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The Effect of Picloram on Somatic Embryogenesis of Different Explants of Strawberry *(Fragaria ananassa* Duch.)

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Authors' contributions

This work was carried out in collaboration between all authors. Author MG designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author MZ managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

The present investigation was conducted to study the effects of different concentrations of picloram on somatic embryogenesis induction, development and maturation of three strawberry (Kurdistan, Paros and Camarosa) cultivars. For this purpose, leaf blade, nodal, petiole, stamen and flower bud calli were cultured on MS medium supplemented with picloram at 0.25, 0.5, 1 and 2 mg/L concentrations. The concentration of growth regulator, cultivar and explant type were found critical to somatic embryogenesis induction, development and maturation. Results obtained from the studies revealed that all explants with the exception of petiole and stamen incubated on medium formed embryonic calli. 2 mg/L picloram yielded the highest percentage of embryonic calli and number of globular-stage embryos and 1 mg/L picloram yielded the highest number of cotyledonary-stage embryos in all types of explants. The leaf explant calli and Paros cultivar were the most responsive to produce somatic embryogenesis induction, development and maturation.

Keywords: Plant regeneration; in vitro culture; growth regulator; callus induction; frequency of embryogenesis.

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1. INTRODUCTION

Cultivated strawberry (*Fragaria* × *ananassa* Duch.) as an octoploid species (2*n* = 8*x* = 56) belonging to the genus *Fragaria* of the family *Rosaceae*, is one of the most important fruit plants for both fresh consumption and food processing [1].

Somatic embryogenesis is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of somatic embryos [2]. The induction of somatic embryogenesis for *in vitro* plant regeneration provides several advantages over organogenesis [3]. Somatic embryogenesis plays an important role in clonal propagation. When integrated with conventional breeding programs and molecular and cell biological techniques, it provides a valuable tool to enhance the pace of genetic improvement of commercial crop species [4]. Somatic embryogenesis provides an excellent morphogenetic system for investigating the cellular and molecular process underlying differentiation [5]. In addition, it also provides the possibility to produce artificial seeds and valuable tools for genetic engineering and germplasm conservation via cryopreservation [6-7]. Somatic embryos can be encapsulated in various gelling systems to form artificial seeds which are easily stored and transported long distance [8]. Somatic embryo resembles the zygotic embryo in various aspects [9] and it is possible to study diverse subjects related to the embryogenesis process using the somatic embryo system. Due to the presence of well-developed root and shoot primordia, somatic embryos germinate easily to produce plantlets without an additional step of rooting [10].

Picloram has been successfully used for somatic embryogenesis in wheat [11], kodo millet [12], and effect of picloram on direct somatic embryo formation in strawberry has been reported recently by Karimi Kurdestani and Karami [13] but it is the first report on indirect somatic embryogenesis using picloram in different explants of strawberry cultivars.

2. MATERIALS AND METHODS

2.1 Callus Induction

The aseptic leaf blade, nodal and petiole (vegetative organs) from fully expended young leaves of 4 to 5 week old plantlets from *in vitro* cultured runner tips were used as explants. The leaf blade (each approximately 4×4 mm), nodal and petiole segments (4-5 mm) were cultured with the abaxial surface in contact with the callus induction media.

Flower buds (4-5 mm diameters) were collected from greenhouse grown stock plants of three strawberry cultivars and washed under running tap water for 30 min to remove surface contaminants. Surface sterilization was done inside the laminar air flow cabinet by dipping the explants for 30 s in 70% (v/v) ethanol and by submerging for 15 min in an aqueous solution of 0.5% (v/v) sodium hypochlorite and three washes in sterile double distilled water. Stamens and receptacles were removed from the flower buds and the flower buds (reproductive organs) were longitudinally cut and used as explant. Vegetative and reproductive organs explants were cultured on MS [14] medium supplemented with naphthalene acetic acid (NAA) at 4 mg/L concentration for callus induction. During callus induction period, all the cultures were incubated at dark in 25 ± 1ºC.

2.2 Somatic Embryogenesis Induction

After four weeks, 50 mg of leaf blade, nodal, petiole, stamen and flower bud calli were cultured on MS medium supplemented with picloram at 0.25, 0.5, 1 and 2 mg/L concentrations. Six calli were cultured in each petridish (100×20 mm) containing 30 ml of medium. The pH of medium was adjusted to 5.8 using 0.1 N NaOH or HCl and medium were solidified with 0.8% agar before autoclaving. The cultures were incubated in a growth chamber under dark at 25±2ºC. After every 4 weeks, to induce somatic embryos, the calli were transferred on to specific media. Data collected were the percentage of embryogenic calli, the number of globular embryos per responding explant as well as the percentage of globular stage embryos developing into cotyledonary stage embryos.

The data were subjected to statistical analysis. Thirty-six explants were used in each treatment, and the experiments were done in six replicates. Factorial analysis of variance was carried out using statistical analysis system (SAS) and differences between means were scored with Duncan's multiple range tests.

2.3 Conversion of Embryos

To regenerate whole plants, cotyledonary somatic embryos were transferred to MS basal medium containing 3% sucrose and 1 mg/L GA_3 and incubated under a 16-h light and 8-h dark photoperiod at 25±1°C. Plantlets developed from somatic embryos were subjected to acclimation, transplanted into plastic bags containing sterile perlite and maintained in a growth chamber under a 16-h light and 8-h dark photoperiod at 25±1ºC and relative humidity of 90–95% for 4 weeks. These plantlets were subsequently transferred into plastic bags containing sterile sand and garden soil (1:1) and maintained in a greenhouse for two weeks. Survival rate of the plants was calculated from number of plants transferred to greenhouse.

3. RESULTS AND DISCUSSION

This experiment was performed to evaluate the effects of the concentration of picloram on mean number of somatic embryos per embryogenic explant and percentage of globular embryos developing into cotyledonary embryos from the different explants of strawberry cultivars (Camarosa, Paros and Kurdistan). For this purpose, leaf blade, nodal, petiole, stamen and flower bud calli derived from MS medium containing 4 mg/L NAA, were cultured on MS medium supplemented with picloram at 0.25, 0.5, 1 and 2 mg/L concentrations.

3.1 Callus Morphology

In this study, two types of callus were observed from the point of view of callus color, texture and morphology. The calli derived from leaf blade, node and flower bud were creamy-yellow, compact and nodular type with proembryogenic structures (embryogenic calli; Fig. 1a). In the embryogenic calli, various stages of somatic embryogenesis were observed simultaneously on the same callus (Fig. 1b) indicating that somatic embryogenesis in strawberry is an asynchronous phenomenon. By contrast, the calli from petiole and stamen explants were white and watery with smooth surface and then turned brown (non-embryogenic calli). Browning of the callus, which has been reported previously [15-16] may be due to the activation of secondary metabolite synthesis. A high frequency of growth and no embryo induction was occurred on this type of calli, therefore we withdraw their analysis.

3.2 Somatic Embryogenesis

3.2.1 Somatic embryogenesis induction

The data obtained in this study indicate that the response of embryogenic calli was greatly dependent on the concentration of picloram. Percentage of embryogenic calli for leaf blade, nodal and flower bud explants were significantly different ($p<0.01$) for different picloram concentrations (Table 1). The means for percentage embryogenic calli ranged from 0% to 43.17%. The highest value (43.17%) was observed for 2 mg/L of picloram while the lowest value (0%) was for 0.25 mg/L picloram. Karimi Kurdestani and Karami [13] also observed similar effects on direct somatic embryogenesis in strawberry.

Data in Table 2 revealed that different concentrations of picloram tested in this trial had a significant effect on the number of globular embryos per explants. Increasing picloram concentration in the medium improved the frequency of globular somatic embryos. According to our results, a medium supplemented with the highest investigated concentration of picloram (2mg/L) proved to be the best for high frequency of globular embryo induction in all tested cultivars. Similarly, medium supplemented with picloram was also found to be favorable for somatic embryogenesis in *Musa acuminata* [17] and feijoa [18].

3.2.2 Somatic embryo development

Globular somatic embryos appeared on the surface of the calli within 3–4 weeks (Fig. 1b). During subculture, these globular embryos further developed into cotyledonary-stage embryos and attained maturity on the same medium (Fig. 1c). Table 3 shows the percentage of globular embryos developing into cotyledonary embryos. The maximum percentage of cotyledonary-stage embryos was recorded four weeks after transfer of globular somatic embryos to MS medium containing picloram.

Analysis of variance showed significant effects of picloram concentrations on the percentage of mature somatic embryos. Increasing picloram concentration from 0.25 to 1 mg/L in the medium increased the number of cotyledonary somatic embryos.

Table 1. Effect of different picloram concentrations on percentage embryogenic calli of various explants of strawberry cultivars

% embryogenic calli of various explants of strawberry cultivars									
Picloram(mg/l)	Leaf blade			nodal			Flower bud		
	Comarosa	Paros	Kurdistan	Comarosa	Paros	Kurdistan	Comarosa	Paros	Kurdistan
0.25	1.87no	0.50o	0.00o	6.00m	0.25o	4.00n	1.75 no	4.17mn	0.00o
0. 5	18.50hi	13.75kl	10.75l	14.75k	17.65i	10.40l	20.07gh	14.50k	9.55l
1	20.75g	23.25ef	17.25ij	15.00k	20.75g	18.62hi	22.97ef	18.65hi	15.47jk
2	41.75a	43.17a	33.00c	36.75b	38.17b	24.02de	25.75d	23.85de	21.75fg

Means having the same letter in columns are not significantly different by Duncan's multiple range test (P < 0.05)

Table 2. Effect of different picloram concentrations on number of globular embryos of various explants of strawberry cultivars

Number of globular embryos per explant of various explants of strawberry cultivars									
Picloram (mg/l)	Leaf blade			Nodal			Flower bud		
	Comarosa	Paros	Kurdistan	Comarosa	Paros	Kurdistan	Comarosa	Paros	Kurdistan
0.25	2.60m	1.80n	0.00q	1.74n	1.30o	0. 80p	0. 80p	0. 80p	0.00q
0. 5	4.20 gh	3.60hjk	3.20kl	4.24gh	3.50jk	2.04n	4.00hi	2.90lm	1.90n
1	5.80bc	5.20de	4.20gh	5.5cd	4.00hi	3.70ij	4.54de	3.70ij	3.00lm
2	7.04a	6.84a	5.94bc	6.04b	4.64fg	4.80ef	5.14de	4.74ef	4.34fgh

Means having the same letter in columns are not significantly different by Duncan's multiple range test (P < 0.05)

Table 3. Effect of different picloram concentrations on percentage of cotyledonary embryos development of various explants of strawberry cultivars

% globular embryos developing into cotyledonary embryos of various explants of strawberry cultivars									
Picloram (mg/l)	Leaf blade			Nodal			Flower bud		
	Comarosa	Paros	Kurdistan	Comarosa	Paros	Kurdistan	Comarosa	Paros	Kurdistan
0.25	16.57rs	24.40mn	19.57pq	22.65no	20.52op	25.65lm	18.67pqr	15.65s	17.62qrs
0. 5	28.60hjk	30.35ghi	32.52fg	26.50klm	29.50hig	32.70fg	26.50klm	35.57e	32.70fg
1	36.17e	43.72a	38.57cd	41.50b	39.67bc	34.52ef	30.72ghi	36.77de	40.57bc
2	31.67gh	35.62e	26.67klm	28.77hjk	21.00op	22.65op	37.72ghi	27.62jkl	31.50gh

Means having the same letter in columns are not significantly different by Duncan's multiple range test (P < 0.05)

The frequency of cotyledonary stage somatic embryos was significantly (P<0.05) greater when 1 mg/L picloram was added to the medium in comparison to 0.25 or 0.5 mg/L. Medium supplemented with 1 mg/L picloram was found superior not only for the optimum growth rate of the embryonic culture (Table 3) but also for the uniformity of embryo development (Fig. 1c). Higher than 1 mg/L picloram content was suboptimal for embryo development as indicated by the decreasing embryo formation frequency. Among the plant growth regulators, auxins and their concentrations play the most important role on the process of somatic embryogenesis [19]. According to Franco et al. [20], somatic embryo formation is possible when auxin is suppressed or replaced by weaker auxin.

In most protocols in which auxins act as efficient inducers of somatic embryogenesis, development of somatic embryos is achieved by reducing or removing auxin from the culture medium. To explain this result, it was proposed that continuous exposition of explants to high exogenous auxin levels interferes with the polar auxin gradient that is normally established during embryogenesis, preventing the correct apical–basal embryo patterning [21-22-23]. High picloram concentration (above 1 mg/L) may similarly be unfavourable for the progression of embryogenesis.

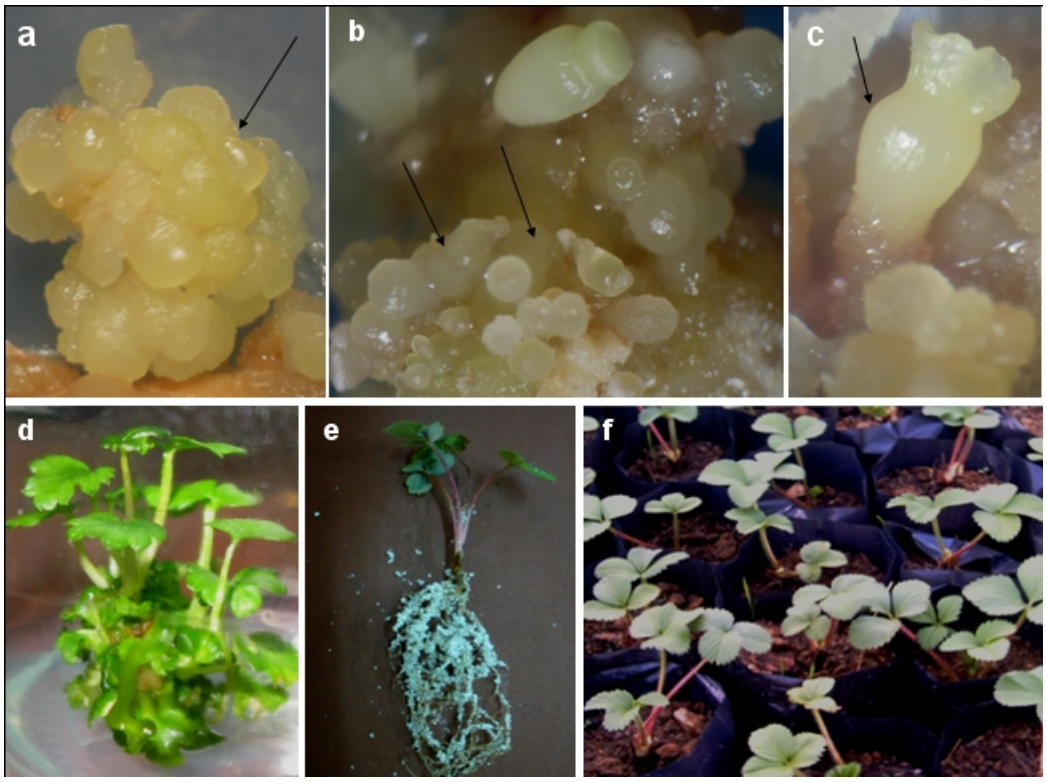

Fig. 1. Somatic embryogenesis and plant regeneration of strawberry (A) Embryonic callus on MS medium containing 2 mg/l picloram; (B) Globular-stage embryos; (C) Cotyledonary-stage embryos; (D) Conversion of somatic embryos to plantlet on MS medium containing 1.0 mg/l GA_3 ; (E) Converted plantlet with well developed roots in pots in the growth chamber; (F) Potted plants in greenhouse

3.3 Effect of Cultivar and Explant

The results obtained in this study indicate that the transition of strawberry calli to the somatic embryogenesis pathway was affected by the interaction between the concentration of picloram, the type of explant and the cultivar. In fact, this kind of interaction is generally considered to play a key role in the success of plant regeneration through indirect somatic embryogenesis. The differences in the responses of the various cultivars to the different auxin concentrations are apparently related to the genetic constitution of the plant material [24]. Our data also show that somatic embryogenesis on the media containing different picloram concentration, exhibit significant differences ($P<0.05$) among the strawberry cultivars. The highest frequency of embryogenic calli and the number of globular embryos per explant were observed for Camarosa followed by Paros and Kurdistan (Table 1, 2). The percentage of cotyledonary-stage embryos was also different among the cultivars. Paros cultivar gave the highest frequency and Comarosa the lowest (Table 3). There is a strong genetic component in the regeneration capacity of different strawberry cultivars [25]. Genotypes within a given species vary greatly in embryogenic capacity [23]. Different responses were also observed in the induction of somatic embryos in different genotype of strawberry by Gerdakaneh et al. [26]. Such differences might reflect differences in the ability to activate key elements of the embryogenic pathway. In the present study, significant genotypic differences were observed for both induction of embryogenic calli and somatic embryos.

The differences in frequencies of somatic embryogenesis among explants were also statistically significant ($P<0.05$). In the embryonic calli phase, only the leaf blade, node and flower bud were effective for the progression of somatic proembryos to globular and cotyledonary stages. Auxin is capable of setting off processes of dedifferentiation and redifferentiation, thus altering cellular determination and endowing new competencies to responsive cells present in the explants [27]. The best explant source for embryo culture was leaf blade, followed by node and flower bud. The different embryogenesis capacity of explants is probably due to the nutritive reserves hormone contents, and the anatomical structure. Similar results have been reported in strawberry [28] and other plant species [29].

3.4 Plant Regeneration

Average germination rate of somatic embryos on MS basal medium containing 3% picroram and 1 mg/L GA_3 was about 43–45% for all cultivars. Plantlets developed from somatic embryos (Fig. 1d) were subjected to acclimation, transplanted into plastic bags and maintained in a growth chamber for four weeks (Fig. 1e). The rooted plants were hardened and transferred to soil with 85% to 90% survival rate (Fig. 1f). The conditions under which most laboratories maintain *in vitro* plant cultures mean high relative humidity and low light. Low CO_2, high sucrose and nutrient containing medium may contribute to a phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field [30-31-32-33-34-35]. Therefore when moved from *in vitro* culture conditions, plantlets must be acclimatized gradually to ambient conditions to avoid mortality that might otherwise occur under an abrupt change in relative humidity, temperature or irradiance.

4. CONCLUSION

Our study demonstrated that the concentration of picloram, cultivar and explant type had important effects on the somatic embryogenesis process and embryos development of the investigated strawberry cultivars.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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