemented media significantly altered the ratio of SEs. ABA at 4 µM and 0.3–10 µM GA_3 caused a significant increase in the percentage of globular SEs up to 95% and 98%, respectively. In agreement with this, Kawade et al. [150] also found a significant increase in the number of SEs from embryogenic calli subcultured on medium

supplemented with 40 µM ABA, and the embryos were also arrested at the globular stage of development. In contrast, 1 µM and 10 µM IAA favored the conversion of globular to bipolar SE on account of the percentage of GEs, which decreased to 59% [99]. In addition, ABA at 1–4 µM + Kin had a positive effect on the development of SEs, as cotyledons were better developed, and fewer malformations occurred [98].

The SEs went through all stages of development, from globular to cotyledonary, and even germinated on medium supplemented with 20 µM NAA + 5 µM GA_3 (Figure 3). However, they became hyperhydrated and malformed if left on this medium at the late cotyledonary or early seedling stage. SEs developed asynchronously so that SEs were present at all stages of development on root explants. The development of the globular embryos to the seedling stage usually took one to two weeks. The cotyledonary SEs did not require PGRs for conversion into plants and hence could be grown on PGR-free medium [84,97,98] although 2.69 µM GA_3 [97,98], 2.86 µM IAA [96] or 5 µM Kin [102] promoted their conversion into plantlets. SE-derived plants retained a rosette form under an SD photoperiod, bolted and flowered under an LD photoperiod, and set viable seeds [45,52,54,55,102]. This is also true for spinach plants obtained by de novo shoot organogenesis [79,83,152,153]. Seeds obtained by in vitro pollination germinated at a high frequency of 83–95% [83,102] and did not require cold stratification [102,152], as numerous seeds germinated while still attached to the mother plant [102]. In addition, the life cycle "from seed-to-seed" of in vitro-cultured SE-derived plants was shortened by two weeks compared to conventional seeds [154], as also observed in *Arabidopsis thaliana* and *Vigna subterranea* [155,156].

Figure 3. Development of spinach somatic embryos from the globular, through the heart-shaped, torpedo, and the cotyledonary stage of development to the seedling stage. From the globular stage to the seedling stage usually took one to two weeks. All embryos were obtained from seedling root sections of the cultivar Matador cultured on medium supplemented with 20 µM NAA + 5 µM GA_3. Unpublished from the authors' laboratory.

In addition, a high survival rate of 96% of SE-derived plants was attained in the cultivar Gyeowoonae, while 77% of the plants grew to maturity and set seed [84]. Nguyen

et al. [84] reported that only 6–7 months elapsed from culture initiation to seed production, and the application of an optimized procedure enabled the successful regeneration of transformed spinach plants [157].

7. Conclusions

Somatic embryogenesis is considered a superior method for the clonal propagation of valuable genotypes. Although spinach has been considered recalcitrant to in vitro regeneration for decades, efficient protocols for SE induction have been established. However, they are not applicable to all cultivars, indicating the wide variation and genotypic dependence of this trait among spinach cultivars. Individual variability in embryogenic capacity behind lot-to-lot variation could also be an explanation for the observed differences among cultivars. Therefore, the effect of genotype must always be considered, as the variations between individuals may be much greater than the variations caused by other factors. Moreover, embryogenic capacity could be significantly improved by several cycles of self-fertilization starting from an individual with moderate embryogenic capacity.

The efficiency of SE induction from root sections of spinach is strongly influenced by the synergistic interaction of NAA, GA_3, and light. Analysis of the GA content revealed that the increased content of bioactive GAs, especially GA_1, correlated with the acquisition of the embryogenic competence of root explants. Thus, it remains to be elucidated how the interaction of NAA and GA_3 triggers an increase in bioactive GAs in embryogenic explants. Further studies on the interaction between these PGRs and light at the level of gene expression and the content of endogenous GAs are currently underway in our laboratory. In this context, the genome assembly of spinach and the establishment of SpinachBase for gene expression analysis are of utmost importance.

In summary, although the protocols developed for the induction of SE in spinach are still genotype dependent, the effects of the major factors influencing somatic embryo induction are now much better understood, and the efficiency of embryogenic capacity has been greatly improved.

Author Contributions: Conceptualization, S.Z.-K.; validation, D.Ć.; formal analysis, J.M. and M.B.; writing—original draft preparation, S.Z.-K.; writing—review and editing, S.Z.-K., J.M. and D.Ć.; visualization, J.M. and M.B.; supervision, S.Z.-K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, grant number 451-03-47/2023-01/200007. The APC was funded by University of Belgrade, Institute for Biological Research "Siniša Stanković"—National Institute of the Republic of Serbia.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, for financial support through Contract № 451-03-47/2023-01/200007.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Roberts, J.L.; Moreau, R. Functional properties of spinach (*Spinacia oleracea* L.) phytochemicals and bioactives. *Food Funct.* **2016**, *7*, 3337–3353. [CrossRef] [PubMed]
2. Morelock, T.E.; Correll, J.C. Spinach. In *Vegetables I: Asteraceae, Brassicaceae, Chenopodiaceae, and Cucurbitaceae*, 1st ed.; Prohens, J., Nuez, F., Eds.; Springer: New York, NY, USA, 2008; pp. 189–218.
3. Shohag, M.J.I.; Wei, Y.Y.; Yu, N.; Zhang, J.; Wang, K.; Patring, J.; He, Z.; Yang, X. Natural variation of folate content and composition in spinach (*Spinacia oleracea*) germplasm. *J. Agric. Food Chem.* **2011**, *59*, 12520–12526. [CrossRef] [PubMed]

4. Salehi, B.; Tumer, T.B.; Ozleyen, A.; Peron, G.; Dall'Acqua, S.; Rajkovic, J.; Naz, R.; Nosheen, A.; Mudau, F.N.; Labanca, F.; et al. Plants of the genus *Spinacia*: From bioactive molecules to food and phytopharmacological applications. *Trends Food Sci. Technol.* **2019**, *88*, 260–273. [CrossRef]
5. Hayes, M.; Pottorff, M.; Kay, C.; Van Deynze, A.; Osorio-Marin, J.; Lila, M.A.; Iorrizo, M.; Ferruzzi, M.G. In vitro bioaccessibility of carotenoids and chlorophylls in a diverse collection of spinach accessions and commercial cultivars. *J. Agric. Food Chem.* **2020**, *68*, 3495–3505. [CrossRef]
6. Rueda, D.; Awika, H.O.; Bedre, R.; Kandel, D.R.; Mandadi, K.K.; Crosby, K.; Avila, C.A. Phenotypic diversity and association mapping of ascorbic acid content in spinach. *Front. Genet.* **2022**, *12*, 752313. [CrossRef]
7. Bhattarai, G.; Shi, A. Research advances and prospects of spinach breeding, genetics, and genomics. *Veg. Res.* **2021**, *1*, 9. [CrossRef]
8. El-Sayed, S.M. Use of spinach powder as functional ingredient in the manufacture of UF-Soft cheese. *Heliyon* **2020**, *6*, e03278. [CrossRef]
9. Czarnowska-Kujawska, M.; Starowicz, M.; Barišić, V.; Kujawski, W. Health-promoting nutrients and potential bioaccessibility of breads enriched with fresh kale and spinach. *Foods* **2022**, *11*, 3414. [CrossRef]
10. Różyło, R.; Piekut, J.; Dziki, D.; Smolewska, M.; Gawłowski, S.; Wójtowicz, A.; Gawlik-Dziki, U. Effects of wet and dry micronization on the GC-MS identification of the phenolic compounds and antioxidant properties of freeze-dried spinach leaves and stems. *Molecules* **2022**, *27*, 8174. [CrossRef]
11. Pateiro, M.; Domínguez, R.; Munekata, P.E.S.; Nieto, G.; Bangar, S.P.; Dhama, K.; Lorenzo, J.M. Bioactive compounds from leaf vegetables as preservatives. *Foods* **2023**, *12*, 637. [CrossRef]
12. Lachnicht, D.; Brevard, P.B.; Wagner, T.L.; DeMars, C.E. Dietary oxygen radical absorbance capacity as a predictor of bone mineral density. *Nutr. Res.* **2002**, *22*, 1389–1399. [CrossRef]
13. Lomnitski, L.; Bergman, M.; Nyska, A.; Ben-Shaul, V.; Grossman, S. Composition, efficacy, and safety of spinach extracts. *Nutr. Cancer* **2003**, *46*, 222–231. [CrossRef] [PubMed]
14. Sarkar, P.; Thirumurugan, K. Modulatory functions of bioactive fruits, vegetables and spices in adipogenesis and angiogenesis. *J. Funct. Food* **2019**, *53*, 318–336. [CrossRef]
15. Ishii, M.; Nakahara, T.; Araho, D.; Murakami, J.; Nishimura, M. Glycolipids from spinach suppress LPS-induced vascular inflammation through eNOS and NK-kB signaling. *Biomed. Pharmacother.* **2017**, *91*, 111–120. [CrossRef]
16. Joseph, J.A.; Shukitt-Hale, B.; Denisova, N.A.; Bielinski, D.; Martin, A.; McEwen, J.J.; Bickford, P.C. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *J. Neurosci.* **1999**, *19*, 8114–8121. [CrossRef]
17. Gutierrez, R.M.P.; Velazquez, E.G. Glucopyranoside flavonoids isolated from leaves of *Spinacia oleracea* (spinach) inhibit the formation of advanced glycation end products (AGEs) and aldose reductase activity (RLAR). *Biomed. Pharmacother.* **2020**, *128*, 110299. [CrossRef]
18. Bautista-Pérez, R.; Cano-Martínez, A.; Gutiérrez-Velázquez, E.; Martínez-Rosas, M.; Pérez-Gutiérrez, R.M.; Jiménez-Gómez, F.; Flores-Estrada, J. Spinach methanolic extract attenuates the retinal degeneration in diabetic rats. *Antioxidants* **2021**, *10*, 717. [CrossRef]
19. Li, Y.; Cui, Y.; Lu, F.; Wang, X.; Liao, X.; Hu, X.; Zhang, Y. Beneficial effects of a chlorophyll-rich spinach extract supplementation on prevention of obesity and modulation of gut microbiota in high-fat diet-fed mice. *J. Funct. Food* **2019**, *60*, 103436. [CrossRef]
20. Li, Y.; Cui, Y.; Hu, X.; Liao, X.; Zhang, Y. Chlorophyll supplementation in early life prevents diet-induced obesity and modulates gut microbiota in mice. *Mol. Nutr. Food Res.* **2019**, *63*, 1801219. [CrossRef]
21. Li, Y.; Lu, F.; Wang, X.; Hu, X.; Liao, X.; Zhang, Y. Biological transformation of chlorophyll-rich spinach (*Spinacia oleracea* L.) extracts under in vitro gastrointestinal digestion and colonic fermentation. *Food Res. Int.* **2021**, *139*, 109941. [CrossRef]
22. Elvira-Torales, L.A.; Periago, M.J.; González-Barrio, R.; Hidalgo, N.; Navarro-González, L.; Gómez-Gallego, C.; Masuero, D.; Soini, E.; Vrhovsek, U.; García-Alonso, F.J. Spinach consumption ameliorates the gut microbiota and dislipaemia in rats with diet-induced non-alcoholic fatty liver disease (NAFLD). *Food Funct.* **2019**, *10*, 2148–2160. [CrossRef] [PubMed]
23. Elvira-Torales, L.A.; Martín-Pozuelo, G.; González-Barrio, R.; Navarro-González, I.; Pallarés, F.J.; Santaella, M.; García-Alonso, J.; Sevilla, A.; Periago-Castón, M.J. Ameliorative effect of spinach on non-alcoholic fatty liver disease induced in rats by a high-fat diet. *Int. J. Mol. Sci.* **2019**, *20*, 1662. [CrossRef] [PubMed]
24. Song, X.; Zhang, X.; Ma, C.; Hu, X.; Chen, F. Rediscovering the nutrition of whole foods: The emerging role of gut microbiota. *Curr. Opin. Food Sci.* **2022**, *48*, 100908. [CrossRef]
25. Mou, B. Evaluation of oxalate concentration in the U.S. spinach germplasm collection. *HortScience* **2008**, *43*, 1690–1693. [CrossRef]
26. Ermer, T.; Eckardt, K.U.; Aronson, P.S.; Knauf, F. Oxalate, inflammasome, and progression of kidney disease. *Curr. Opin. Nephrol. Hypertens.* **2016**, *25*, 363–371. [CrossRef]
27. Hord, N.G.; Tang, Y.; Bryan, N.S. Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *Am. J. Clin. Nutr.* **2009**, *90*, 1–10. [CrossRef]
28. Camp, N.E. Methemoglobinemia. *J. Emerg. Nurs.* **2007**, *33*, 172–174. [CrossRef]
29. Cho, M.J.; Howard, L.R.; Prior, R.L.; Morelock, T. Flavonoid content and antioxidant capacity of spinach genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* **2008**, *88*, 1099–1106. [CrossRef]
30. Ribera, A.; Bai, Y.; Wolters, A.M.A.; Van Treuren, R.; Kik, C. A review on the genetic resources, domestication and breeding history of spinach (*Spinacia oleracea* L.). *Euphytica* **2020**, *216*, 48. [CrossRef]

31. Tao, H.; Li, L.; He, Y.; Zhang, X.; Zhao, Y.; Wang, Q.; Hong, G. Flavonoids in vegetables: Improvement of dietary flavonoids by metabolic engineering to promote health. *Crit. Rev. Food Sci. Nutr.* **2022**, 1–15. [CrossRef]
32. Das, A.; Rout, B.M.; Datta, S.; Singh, S.; Munshi, A.D.; Dey, S.S. Spinach (*Spinacia oleracea* L.) breeding: From classical to genomics-centric approach. In *Smart Plant Breeding for Vegetable Crops in Post-Genomics Era*, 1st ed.; Singh, S., Sharma, D., Sharma, S.K., Singh, R., Eds.; Springer: Singapore, 2023; pp. 117–142.
33. Ribera, A.; van Treuren, R.; Kik, C.; Bai, Y.; Wolters, A.M.A. On the origin and dispersal of cultivated spinach (*Spinacia oleracea* L.). *Genet. Resour. Crop. Evol.* **2021**, *68*, 1023–1032. [CrossRef]
34. Van Treuren, R.; de Groot, L.; Hisoriev, H.; Khassanov, F.; Farzaliyev, V.; Melyan, G.; Gabrielyan, I.; van Soest, L.; Tulmans, C.; Courand, D.; et al. Acquisition and regeneration of *Spinacia turkestanica* and *S. tetrandra* to improve a spinach gene bank collection. *Genet. Resour. Crop Evol.* **2020**, *67*, 549–559. [CrossRef]
35. Xu, C.; Jiao, C.; Sun, H.; Cai, X.; Wang, X.; Ge, C.; Zheng, Y.; Liu, W.; Sun, X.; Xu, Y.; et al. Draft genome of spinach and transcriptome diversity of 120 *Spinacia* accessions. *Nat. Commun.* **2017**, *8*, 15275. [CrossRef] [PubMed]
36. Cai, X.; Sun, X.; Xu, C.; Sun, H.; Wang, X.; Ge, C.; Zhang, Z.; Wang, Q.; Fei, Z.; Jiao, C.; et al. Reference genome and resequencing of 305 accessions provide insights into spinach evolution, domestication and genetic basis of agronomic traits. *Nat. Commun.* **2021**, *12*, 7246. [CrossRef]
37. Collins, K.; Zhao, K.; Chen, J.; Xu, C.; Cai, X.; Wang, X.; Ge, C.; Dai, S.; Wang, Q.; Wang, Q.; et al. SpinachBase: A central portal for spinach genomics. *Database* **2019**, *2019*, baz072. [CrossRef]
38. Irish, B.M.; Correll, J.C.; Feng, C.; Bentley, T.; de los Reyes, B.G. Characterization of a resistance locus (*Pfs-1*) to the spinach downy mildew pathogen (*Peronospora farinosa* f. sp. *spinaciae*) and development of a molecular marker linked to *Pfs-1*. *Phytopathology* **2008**, *98*, 894–900. [CrossRef]
39. Bhattarai, G.; Shi, A.; Feng, C.; Dhillon, B.; Mou, B.; Correll, J. Genome wide association studies in multiple spinach breeding populations refine downy mildew race 13 resistance genes. *Front. Plant Sci.* **2020**, *11*, 563187. [CrossRef]
40. Bhattarai, G.; Shi, A.; Kandel, D.R.; Solís-Gracia, N.; da Silva, J.A.; Avila, C.A. Genome-wide simple sequence repeats (SSR) markers discovered from whole-genome sequence comparisons of multiple spinach accessions. *Sci. Rep.* **2021**, *11*, 9999. [CrossRef]
41. Hirakawa, H.; Toyoda, A.; Itoh, T.; Suzuki, Y.; Nagano, A.J.; Sugiyama, S.; Onodera, Y. A spinach genome assembly with remarkable completeness, and its use for rapid identification of candidate genes for agronomic traits. *DNA Res.* **2021**, *28*, dsab004. [CrossRef]
42. She, H.; Xu, Z.; Zhang, H.; Li, G.; Wu, J.; Wang, X.; Li, Y.; Liu, Z.; Qian, W. Identification of a male-specific region (MSR) in *Spinacia oleracea*. *Hortic. Plant J.* **2021**, *7*, 341–346. [CrossRef]
43. Liu, Z.; Lu, T.; Feng, C.; Zhang, H.; Xu, Z.; Correll, J.C.; Qian, W. Fine mapping and molecular marker development of the *Fs* gene controlling fruit spines in spinach (*Spinacia oleracea* L.). *Theor. Appl. Genet.* **2021**, *134*, 1319–1328. [CrossRef] [PubMed]
44. Janick, J.; Stevenson, E.C. Environmental influences on sex expression in monoecious lines of spinach. *Proc. Am. Soc. Hortic. Sci.* **1955**, *75*, 416–422.
45. Ćulafić, L.; Nešković, M. Effect of growth substances on flowering and sex expression in isolated apical buds of *Spinacia oleracea*. *Physiol. Plant.* **1980**, *48*, 588–591. [CrossRef]
46. Hata, N.; Murakami, K.; Yoshida, Y.; Masuda, M. Effect of temperature on expression of gynomonoecy in selfed-seed populations of *Spinacia oleracea* L. *J. Jpn. Soc. Hortic. Sci.* **2005**, *74*, 228–233. [CrossRef]
47. Onodera, Y.; Yonaha, I.; Niikura, S.; Yamazaki, S.; Mikami, T. Monoecy and gynomonoecy in *Spinacia oleracea* L.: Morphological and genetic analyses. *Sci. Hortic.* **2008**, *118*, 266–269. [CrossRef]
48. Golenberg, E.M.; West, N.W. Hormonal interactions and gene regulation can link monoecy and environmental plasticity to the evolution of dioecy in plants. *Am. J. Bot.* **2013**, *100*, 1022–1037. [CrossRef]
49. Cossard, G.G.; Pannell, J.R. Enhanced leaky sex expression in response to pollen limitation in the dioecious plant *Mercurialis annua*. *J. Evol. Biol.* **2021**, *34*, 416–422. [CrossRef]
50. Chailakhyan, M.K.; Khryanin, V.N. Effect of growth regulators and role of roots in sex expression in spinach. *Planta* **1978**, *142*, 207–210. [CrossRef]
51. Hata, N.; Murakami, K.; Yoshida, Y.; Masuda, M. Effect of photoperiod after bolting on the expression of gynomonoecy in *Spinacia oleracea* L. *J. Jpn. Soc. Hortic. Sci.* **2006**, *75*, 141–147. [CrossRef]
52. Komai, F.; Shikazono, N.; Tanaka, A. Sexual modification of female spinach seeds (*Spinacia oleracea* L.) by irradiation with ion particles. *Plant Cell Rep.* **2003**, *21*, 713–717. [CrossRef]
53. Al-Khayri, J.M.; Huang, F.H.; Morelock, T.E.; Busharar, T.A. Spinach tissue culture improved with coconut water. *HortScience* **1992**, *27*, 357–358. [CrossRef]
54. Komai, F.; Masuda, K.; Ishizaki, T.; Harada, T. Sex expression in plants regenerated from the root callus of female and male spinach (*Spinacia oleracea*). *Plant Sci.* **1999**, *146*, 35–40. [CrossRef]
55. Komai, F.; Masuda, K. Plasticity in sex expression of spinach (*Spinacia oleracea*) regenerated from root tissues. *Plant Cell Tissue Organ Cult.* **2004**, *78*, 285–287. [CrossRef]
56. Onodera, Y.; Itaru, Y.; Hiroki, M.; Tanaka, A.; Niikura, S.; Yamazaki, S.; Mikami, T. Mapping of the genes for dioecism and monoecism in *Spinacia oleracea* L.: Evidence that both genes are closely linked. *Plant Cell Rep.* **2011**, *30*, 965–971. [CrossRef]
57. Yamamoto, K.; Oda, Y.; Haseda, A.; Fujito, S.; Mikami, T.; Onodera, Y. Molecular evidence that the genes for dioecism and monoecism in *Spinacia oleracea* L. are located at different loci in a chromosomal region. *Heredity* **2014**, *112*, 317–324. [CrossRef]

58. Deng, C.L.; Zhang, W.L.; Cao, Y.; Wang, S.J.; Li, S.F.; Gao, W.J.; Lu, L.D. Rapid cloning and bioinformatic analysis of spinach Y chromosome-specific EST sequences. *J. Genet.* **2015**, *94*, 705–713. [CrossRef]
59. Okazaki, Y.; Takahata, S.; Hirakawa, H.; Suzuki, Y.; Onodera, Y. Molecular evidence for recent divergence of X-and Y-linked gene pairs in *Spinacia oleracea* L. *PLoS ONE* **2019**, *14*, e0214949. [CrossRef]
60. Qian, W.; Fan, G.; Liu, D.; Zhang, H.; Wang, X.; Wu, J.; Xu, Z. Construction of a high-density genetic map and the X/Y sex-determining gene mapping in spinach based on largescale markers developed by specific-locus amplified fragment sequencing (SLAF-seq). *BMC Genom.* **2017**, *18*, 276. [CrossRef]
61. Ma, X.; Yu, L.; Fatima, M.; Wadlington, W.H.; Hulse-Kemp, A.M.; Zhang, X.; Zhang, S.; Xu, X.; Wang, J.; Huang, H.; et al. The spinach YY genome reveals sex chromosome evolution, domestication, and introgression history of the species. *Genome Biol.* **2022**, *23*, 75. [CrossRef]
62. West, N.W.; Golenberg, E.M. Gender-specific expression of GIBBERELLIC ACID INSENSITIVE is critical for unisexual organ initiation in dioecious *Spinacia oleracea*. *New Phytol.* **2018**, *217*, 1322–1334. [CrossRef]
63. Lenaerts, B.; Collard, B.C.Y.; Demont, M. Review: Improving global food security through accelerated plant breeding. *Plant Sci.* **2019**, *287*, 110207. [CrossRef]
64. Germanà, M.A. Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Rep.* **2011**, *30*, 839–857. [CrossRef]
65. George, E.F. Plant tissue culture procedure—Background. In *Plant Propagation by Tissue Culture: The Background*, 1st ed.; George, E.F., Hall, M.A., De Klerk, G.J., Eds.; Springer: Dordrecht, The Netherlands, 2008; Volume 1, pp. 1–28.
66. von Arnold, S.; Sabala, I.; Bozhkov, P.; Dyachok, J.; Filonova, L. Developmental pathways of somatic embryogenesis. *Plant Cell Tissue Organ Cult.* **2002**, *69*, 233–249. [CrossRef]
67. Davey, M.R.; Anthony, P.; Power, B.; Lowe, K.C. Leafy vegetables. In *Compendium of Transgenic Crop Plants: Transgenic Vegetable Crops*, 1st ed.; Kole, C., Hall, T.C., Eds.; Wiley-Blackwell: Oxford, UK, 2008; Volume 6, pp. 217–248.
68. Al-Khayri, J.M.; Huang, F.H.; Morelock, T.E.; Busharar, T.A.; Gbur, E.E. Genotype—Dependent response of spinach cultivars to in vitro callus induction and plant regeneration. *Plant Sci.* **1991**, *78*, 121–126. [CrossRef]
69. Komai, F.; Okuse, I.; Harada, T. Somatic embryogenesis and plant regeneration in culture of root segments of spinach (*Spinacia oleracea* L.). *Plant Sci.* **1996**, *13*, 203–208. [CrossRef]
70. Ishizaki, T.; Komai, F.; Masuda, K. Screening for strongly regenerative genotypes of spinach in tissue culture using subcultured root explants. *Plant Cell Tissue Organ Cult.* **2001**, *67*, 251–255. [CrossRef]
71. Belić, M.; Zdravković-Korać, S.; Uzelac, B.; Ćalić, D.; Pavlović, S.; Milojević, J. Variability in somatic embryo-forming capacity of spinach. *Sci. Rep.* **2020**, *10*, 19290. [CrossRef]
72. Nešković, M.; Radojević, L. The growth of and morphogenesis in tissue cultures of *Spinacia oleracea* L. *Bull. Inst. Jard Bot. Belgrade Univ.* **1973**, *8*, 35–37.
73. Kondo, K.; Nadamitsu, S.; Tanaka, R.; Taniguchi, K. Micropropagation of *Spinacia oleracea* L. through culture of shoot primordia. *Plant Tissue Cult. Lett.* **1991**, *8*, 1–4. [CrossRef]
74. Sasaki, H. Callus and organ formation in tissue cultures of spinach (*Spinacia oleracea* L.). *J. Jpn. Soc. Hortic. Sci.* **1989**, *58*, 149–153. [CrossRef]
75. Sasaki, H.; Arato, T.; Takahashi, K.; Kobori, T. Regeneration of spinach plant from hypocotyl tissue cultured in vitro. *J. Hokkaido Univ. Educ.* **1994**, *45*, 31–35.
76. Satoh, T.; Abe, T.; Sasahara, T. Plant regeneration from hypocotyl-derived calli of spinach (*Spinacia oleracea* L.) and anatomical characteristics of regenerating calli. *Plant Tissue Cult. Lett.* **1992**, *9*, 176–183. [CrossRef]
77. Mii, M.; Nakano, K.; Okuda, K.; Lizuka, M. Shoot regeneration from spinach hypocotyl segments by short term treatment with 5,6-dichloro-indole-3-acetic acid. *Plant Cell Rep.* **1992**, *11*, 58–61. [CrossRef]
78. Molvig, L.; Rose, R.J. A regeneration protocol for *Spinacia oleracea* using gibberellic acid. *Aust. J. Bot.* **1994**, *42*, 763–769. [CrossRef]
79. Xiao, X.G.; Branchard, M. In vitro high frequency plant regeneration from hypocotyl and root segments of spinach by organogenesis. *Plant Cell Tissue Organ Cult.* **1995**, *42*, 239–244. [CrossRef]
80. Shojaei, T.R.; Salari, V.; Ramazan, D.; Ehyaei, M.; Gharechahi, J.; Chaleshtori, R.M. The effect of plant growth regulators, explants and cultivars on spinach (*Spinacia oleracea* L.) tissue culture. *Afr. J. Biotechnol.* **2010**, *9*, 4179–4185.
81. Zhang, H.X.; Zeevaart, J. An efficient *Agrobacterium tumefaciens*-mediated transformation and regeneration system for cotyledons of spinach (*Spinacia oleracea* L.). *Plant Cell Rep.* **1999**, *18*, 640–645. [CrossRef]
82. Geekiyanage, S.; Takase, T.; Watanabe, S.; Fukai, S.; Kiyouse, T. The combined effect of photoperiod, light intensity and GA_3 on adventitious shoot regeneration from cotyledons of spinach (*Spinacia oleracea* L.). *Plant Biotechnol.* **2006**, *23*, 431–435. [CrossRef]
83. Knoll, K.; Short, K.; Curtis, I.; Power, J.B.; Davey, M.R. Shoot regeneration from cultured root explants of spinach (*Spinacia oleracea* L.): A system for *Agrobacterium* transformation. *Plant Cell Rep.* **1997**, *17*, 96–101. [CrossRef]
84. Nguyen, Q.V.; Sun, H.J.; Boo, K.H.; Lee, D.; Lee, J.H.; Lim, P.O.; Lee, H.Y.; Riu, K.Z.; Lee, D.S. Effect of plant growth regulator combination and culture period on in vitro regeneration of spinach (*Spinacia oleracea* L.). *Plant Biotechnol. Rep.* **2013**, *7*, 99–108. [CrossRef]
85. Al-Khayri, J.M.; Huang, F.H.; Morelock, T.E. Regeneration of spinach from leaf callus. *HortScience* **1991**, *26*, 913–914. [CrossRef]
86. Al-Khayri, J.M.; Huang, F.H.; Morelock, T.E.; Busharar, T.A. Stimulation of shoot regeneration in spinach callus by gibberellic acid. *HortScience* **1992**, *27*, 1046. [CrossRef]

87. Al-Khayri, J.M.; Huang, F.H.; Morelock, T.E.; Busharar, T.A. In vitro plant regeneration of spinach from mature seed-derived callus. *Vitr. Cell Dev. Biol.-Plant* **1992**, *28*, 64–66. [CrossRef]
88. Goto, T.; Miyazaki, M. Plant regeneration from mesophyll protoplasts of *Spinacia oleracea* L. *Plant Tissue Cult. Lett.* **1992**, *9*, 15–21. [CrossRef]
89. Goto, T.; Miyazaki, M.; Oku, M. Improved procedure for protoplast culture and plant regeneration of spinach (*Spinacia oleracea* L.). *J. Jpn. Soc. Hortic. Sci.* **1996**, *65*, 349–354. [CrossRef]
90. Goto, T.; Miyazaki, M.; Oku, M. Varietal variations in plant regenerative potential from protoplasts in spinach (*Spinacia oleracea* L.). *J. Jpn. Soc. Hortic. Sci.* **1998**, *67*, 503–506. [CrossRef]
91. Komai, F.; Kiyoshi, K.; Harada, T.; Okuse, I. Plant regeneration from adventitious roots of spinach (*Spinacia oleracea* L.) grown from protoplasts. *Plant Sci.* **1996**, *120*, 89–94. [CrossRef]
92. Dalton, C.C.; Street, H.E. The role of the gas phase in the greening and growth of illuminated cell suspension cultures of spinach (*Spinacia oleracea* L.). *Vitr. Cell Dev. Biol.-Plant* **1976**, *12*, 485–494. [CrossRef]
93. Keleş, D.; Özcan, C.; Pınar, H.; Ata, A.; Denli, N.; Yücel, N.K.; Taşkın, H.; Büyükalaca, S. First report of obtaining haploid plants using tissue culture techniques in spinach. *HortScience* **2016**, *51*, 742–749. [CrossRef]
94. Nešković, M.; Ćulafić, L. Spinach (*Spinacia oleracea* L.). In *Crops II. Biotechnology in Agriculture and Forestry*, 1st ed.; Bajaj, Y.P.S., Ed.; Springer: Berlin/Heidelberg, Germany, 1988; Volume 6, pp. 370–383.
95. Al-Khayri, J.M. Micropropagation of *Spinacia oleracea* L. (Spinach). In *High-Tech and Micropropagation V. Biotechnology in Agriculture and Forestry*, 1st ed.; Bajaj, Y.P.S., Ed.; Springer: Berlin/Heidelberg, Germany, 1997; Volume 39, pp. 173–200.
96. Xiao, X.G.; Branchard, M. Embryogenesis and plant regeneration of spinach (*Spinacia oleracea* L.) from hypocotyl segments. *Plant Cell Rep.* **1993**, *13*, 69–71. [CrossRef]
97. Zdravković-Korać, S.; Nešković, M. Somatic embryogenesis in spinach (*Spinacia oleracea* L.). *Arch. Biol. Sci.* **1993**, *45*, 57–58.
98. Zdravković-Korać, S.; Nešković, M. Organogenesis and somatic embryogenesis in *Spinacia oleracea* tissue culture. *Arch. Biol. Sci.* **1998**, *50*, 183–188.
99. Zdravković-Korać, S.; Nešković, M. Induction and development of somatic embryos from spinach (*Spinacia oleracea*) leaf segments. *Plant Cell Tissue Organ Cult.* **1998**, *55*, 109–114. [CrossRef]
100. Komai, F.; Okuse, I.; Harada, T. Effective combinations of plant growth regulators for somatic embryogenesis from spinach root segments. *J. Jpn. Soc. Hortic. Sci.* **1996**, *65*, 559–564. [CrossRef]
101. Leguillon, S.; Charles, G.; Branchard, M. Plant regeneration from thin cell layers in *Spinacia oleracea*. *Plant Cell Tissue Organ Cult.* **2003**, *74*, 257–265. [CrossRef]
102. Milojević, J.; Tubić, L.; Zdravković-Korać, S.; Dragićević, I.; Ćalić-Dragosavac, D.; Vinterhalter, B. Increased regeneration capacity in spinach lines obtained by in vitro self-fertilisation. *Sci. Hortic.* **2011**, *130*, 681–690. [CrossRef]
103. Komai, F.; Okuse, I.; Harada, T. Histological identification of somatic embryogenesis in spinach root tissue pieces. *Plant Tissue Cult. Lett.* **1995**, *12*, 313–315. [CrossRef]
104. Komai, F.; Okuse, I.; Saga, K.; Harada, T. Improvement on the efficiency of somatic embryogenesis from spinach root tissues by applying various sugars. *J. Jpn. Soc. Hortic. Sci.* **1996**, *65*, 67–72. [CrossRef]
105. Belić, M.; Zdravković-Korać, S.; Janošević, D.; Savić, J.; Todorović, S.; Banjac, N.; Milojević, J. Gibberellins and light synergistically promote somatic embryogenesis from the in vitro apical root sections of spinach. *Plant Cell Tissue Organ Cult.* **2020**, *142*, 537–548. [CrossRef]
106. Ishizaki, T.; Megumi, C.; Komai, F.; Masuda, K.; Oosawa, K. Accumulation of a 31-kDa glycoprotein in association with the expression of embryogenic potential by spinach callus in culture. *Physiol. Plant.* **2002**, *114*, 109–115. [CrossRef]
107. Wu, H.; Chen, B.; Fiers, M.; Wrobel-Marek, J.; Kodde, J.; Groot, S.P.C.; Angenent, G.; Feng, H.; Bentsink, L.; Boutilier, K. Seed maturation and post-harvest ripening negatively affect arabidopsis somatic embryogenesis. *Plant Cell Tissue Organ Cult.* **2019**, *139*, 17–27. [CrossRef]
108. Milojević, J.; Tubić, L.; Nolić, V.; Mitić, N.; Ćalić-Dragosavac, D.; Vinterhalter, B.; Zdravković-Korać, S. Hygromycin promotes somatic embryogenesis in spinach. *Plant Cell Tissue Organ Cult.* **2012**, *109*, 573–579. [CrossRef]
109. Milojević, J.; Tubić, L.; Pavlović, S.; Mitić, N.; Ćalić, D.; Vinterhalter, B.; Zdravković-Korać, S. Long days promote somatic embryogenesis in spinach. *Sci. Hortic.* **2012**, *142*, 32–37. [CrossRef]
110. Milić, M.; Savić, J.; Tubić, L.; Devrnja, N.; Ćalić, D.; Zdravković-Korać, S.; Milojević, J. Expression of the gene for ribosome-inactivating protein, *SoRIP2*, as a tool for the evaluation of somatic embryogenesis in spinach. *Plant Cell Tissue Organ Cult.* **2017**, *129*, 483–491. [CrossRef]
111. White, P.R. *A Handbook of Plant Tissue Culture*, 1st ed.; Jaques Cattell Press: Lancaster, PA, USA, 1943; pp. 1–277.
112. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
113. Gamborg, O.L.; Miller, R.A.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **1968**, *50*, 151–158. [CrossRef]
114. Nitsch, J.P. Experimental androgenesis in *Nicotiana*. *Phytomorphology* **1969**, *19*, 389–404.
115. Schenk, R.U.; Hildebrandt, A.C. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* **1972**, *50*, 199–204. [CrossRef]

116. Gaj, M.D. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. *Plant Growth Regul.* **2004**, *43*, 27–47. [CrossRef]
117. Nešković, M.; Petrović, J.; Radojević, L.; Vujičić, R. Stimulation of growth and nucleic acid biosynthesis at low concentration of abscisic acid in tissue culture of *Spinacia oleracea*. *Physiol. Plant.* **1977**, *39*, 148–154. [CrossRef]
118. Ishizaki, T.; Hoshino, Y.; Masuda, K.; Oosawa, K. Explants of Ri-transformed hairy roots of spinach can develop embryogenic calli in the absence of gibberellic acid, an essential growth regulator for induction of embryogenesis from nontransformed roots. *Plant Sci.* **2002**, *163*, 223–231. [CrossRef]
119. Dehio, C.; Grossmann, K.; Schell, J.; Schmülling, T. Phenotype and hormonal status of transgenic tobacco plants overexpressing the *rolA* gene of *Agrobacterium rhizogenes* T-DNA. *Plant Mol. Biol.* **1993**, *23*, 1199–1210. [CrossRef] [PubMed]
120. Schen, W.H.; Petit, A.; Guern, J.; Tempé, J. Hairy roots are more sensitive to auxin than normal roots. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 3417–3421. [CrossRef] [PubMed]
121. Spanò, L.; Mariotti, D.; Cardarelli, M.; Branca, C.; Costantino, P. Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. *Plant Physiol.* **1988**, *87*, 479–483. [CrossRef]
122. Ishizaki, T.; Komai, F.; Msuda, K.; Megumi, C. Exogenous ethylene enhances formation of embryogenic callus and inhibits embryogenesis in cultures of explants of spinach roots. *J. Am. Soc. Hortic. Sci.* **2000**, *125*, 21–24. [CrossRef]
123. Chin, D.P.; Bao, J.H.; Mii, M. Transgenic spinach plants produced by *Agrobacterium*-mediated method based on the low temperature-dependent high plant regeneration ability of leaf explants. *Plant Biotechnol.* **2009**, *26*, 243–248. [CrossRef]
124. Zdravković-Korać, S.; Belić, M.; Ćalić, D.; Milojević, J. Trichostatin and dimethyl sulfoxide enhance somatic embryogenesis from root apices of spinach. In *Book of Abstracts of the 4th International Conference on Plant Biology and 23rd SPPS Meeting, Belgrade, Serbia, 6–8 October 2022*; Milutinović, M., Ed.; Serbian Plant Physiology Society: Belgrade, Serbia, 2022; p. 25.
125. Belić, M. Synergistic Effect of Gibberellin and Light on the Induction of Somatic Embryogenesis from Lateral Roots of Spinach (*Spinacia oleracea* L.) in vitro. Ph.D. Thesis, University of Belgrade, Belgrade, Serbia, 2021.
126. Belić, M.; Tarkowská, D.; Zdravković-Korać, S.; Milojević, J. Enhanced Metabolism of Gibberellins is Involved in the Induction of Somatic Embryogenesis from Root Apices of Spinach. In *Book of Abstracts of the 4th International Conference on Plant Biology and 23rd SPPS Meeting, Belgrade, Serbia, 6–8 October 2022*; Milutinović, M., Ed.; Serbian Plant Physiology Society: Belgrade, Serbia, 2022; p. 26.
127. Belić, M.; Zdravković-Korać, S.; Milojević, J. Paclobutrazol and GA_3 Synergistically Promote Somatic Embryogenesis from Root Apices of Spinach. In *Book of Abstracts of the 4th International Conference on Plant Biology and 23rd SPPS Meeting, Belgrade, Serbia, 6–8 October 2022*; Milutinović, M., Ed.; Serbian Plant Physiology Society: Belgrade, Serbia, 2022; p. 27.
128. Desta, B.; Amare, G. Paclobutrazol as a plant growth regulator. *Chem. Biol. Technol. Agric.* **2021**, *8*, 1. [CrossRef]
129. Maheshwari, C.; Garg, N.K.; Hasan, M.V.P.; Meena, N.L.; Singh, A.; Tyagi, A. Insight of PBZ mediated drought amelioration in crop plants. *Front. Plant Sci.* **2022**, *13*, 1008993. [CrossRef]
130. Nagar, S.; Singh, V.P.; Arora, A.; Dhakar, R.; Singh, N.; Singh, G.P.; Meena, S.; Kumar, S.; Ramakrishnan, R.S. Understanding the role of gibberellic acid and paclobutrazol in terminal heat stress tolerance in wheat. *Front. Plant Sci.* **2021**, *12*, 692252. [CrossRef]
131. Talon, M.; Zeevaart, J.A.D.; Gage, D.A. Identification of gibberellins in spinach and effects of light and darkness on their levels. *Plant Physiol.* **1991**, *97*, 1521–1526. [CrossRef]
132. Zeevaart, J.A.D.; Gage, D.A.; Talon, M. Gibberellin A1 is required for stem elongation in spinach. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 7401–7405. [CrossRef] [PubMed]
133. Wu, K.; Li, L.; Gage, D.; Zeevaart, J.A.D. Molecular cloning and photoperiod-regulated expression of *gibberellin 20-oxidase* from the long day plant spinach. *Plant Physiol.* **1996**, *110*, 547–554. [CrossRef]
134. Lee, D.J.; Zeevaart, J.A.D. Molecular cloning of *GA2-oxidase3* from spinach and its ectopic expression in *Nicotiana sylvestris*. *Plant Physiol.* **2005**, *138*, 243–254. [CrossRef] [PubMed]
135. Lee, D.J.; Zeevaart, J.A.D. Regulation of *gibberellin 20-oxidase1* expression in spinach by photoperiod. *Planta* **2007**, *226*, 35–44. [CrossRef]
136. Hedden, P.; Phillips, A. Gibberellin metabolism: New insights revealed by the genes. *Trends Plant Sci.* **2000**, *5*, 523–530. [CrossRef] [PubMed]
137. Yamaguchi, S.; Kamiya, Y. Gibberellin biosynthesis: Its regulation by endogenous and environmental signals. *Plant Cell Physiol.* **2000**, *3*, 251–257. [CrossRef] [PubMed]
138. Graebe, J.E. Gibberellin biosynthesis and control. *Annu. Rev. Plant Physiol.* **1987**, *38*, 419–465. [CrossRef]
139. Lardon, R.; Wijnker, E.; Keurentjes, J.; Geelen, D. The genetic framework of shoot regeneration in Arabidopsis comprises master regulators and conditional fine-tuning factors. *Commun. Biol.* **2020**, *3*, 549. [CrossRef]
140. Lardon, R.; Geelen, D. Natural variation in plant pluripotency and regeneration. *Plants* **2020**, *24*, 1261. [CrossRef]
141. Motte, H.; Vercauteren, A.; Depuydt, S.; Landschoot, S.; Geelen, D.; Werbrouck, S.; Goormachtig, S.; Vuylsteke, M.; Vereecke, D. Combining linkage and association mapping identifies RECEPTOR-LIKE PROTEIN KINASE1 as an essential Arabidopsis shoot regeneration gene. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 8305–8310. [CrossRef]
142. Luichtl, M.; Fiesselmann, B.S.; Matthes, M.; Yang, X.; Peis, O.; Brunner, A.; Torres-Ruiz, R.A. Mutations in the Arabidopsis RPK1 gene uncouple cotyledon anlagen and primordia by modulating epidermal cell shape and polarity. *Biol. Open* **2013**, *2*, 1093–1102. [CrossRef] [PubMed]

143. Jha, P.; Ochatt, S.J.; Kumar, V. *WUSCHEL*: A master regulator in plant growth signaling. *Plant Cell Rep.* **2020**, *39*, 431–444. [CrossRef] [PubMed]
144. Rose, R.J. Somatic embryogenesis in the *Medicago truncatula* model: Cellular and molecular mechanisms. *Front. Plant Sci.* **2019**, *10*, 267. [CrossRef] [PubMed]
145. Orłowska, A.; Kępczyńska, E. Identification of *Polycomb Repressive Complex1*, *Trithorax group* genes and their simultaneous expression with *WUSCHEL*, *WUSCHEL-related Homeobox5* and *SHOOT MERISTEMLESS* during the induction phase of somatic embryogenesis in *Medicago truncatula* Gaertn. *Plant Cell Tissue Organ Cult.* **2018**, *134*, 345–356. [CrossRef]
146. Lombardi-Crestana, S.; da Silva Azevedo, M.; Ferreira e Silva, G.F.; Pino, L.E.; Appezzato-da-Glória, B.; Figueira, A.; Silveira Nogueira, F.T.; Pereira Peres, L.E. The tomato (*Solanum lycopersicum* cv. Micro-Tom) natural genetic variation Rg1 and the DELLA mutant procera control the competence necessary to form adventitious roots and shoots. *J. Exp. Bot.* **2012**, *63*, 5689–5703. [CrossRef]
147. Nishimura, A.; Ashikari, M.; Lin, S.; Takashi, T.; Angeles, E.R.; Yamamoto, T.; Matsuoka, M. Isolation of a rice regeneration quantitative trait loci gene and its application to transformation systems. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 11940–11944. [CrossRef]
148. Zhang, H.; Zhang, T.T.; Liu, H.; Shi, D.Y.; Wang, M.; Bie, X.M.; Li, X.G.; Zhang, X.S. Thioredoxin-mediated ROS homeostasis explains natural variation in plant regeneration. *Plant Physiol.* **2018**, *176*, 2231–2250. [CrossRef]
149. Lim, T.S.; Chitra, T.R.; Tay, B.H.; Pua, E.C.; Yu, H. Molecular characterization of *Arabidopsis* and *Brassica juncea* Cu/Znsuperoxide dismutases reveals their regulation of shoot regeneration. *J. Plant Growth Regul.* **2008**, *27*, 99–109. [CrossRef]
150. Kawade, K.; Ishizaki, T.; Masuda, K. Differential expression of ribosome-inactivating protein genes during somatic embryogenesis in spinach (*Spinacia oleracea*). *Physiol. Plant.* **2008**, *134*, 270–281. [CrossRef]
151. Kawade, K.; Masuda, K. Transcriptional control of two ribosome—Inactivating protein genes expressed in spinach (*Spinacia oleracea*) embryos. *Plant Physiol. Biochem.* **2009**, *47*, 327–334. [CrossRef]
152. Al-Khayri, J.M.; Huang, F.H.; Morelock, T.E.; Lane, F.E. In vitro flowering in regenerated shoots of spinach. *HortScience* **1991**, *26*, 1422. [CrossRef]
153. Al-Khayri, J.M.; Huang, F.H.; Morelock, T.E.; Busharar, T.A. In vitro seed production from sex-modified male spinach plants regenerated from callus cultures. *Sci. Hortic.* **1992**, *52*, 277–282. [CrossRef]
154. Milojević, J. Expression of Gene Encoding Ribosome-Inactivating Protein (*SoRIP2*) as a Marker for the Analysis of Spinach (*Spinacia oleracea* L. cv. Matador) Embryogenic Potential In Vitro. Ph.D. Thesis, University of Belgrade, Belgrade, Serbia, 2015.
155. Ochatt, S.J.; Sangwan, R.S.; Marget, P.; Ndong, Y.A.; Rancillac, M.; Perney, P. New approaches towards the shortening of generation cycles for faster breeding of protein legumes. *Plant Breed.* **2002**, *121*, 436–440. [CrossRef]
156. Ochatt, S.J.; Sangwan, R.S. In vitro shortening of generation time in *Arabidopsis thaliana*. *Plant Cell Tissue Organ Cult.* **2008**, *93*, 133–137. [CrossRef]
157. Nguyen, Q.V.; Boo, K.H.; Sun, H.J.; Cao, D.V.; Lee, D.; Ko, S.H.; Kang, S.; Yoon, S.; Kim, S.C.; Park, S.P.; et al. Evaluation of factors influencing *Agrobacterium*-mediated spinach transformation and transformant selection by EGFP fluorescence under low-selective pressure. *Vitr. Cell Dev. Biol.-Plant* **2013**, *49*, 498–509. [CrossRef]