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In Vitro Plant Regeneration of *Sulla coronaria* from Floral Explants as a Biotechnological Tool for Plant Breeding

Monica Auteri [1], Angela Carra [2], Giuseppe Di Miceli [1,*], Nicolò Iacuzzi [1], Alessandro Albano [3], Nicoletta Lala [1], Antonio Motisi [2] and Caterina Catalano [2,4]

[1] Department of Agricultural, Food and Forest Sciences, University of Palermo, Viale delle Scienze, Building 4, 90128 Palermo, PA, Italy; monica.auteri@unipa.it (M.A.); nicolo.iacuzzi@unipa.it (N.I.); nicoletta.lala@unipa.it (N.L.)

[2] National Research Council of Italy (CNR), Institute of Biosciences and BioResources (IBBR), Via Ugo La Malfa 153, 90146 Palermo, PA, Italy; angela.carra@cnr.it (A.C.); antonio.motisi@ibbr.cnr.it (A.M.); caterina.catalano@cnr.it (C.C.)

[3] Department of Economics, Business and Statistics, University of Palermo, Viale delle Scienze, Building 13, 90128 Palermo, PA, Italy; alessandro.albano@unipa.it

[4] National Research Council of Italy (CNR), Institute of Agricultural Biology and Biotechnology (IBBA), Via Alfonso Corti 12, 20133 Milano, MI, Italy

* Correspondence: giuseppe.dimiceli@unipa.it

Abstract: *Sulla coronaria* L. Medik., a biennial forage legume typical of the Mediterranean basin, plays a significant role in foraging systems due to its high nutritional value, ability to increase ruminant live weight, and potential to reduce methane emissions. However, its allogamous nature complicates genetic improvement and the development of new varieties with desired traits. Given these challenges, this study aims to develop, for the first time, a successful protocol for the in vitro meristematic shoot regeneration of *S. coronaria*. The experiment utilizes four different flower explants (anther with filament, ovary, petals, and whole immature flower) collected from twenty distinct *S. coronaria* biotypes with three plant growth regulator (PGR) combinations and under both light and dark conditions. In terms of the regeneration response, the key factors appear to be the combinations of PGRs and the type of explant used. The interactions between all the factors do not seem to be significant.

Keywords: forage crop; factors affecting regeneration; biotypes; PGR combinations; light conditions

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1. Introduction

Sulla coronaria (L.) Medik. [1], also known as *Hedysarum coronarium* L., is a biennial forage legume commonly found in arable farming systems and natural environments throughout the Mediterranean region [2–4]. This plant plays a significant role in foraging systems, with its cultivation spanning from Portugal and Greece to North Africa (Morocco, Algeria, Tunisia), as well as Egypt, Turkey, and Lebanon [5]. It is also found in certain regions in New Zealand [6], Australia [7,8], and California [9]. Its geographical spread on a global scale was facilitated by the identification and selection of the *Rhizobium sullae* strain (WSM1592), as highlighted by Yates et al. [7]. This advancement was followed by the production and commercialization of the inoculum, which played a crucial role in its widespread cultivation. It is known for its excellent forage characteristics, such as an exceptional grazing resource, as well as its suitability for making hay, silage, and dehydrated products [10]. It has always been studied for its remarkable ability to increase milk production, primarily due to its high nutritional value [11] and moderate content of total phenolic compounds, especially proanthocyanidins, known as condensed tannins [12–15]. These secondary metabolites have demonstrated potential benefits for ruminant health by improving production performance. Such benefits include increased live weight, enhanced milk

quality, and a reduction in methane emissions [16]. *S. coronaria* is primarily an allogamous species [17], a trait that influences the genetic variability of natural populations and seed production [10]. This allogamous nature complicates genetic improvement efforts aimed at developing new varieties with desired traits. Additionally, it affects the conservation of existing varieties, particularly in their natural habitats. In Italy, since 1970, when the first two varieties of sulla were added to the National Register of Varieties, a total of 15 applications for registration have been submitted. Among these, five have been rejected, one is currently in the registration phase, one has been canceled, and six are in the final stage of registration termination. As a result, only two varieties are currently registered. This scenario highlights a challenge in promoting the development and maintenance of improved varieties, given the complexity of managing the seed business. The difficulties associated with seed production are reflected by the limited interest in the production of improved varieties. Currently, much of the genetic material used for sowing *S. coronaria* meadows still comes from local landraces. This reliance on traditional genetic material highlights the ongoing challenges in advancing and commercializing new, improved *S. coronaria* varieties. However, it is also widely recognized that the effects of biotic and abiotic stresses can reduce the availability of plant resources and lead to a decrease in biomass, seeds [18], and productivity in forage crops [19]. In this context, biotechnologies offer a promising alternative to conventional breeding, aiding in the development of stress-resistant genotypes and enhancing crop yield and quality [20,21]. Plant biotechnology has proven effective in plant breeding through methods such as genomic selection, DNA-based markers, and genome editing. In vitro shoot regeneration and propagation allow for the production of new individuals from minimal starting material, thereby eliminating seasonal constraints [22]. The efficiency of in vitro plant regeneration is influenced by various factors, including genotypes, plant growth regulators (PGRs), light conditions, explant type, and the characteristics of the donor plant [23]. By manipulating these factors, the production of new varieties can be significantly enhanced [24]. Regarding the regeneration of *S. coronaria*, only one experiment, conducted by Arcioni et al. [25], has been reported, in which they successfully obtained regenerates from protoplasts derived from hypocotyls and cotyledons. To the best of our knowledge, no other studies on the regeneration of *S. coronaria* have been published. Therefore, the aim of this study was to develop a successful protocol for obtaining in vitro regeneration of meristematic shoots from floral explants. The ability to regenerate new individuals from four explant types (anther with filament, ovary, petal, and whole immature flower) was compared in 20 different biotypes of *S. coronaria*. Explants were cultivated in vitro under three different combinations of PGRs and under light and dark conditions.

2. Materials and Methods

2.1. Sample Collection

The flowers used in this study were collected in March 2023 in twenty different sites located in Sicily, Italy (Figure 1), and samples were kept under refrigeration until culture.

The sites were assigned to 4 different habitats (HAB1, HAB3, HAB4, HAB5) [10]. Sites were specified with 2 letters indicating the province of sampling, followed by a sequential number: Agrigento (AG), Caltanissetta (CL), Enna (EN), Messina (ME), Palermo (PA), and Trapani (TP). HAB1 denotes the mountainous area, and includes 4 sites (ME1, EN1, PA6, and PA7). HAB3 includes 4 sites (CL1, CL2, AG1, and PA5) in the inland hilly area, while HAB4 refers to the sites CL3, AG2, AG3, and AG4, which are located on the southern coast. Finally, HAB5 comprises 8 sites (AG5, PA1, PA2, PA3, PA4, TP1, TP2, and TP3), which are found in the western hilly area.

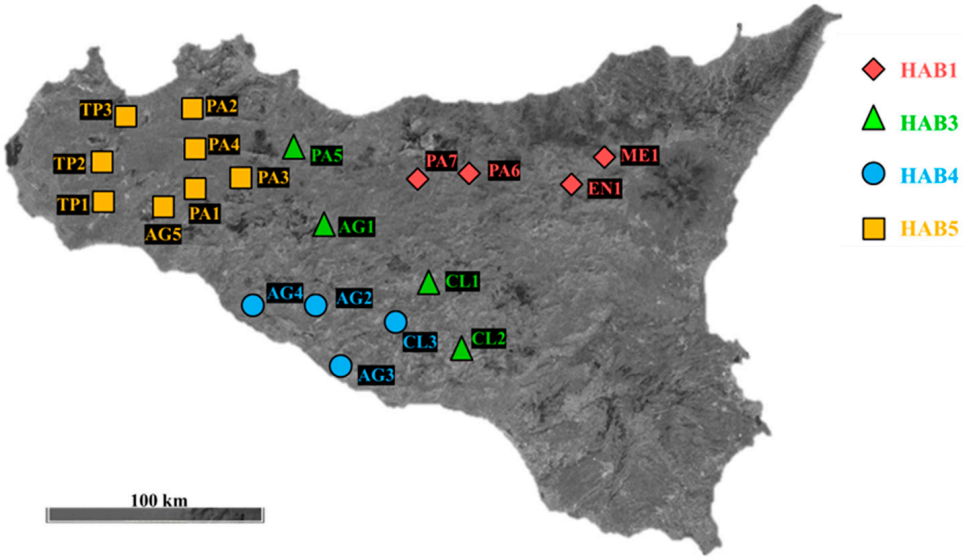

Figure 1. Geographical locations of the 20 sampling sites of the *Sulla coronaria* landraces. The 2 letters indicate the province of sampling: AG: Agrigento, PA: Palermo, TP: Trapani, CL: Caltanissetta, EN: Enna, ME: Messina, followed by a sequential number. Different symbols indicate the habitats assigned according to Ruisi et al. [10].

2.2. Plant Materials

Cultures were initiated from four different explants, anther with filament (Ant), ovary (Ov), petals (Pet) and whole flower (Wf) (Figure 2) dissected from immature flowers, which were collected 15 days before anthesis from both wild plants and different biotypes.

Figure 2. Floral explants used in this study. Anthers with filament (**a**), ovary (**b**), petals (**c**), whole flower (**d**).

Whole inflorescences were surface sterilized as described by Catalano et al. [26], with slight modifications. The inflorescences were first washed by immersion for 10 min in distilled water containing 0.15 mL/100 mL (approximately 3 drops) of Tween-20 under gentle shaking. Then, the flowers were disinfected under laminar flow with ethanol 70% (*v*/*v*) for 1 min and sodium hypochlorite 0.6% (*w*/*v* in water) for 7 min. The explants were then rinsed three times for 5 min in sterile distilled water. Only close flowers were used.

2.3. Culture Conditions

The explants were cultured on MS solidified medium [27] supplemented with 30 g L^{-1} of sucrose; the pH of the medium was adjusted to 5.7 ± 0.1 with 1N NaOH, and 7 g L^{-1} of agar was added to solidify the medium before autoclaving (20 min, 120 °C). The explants were plated under three different PGR combinations, as reported in Table 1.

Table 1. PGR combinations used in this study.

PGR Combinations	Ref.
(S.c. 1) 5 µM N-(2-chloro-4-pyridyl)-N′-phenylurea (4-CPPU) + 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D)	[26]
(S.c. 2) 8.8 µM 6-banzylaminopurine (BA) + 1.07 µM 1-Naphthalene acetic acid (NAA)	[27]
(S.c. 3) 10 µM naphthoxyacetic acid (NOA) + 4.4 µM 6-banzylaminopurine (BA)	[26]

All the PGRs were filter sterilized and added to the medium after autoclaving. Petri dishes (60 × 10 mm) were filled with 8 mL of medium and sealed with Parafilm MTM. All chemicals used in this experiment were purchased from Duchefa Biochemie, Haarlem, The Netherlands.

Four different explants were cultured (five explants per Petri dish); each treatment consisted of five replications. All the explants were incubated in a climatic chamber at 25 ± 1 °C. To evaluate the influence of light, half of the explants were incubated in dark conditions; the others were incubated under a 16 h per day length and a photosynthetic photon flux of 50 µmol m^{-2} s^{-1} provided by an Osram cool white 18 W fluorescent lamp. The explants producing callus with green spots were transferred to hormone-free MS medium and cultured for an additional month to allow for proliferation and development.

2.4. Data Collection

The explants were checked at 15-day intervals. Non-responsive explants, callus, and regenerates were evaluated as percentage of all the conditions simultaneously applied.

The effects of biotype, PGR combinations, explant type, and light conditions on callus production and the regeneration of new individuals were evaluated.

2.5. Statistical Analysis

The dataset includes multiple explants collected from 20 plants, with each explant displaying one of three possible outcomes: callus formation, meristematic shoot formation, or no response. To investigate the factors influencing these outcomes, we modeled two binary responses using logistic regression. Model 1 examined the presence vs. absence of callus formation, and model 2 analyzed the presence vs. absence of meristematic shoot formation. Since the explants represent repeated measurements taken from the same plant, it is important to account for the hierarchical structure of the data by incorporating a random effect to capture potential plant-level variability. If such variability exists and is ignored, it could result in biased parameter estimates and underestimated standard errors, as the explants from the same plant may not be independent.

To address this issue, we initially applied mixed-effects logistic regression models [28–31] for both responses (callus formation and meristematic shoot formation), incorporating plant-level random intercepts to account for potential heterogeneity among plants. In these models, biotype was included both as a fixed effect and in the random effects structure, while the other explanatory variables, such as PGR combination (3 levels), explant type (4 levels), and light condition (2 levels), were just included in the fixed part.

Contaminated explants were excluded from the analysis, as contamination was attributed to the disinfection method rather than the experimental conditions applied.

For each response, we performed model selection using the Akaike information criterion (AIC) and Bayesian information criterion (BIC) [32,33].

The general model specification, assuming no interactions between the variables, is provided by the following equation:

$$\begin{aligned}\log\left(\frac{\Pr(Y_{ij}=1)}{\Pr(Y_{ij}=0)}\right) &= \beta_0 + \boldsymbol{\beta_1}(\text{Biotype})_{ij} + \boldsymbol{\beta_2}(\text{PGR combination})_{ij} + \boldsymbol{\beta_3}(\text{Explant type})_{ij} \\ &+ \beta_4(\text{Light condition})_{ij} + u_i\end{aligned} \quad (1)$$

where Y_{ij} represents the binary response for the j-th explant of the i-th plant (i.e., callus formation in model 1 and meristematic shoot formation in model 2). Moreover, β_0 is the fixed intercept, while $\boldsymbol{\beta_1}, \boldsymbol{\beta_2}, \boldsymbol{\beta_3}, \beta_4$ represent the fixed coefficients for the explanatory variables: biotype, PGR combination, explant type, and light condition, respectively. The term u_i, i.e., the random effect associated with plant i, captures the variability between individual plants. This allows the model to account for the non-independence of observations from the same plant.

The interpretation of the model's output, particularly the estimated regression coefficients, depends on the choice of the baseline for each categorical explanatory variable. Indeed, in all factors, one level is chosen as the reference category, and the model's coefficients reflect the comparison of other levels against this baseline.

We reported both the significance and the direction of the regression coefficients for each variable. The symbols "+" and "-" were used to indicate whether a variable had a positive or negative effect on the response (callus formation or meristematic shoots). Statistical significance was denoted as follows: * for $\alpha \leq 0.05$, ** for $\alpha \leq 0.01$, and *** for $\alpha \leq 0.001$. Statistical analysis was performed using R version 4.4.1 (14 June 2024).

3. Results

Regeneration responses were recorded after approximately 20 days from culture. A callus formation (Figure 3a) was recorded before the appearance of shoots under all the combinations tested (Figure 3b,c).

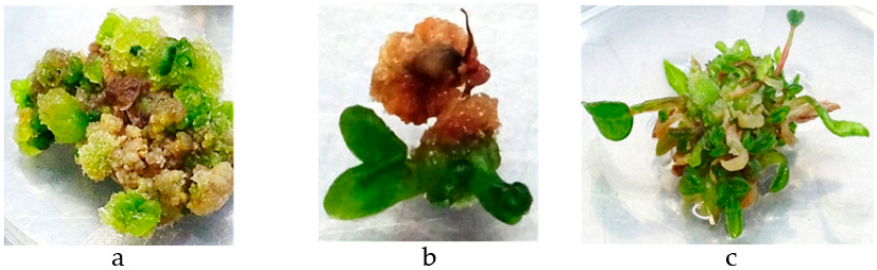

Figure 3. Callus (**a**) and shoots (**b**,**c**) obtained from whole flower and ovaries. Respectively, (**a**) was obtained from ME1 cultured on S.c. 1 medium, whole flower, light conditions; (**b**,**c**) from TP1 and TP3 both from ovary, cultured on S.c. 3 medium, incubated in light regime. Pictures were taken after 1 month of culture.

3.1. Results of the Models

The results of the percentages of callus, meristematic shoots, and non-responsive explants, under all test conditions, are reported in Table S1. Some observations have been excluded due to contamination of all the explants.

3.1.1. Callus Production

For callus production, we initially fitted the mixed-effects logistic regression model Equation (1), where Y_{ij} represents the binary outcome, with $Y_{ij} = 1$ if the j-th explant from the i-th plant exhibits *callus* formation, and $Y_{ij} = 0$ otherwise. The models assumes that the response is Bernoulli distributed, that is:

$$Y_{ij} \sim Bernoulli(p_{ij}).$$

Here, p_{ij} represents the probability that the j-th explant from the i-th plant exhibits callus formation. Initially, the model included both fixed effects (biotype, PGR combination, explant type, and light conditions) and random intercepts to account for plant-level variability. However, after fitting the model, the variance associated with the random intercept was found to be near zero, indicating that plant-level heterogeneity was negligible. As a result, we excluded the random intercept in the final model, thus fitting a standard

logistic regression model. Model selection on the fixed part was based on AIC and BIC, which favored the model containing only the main effects. Including interaction terms between factors led to an increase in both AIC and BIC values, indicating a poorer model fit. Additionally, the coefficients for the interaction terms were not statistically significant, further justifying their exclusion. Thus, the final model is defined as:

$$\log\left(\frac{\Pr(Y_j=1)}{\Pr(Y_j=0)}\right) = \beta_0 + \boldsymbol{\beta_1}(\text{Biotype})_j + \boldsymbol{\beta_2}(\text{PGR combination})_j + \boldsymbol{\beta_3}(\text{Explant type})_j + \beta_4(\text{Light condition})_j \quad (2)$$

The baseline for the final model was set as: Biotype = "AG1", PGR combination = "S.c. 1", Explant type = "Ant", and Light condition = "Dark". The results are summarized in Table 2.

Table 2. Effects of biotypes, PGR combinations, explant type, and light conditions on callus formation.

Variables	*Category*	*Significance for Callus Production*	*Type of Effect*
Biotypes	AG1	Reference category	
	AG2	***	-
	AG3	**	-
	AG4	***	-
	AG5	**	-
	CL1	***	-
	CL2	***	-
	CL3	**	-
	EN1	***	-
	ME1	***	-
	PA1	***	-
	PA2	***	-
	PA3	*	-
	PA4	***	-
	PA5	***	-
	PA6	*	-
	PA7	***	-
	TP1	***	-
	TP2	**	-
	TP3	***	-
PGR Combinations	S.c. 1	Reference category	
	S.c. 2	n.s.	n.s.
	S.c. 3	**	-
Explant Type	Ant	Reference category	
	Ov	***	-
	Pet	***	-
	Wf	***	-
Light Conditions	Dark	Reference category	
	Light	*	-

The baseline model is Biotype = "AG1", PGR combinations = "S.c. 1", Explant = "Ant", and Light condition = "Dark". Significance levels: n.s. = not significant; * = significant at $\alpha \leq 0.05$, ** = significant at $\alpha \leq 0.01$, *** = significant at $\alpha \leq 0.001$. +: positive interaction; -: negative interaction. Ant: Anther, Ov: Ovary, Pet: Petals, Wf: Whole flower.

Table 2 shows that the Biotype AG1, which was used as the reference category, is the most favorable for callus formation. Due to the corner-point parameterization, all other biotypes showed negative coefficients compared to AG1, indicating that they produced fewer *calli*. These negative coefficients were statistically significant for all other biotypes, suggesting a clear difference in callus production across biotypes. PGR combinations also play a role, with S.c. 3 reducing callus formation significantly compared to S.c. 1, while S.c. 2 does not show significant differences compared to the baseline. The explant type is a key factor in affecting the probability of callus formation. Specifically, Ant (baseline)

shows the highest probability of callus formation, while Ov, Pet, and Wf are associated with significantly lower odds of callus production. Finally, light conditions have a small but significant negative effect, indicating that light exposure decreases the probability of callus formation. All estimates reflect the difference in log-odds of callus production between a given level of a factor and its reference level. Hence, AG1 biotype, Ant explants, S.c. 1 PGR combination, and no light exposure represent the most favorable conditions for callus production according to this model.

3.1.2. Meristematic Shoots

For meristematic shoots, we initially fitted the mixed-effect logistic regression model defined in Equation (1), where Y_{ij} is 1 if the j-th explant from the i-th plant exhibits meristematic shoot formation, and 0 otherwise. The models assumes that the response is Bernoulli distributed, that is:

$$Y_{ij} \sim Bernoulli(p_{ij}).$$

where p_{ij} represents the probability that the explant exhibits meristematic shoots. This probability was modeled as a function of the fixed effects (such as biotype, PGR combination, explant type, and light conditions) and random effects (biotype) to account for plant-level variability. During model selection for the fixed effects, the variables biotype and light condition were found to be non-significant. Excluding these variables led to improvements in AIC and BIC, indicating a better model fit. However, the variance associated with the random intercept for biotype was remarkable (equals to 3.455), indicating non-negligible heterogeneity across plants, thus justifying the inclusion of the random effect in the final model. Thus, the final model includes only the significant main effects of PGR combination and explant type, along with the random intercept for plant-level variability. It is important to note that while the variable biotype did not show significance as a fixed effect, it proves valuable as a random effect due to the variability observed in the response across different biotypes. This phenomenon can be attributed to the substantial variance of the random component associated with biotype, suggesting that while individual biotypes may not consistently influence shoot formation, the differences among biotypes still contribute to the overall model by accounting for unobserved heterogeneity. Moreover, although meristematic shoots were observed in only seven biotypes, the model includes all biotypes for analysis. Indeed, including all biotypes in the model ensures a robust analysis without prematurely excluding any groups based on limited outcomes. As regards model selection, when interaction terms between covariates were included, both the AIC and BIC values increased, indicating a decline in model performance. Moreover, the interaction terms were not statistically significant, further justifying their exclusion. Thus, the final model is specified as follows:

$$\log\left(\frac{\Pr(Y_{ij}=1)}{\Pr(Y_{ij}=0)}\right) = \beta'_0 + \boldsymbol{\beta'_1}(\text{PGR combination})_{\boldsymbol{ij}} + \boldsymbol{\beta'_3}(\text{Explant type})_{\boldsymbol{ij}} + u_i \quad (3)$$

The model's baseline was set at: PGR combination = "S.c. 1", Explant type = "Ant". The results of the model are summarized in Table 3.

Notably, the PGR combinations demonstrated a significant impact on the response, with both S.c. 2 and S.c. 3 showing significant positive effects on shoot formation. Specifically, the results indicate that using the PGR combination S.c. 1 results in fewer meristematic shoots compared to S.c. 2 and S.c. 3, which produced the highest number of shoots. Additionally, the type of explant significantly influenced shoot development, with petals showing a notable negative effect, suggesting that they are less effective for shoot regeneration compared to the reference condition. In contrast, other explant types, such as ovary and whole flower, did not exhibit significant differences compared to the baseline.

Table 3. Effects of PGR combinations and explant type on meristematic shoots.

Variables	Category	Significance for Meristematic Shoots	Type of Effect
PGR Combinations	S.c. 1	Reference category	
	S.c. 2	*	+
	S.c. 3	**	+
Explant Type	Ant	Reference category	
	Ov	n.s.	n.s.
	Pet	*	-
	Wf	n.s.	n.s.

The baseline model is PGR = "S.c. 1", Explant type = "Ant". Significance levels: n.s. = not significant; * = $\alpha \leq 0.05$; ** = $\alpha \leq 0.01$.

4. Discussion

We report, for the first time to our knowledge, an experimental procedure for meristematic shoot regeneration of *S. coronaria* starting from flower explants. Although using flowers imposes a temporal limitation on collection, flower tissues are easy to sterilize and have demonstrated high regenerative potential in several species [34–39]. Additionally, immature tissues have been identified as the most suitable explant for somatic embryogenesis and in vitro regeneration in many species, including *Leguminosae* [40].

In our experimental procedure, callus and shoots were obtained under all the combinations tested. The best performance in meristematic shoot production was recorded with medium S.c. 3 (10 µM NOA + 4.4 µM BA) and medium S.c. 2 (8.8 µM BA + 1.07 µM NAA). In both cases, a combination of auxin and cytokinin was used to induce regeneration. Auxins and cytokinins together determine the regeneration response due to their crucial roles in cell cycle regulation and cell division [41]. The presence of auxin NAA is generally essential for initiating competent callus for plant regeneration [42]. Both NOA and NAA have been noted for their beneficial impact on promoting significant regeneration rates in various species, including grapevine [34,38] and sesame [43]. BA has also been widely used for in vitro regeneration processes, yielding successful results [44]. The addition of different concentrations of BA in combination with auxins such as NAA to different media significantly affects callus induction and tissue quality [45]. Our results confirm previous reports, but concerning *S. coronaria*, they are novel.

The effectiveness of most tissue culture studies is largely dependent on the type of explant [46], even though among all the explants tested, only the petals showed a negative effect on the production of meristematic shoots. Several authors have reported the effectiveness of anthers, which are quite simple to handle, especially in the early stages of development when the ability to respond positively is greater [47]. In vitro regeneration from anthers has been documented in other plant species, suggesting that these explants might be more competent for regeneration compared to other tissue types [48–50]. Genotype is a crucial factor influencing how plants respond to tissue culture, with variations observed across species, cultivars, and even among individuals within the same species. Certain genotypes exhibit a higher propensity for in vitro regeneration, as demonstrated in various species such as *Crocus* sp., *Rosa* sp., *Glycine max* L. Merr., *Feijoa sellowiana* O. Berg, and *Carthamus tinctorius* L. [51]. In our study, shoots were obtained in only seven *S. coronaria* biotypes tested, but based on statistical analysis, this does not appear to be a significant factor. Environmental conditions influence the regeneration outcome, and the light regime (e.g., dark vs. light exposure) usually affects both the type of callus formed and subsequent regeneration [24]. Some species require dark treatments to improve regeneration percentages, as reported for grapevine [52], while for other species, continuous light presence is important in every regeneration phase [53]. In our study, no significant differences were found between explants incubated in the dark and those in light regarding meristematic shoot production, but this seems to be important for callus production. Interactions between factors can strongly impact the process [54]. Unfortunately, not all interactions in

the regeneration process are well documented in the literature. Fiuk & Rybczyński [51] emphasized the importance of considering the interaction between genotype and PGR combinations, but based on our data, this does not appear to be valid for shoot formations in *S. coronaria*. Additionally, genotype and PGR combinations seem to be primary sources of variation in the in vitro culture responses of cereals and forages [55], as we observed for PGR combinations. Pinto et al. [56] reported no statistically significant interaction between light conditions and culture media, which is in line with our study. However, the triple interaction between genotype, explant, and culture medium was investigated by Bedir et al. [46], who found that each explant from each genotype responded differently to each culture medium, contrary to our results in which there were no significant interactions.

5. Conclusions

This study is the first to explore the potential for in vitro regeneration from floral explants of *S. coronaria*, providing a fundamental contribution to improve the knowledge of the factors involved in the process. Four different factors—biotype, light conditions, PGR combinations, and explant type—were tested for their effects on the in vitro regeneration of sulla. Regarding the development of meristematic shoots, only the PGR combinations and the explant types appear to be important for the regeneration process. In particular, the use of PGR combinations S.c. 2 and S.c. 3 showed a positive effect on the process, while using petals as explants had a negative effect. These results could be useful for the selection of new varieties and to better understand, with breeding techniques such as DNA-based markers, which genes are responsible for the desired trait. Research is currently ongoing to verify these data, while additional analyses are necessary to evaluate the ploidy and the genetic assessments of regenerates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14112667/s1, Table S1: Percentages of callus, meristematic shoots and non-responsive explants for all combinations tested.

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