Media and hormones influence in micropropagation success of blackberry cv. 'Chester'

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Abstract

Berry fruits are a rich source for human nutrition and play a major role for the livelihood of small producers. Among hybrid blackberries, the cultivar 'Chester' has good fruit quality but traditional propagation methods show limiations. The advantages of micropropagation exploited by the breeding industry in many plant species need to be developed for the cultivar 'Chester'. This work aimed to find better conditions in the stages of in vitro establishment, shoot proliferation and rooting for the micropropagation of this cultivar. Plant material was collected at different developmental stages. Experiments evaluated sterilization procedures, shoot multiplication, elongation, rooting and acclimation. Actively growing new shoots taken in spring time allowed to obtain in vitro culture establishment of axenic tissues. Shoot multiplication rate was highest (5.2) on MS medium.

The media had a stark incluence on rooting with a higher proportion of rooted shoots on WPM and DKW. The highest number of roots per shoot (5.4) and higher root length were observed on WPM. The results stress the importance of evaluating different culture media for specific cultivars under micropropagation. The protocol described permits the micropropagation of blackberry cv. 'Chester', opening a new alternative for plant production in the difficult propagation of this cultivar.

Keywords: *Rubus, in vitro* culture, tissue culture, shoot, growth, root.

Introduction

Among berry fruits, strawberry, raspberry, blackberry, blueberry and mulberry are included. They are fleshy, colorful and attractive fruits produced from a single flower. They are very rich in terms of human nutrition, since they contain anthocyanins, phenolic substances and vitamins such as C and E. Berry plants are a genetically very diverse group and play a major role in modern society and economy[2,6,15,19].

The berry industry is a profitable and significant activity for the livelihood of small producers in Central South Chile. Among the crops produced, blackberries are one of the most important in terms of area and economic value in this area. This group of berries is more productive than raspberries (*Rubus idaeus*) and the harvest extends for two or three weeks in Chile, allowing the producer to finish the task in a short period of time, leaving available workforce for raspberry harvest[11,26]. The main area in Chile planted with blackberry is the Region of Maule with ca. 1000 ha[14]. Local small producers consider that among hybrid blackberries, the cultivar 'Chester' has good fruit quality and advantageous plant habit. This cultivar was released in 1985 by ISDA-ARS[13].

Blackberry plant habit can be prostrate ('Marion', 'Evergreen Thornless', 'Boysen'), semi-erect ('Chester Thornless', 'Thornfree', 'Triple Crown') or erect ('Navajo'), where semi-erect cultivars represent ca. 50% of the world blackberry production.

'Chester' cultivar is characterized with big sweet fruits of good quality and high yield potential with tolerance to pests and diseases. The fruit has late maturation (at the beginning of January in Chile) and good postharvest performance. The plant is semi-erect needing a supporting system. Traditional methods of plant propagation do not work well for this cultivar, as it is the case for several blackberries[10].

Micropropagation is the rapid vegetative propagation of plants under *in vitro* conditions of high light intensity, controlled temperature and a defined nutrient medium. The technique has been applied to a substantial number of plant species that are economically important and vegetatively propagated. Plant micropropagation has a number of advantages over traditional techniques with the main one being the production of many plants that are clones of each other. Additionally, micropropagation can be used to produce disease-free plants[1,3].

The advantages of micropropagation exploited by the breeding industry in many plant species are not well developed for blackberries including the cultivar 'Chester'. This work aimed to find better conditions in the stages of *in vitro* establishment, shoot proliferation and rooting for the micro propagation of this cultivar.

Material and Methods

Plant material (*Rubus fruticosus* L. cv. 'Chester') was collected from a commercial crop in Romeral (34°59'19" Lat S, 71°02'23" Long W; Curicó, Region of Maule - Chile), a traditional area for the production of blackberries and strawberries in the country. Material was collected at different developmental stages from dormant buds and canes in June and August (winter), to active shoots in October

(spring time). The material was washed in running tap water for 15 min and dipped in methanol (70% v/v) for 5 min with 2 drops of tween 20, then rinsed three times with distilled water. Plant tissues were placed in MS medium[21] with 30 g L^{-1} sucrose and 8 g L^{-1} agar at pH 5.7 and incubated at 25 °C in a 16 h light photoperiod.

Experiments evaluated the use of a fungicide (Benomyl) at 0, 0.5, 1 or 2 g L^{-1} for 5, 10 or 15 min and a sterilization procedure using sodium hypochlorite at concentrations ranging from 0.1 to 5% for 5, 10 or 15 min. In a first experiment, each treatment had 5 jars with 5 buds each. In experiments 2 and 3, each treatment had 3 jars with 3 and 1 cane respectively. In experiment 4, each treatment had 4 jars with 1 cane (1 bud) and experiment 5 had 7 jars per treatment with one shoot with a single bud (Table 1).

Shoot multiplication was assessed using material obtained in experiment 5, incremented in MS medium supplemented with 2 mg L^{-1} BAP. In experiment 6, shoots were cultured on MS medium with 20 g L^{-1} sucrose and 2 mg L^{-1} BAP evaluating 0, 0.1 and 0.5 mg L^{-1} NAA, subculturing every 30 days, using 7 jars with 5 shoot each per treatment. BAP concentrations (0.7, 1.4 and 2 mg L^{-1}) were evaluated in experiment 7 on MS medium, with 6 jars with 5 shoots each per treatment.

Shoot elongation and rooting were evaluated in experiment 8, using culture media MS, WPM[17] and DKW[9] supplemented with 0.5 mg L^{-1} GA_3. Evaluation was made after two subcultures using 6 jars with 5 shoots each per treatment.

Plantlets (n=17) were acclimatized in pots using compost in a shaded environment, the pot covered with a transparent plastic bag progressively opened by cuttings until the complete removal of the bag after a week. Surviving plants were counted after 3 months. Shoot multiplication rate, oxidation, callus, hyperhydricity, shoot length, leaf number, number of shoots with roots and number of roots per shoot were evaluated and analyzed using one way ANOVA and Tukey test at 5%.

Results and Discussion

Establishment of cultures: Explants sterilized and introduced from dormant material suffered extensive contamination. No axenic cultures could be obtained from this material due to the development of microorganisms, especially the rapid spread of fungal mycelia. Fungicide in swollen buds allowed to delay the fungus development for 1-2 d, but eventually the material was lost.

Actively growing new shoots were incubated *in vitro* and stayed free of microorganisms for 50 d, a longer time than any of the material evaluated (Fig. 1A). Active stages of seasonal growth are expected to carry lower quantities of microorganisms[20,22,23], supporting the observations that physiological status or phenological stages are crucial for a successful establishment of tissues *in vitro*. Young and less differentiated shoots will enhance probabilities of achieving *in vitro* culture establishment of axenic tissues.

Our results are in line with observations by Wu et al[28] who recommended the use of *Rubus* material from canes of less than two years, with old structures leading to floral shoots and abundant presence of microorganisms. Moreover, Bobrowski et al[5] and Tsao et al[27] worked with material in active growth.

Using shoots in active growth (experiment 5), axenic material was obtained sterilizing with 1.5% NaClO for 5 min. Similar conditions have been reported for the establishment of material by Fernández and Clark[10], Wu et al[28] and Mroginski et al[20]. We observed a rather low proportion of axenic cultures in relation to the material initially inoculated, which could be at least in part explained by the use of material from plants in a commercial orchard without additional treatments to the plant. However, this procedure is a direct and rapid way of acquiring plant material.

Shoot multiplication: The concentration of NAA seemed to had no effect on shoot multiplication rate (ANOVA, p=0.159), which ranged from 2.3 to 4.0. Gajdošová et al[12] observed the highest rate (2.6) after 60 days of culture on MS medium supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IBA, noting differences among cultivars. The latter observation was also made by Clark and Finn[7]. In the present study, shoot oxidation was not statistically influenced by auxin concentration in the medium (ANOVA, p=0.062; Fig. 1C), although some tendency to increase with the concentration may be present, which would require further investigation. In agreement with this observation, Azofeifa[4] found that oxidation was promoted by higher concentrations of auxin in the medium. In our study, some callus developed at the basal end of the shoot in all treatments.

Cytokinin presence (BAP) in the medium showed influence on shoot multiplication rate (Fig. 1B and Fig. 2A) with the highest rate (5.2) at 1.4 mg L^{-1} BAP (Tukey, $p < 0.05$). Wu et al[28] also noted the use of cytokinin for shoot multiplication in *Rubus* is determinant and was better at 1.0 mg L^{-1}. Higher rates of shoot multiplication in *Rubus* have been reported for longer incubation times in previous studies[5,8,18]. Hyperhydricity was not a problem in the present study, although Wu et al[28] observed a higher presence of hyperhydricity with 2 mg L^{-1} BAP. Oxidation (<30%) was present in shoots of all treatments but its influence on multiplication was considered very low.

Elongation and rooting: Shoot height was influenced by media (ANOVA, p=0.003; Tukey, $p < 0.05$), with smaller shoots on MS medium. Sigarroa and García[25] made similar observations while Doina et al[8] concluded that DKW was the best medium for shoot multiplication. The number of leaves per shoot was also influenced by media (ANOVA, p=0.004;

Tukey, $p < 0.05$) with a smaller number of leaves on MS medium in contrast to observations made by Leitzke et al[16].

WPM medium produced 5.4 roots per shoot (Fig. 1E) higher than on DKW or MS medium (ANOVA, $p < 0.001$; Tukey, $p < 0.05$). Additionally, a higher root length was produced on WPM medium than on DKW or MS (Fig. 1F and fig. 2B). The media also had a stark influence on rooting (Fig. 1D) with a higher proportion of rooted shoots for WPM and DKW (ANOVA, $p < 0.001$; Tukey, $p < 0.05$). Leitzke et al[16] observed 2 roots per shoot cultured on MS medium while Sigarroa and García[25] did not recommend MS medium for elongation and rooting.

Considering these results and that elongation and rooting were simultaneously observed in this experience, WPM should be recommended for shoot elongation and rooting of blackberry cv. 'Chester'. Meng et al[18] also observed that WPM medium was better for blackberry culture than other media tested. Oxidation at the base of the shoot was observed in the first culture cycle, but was absent in the second.

The present results as well as those reviewed by Clark and Finn[7] stress the importance of evaluating different culture media for specific cultivars under micropropagation. Plant survival after acclimatization was high (88.2%; Fig. 2C) considering that the procedure was applied in summer with non-optimal conditions (high temperature and low air humidity).

According to the literature, survival during acclimatization is affected by the stress on plants due to a sudden increase of transpiration rate[22]. *In vitro* plants are characterized by a reduced functionality of their stomata and a thin cuticle[20]. In addition to temperature and humidity, substrate has also influence on survival during acclimatization[23,24]. However, we consider that in our experiment, environmental conditions had a greater effect than substrate on plant survival.

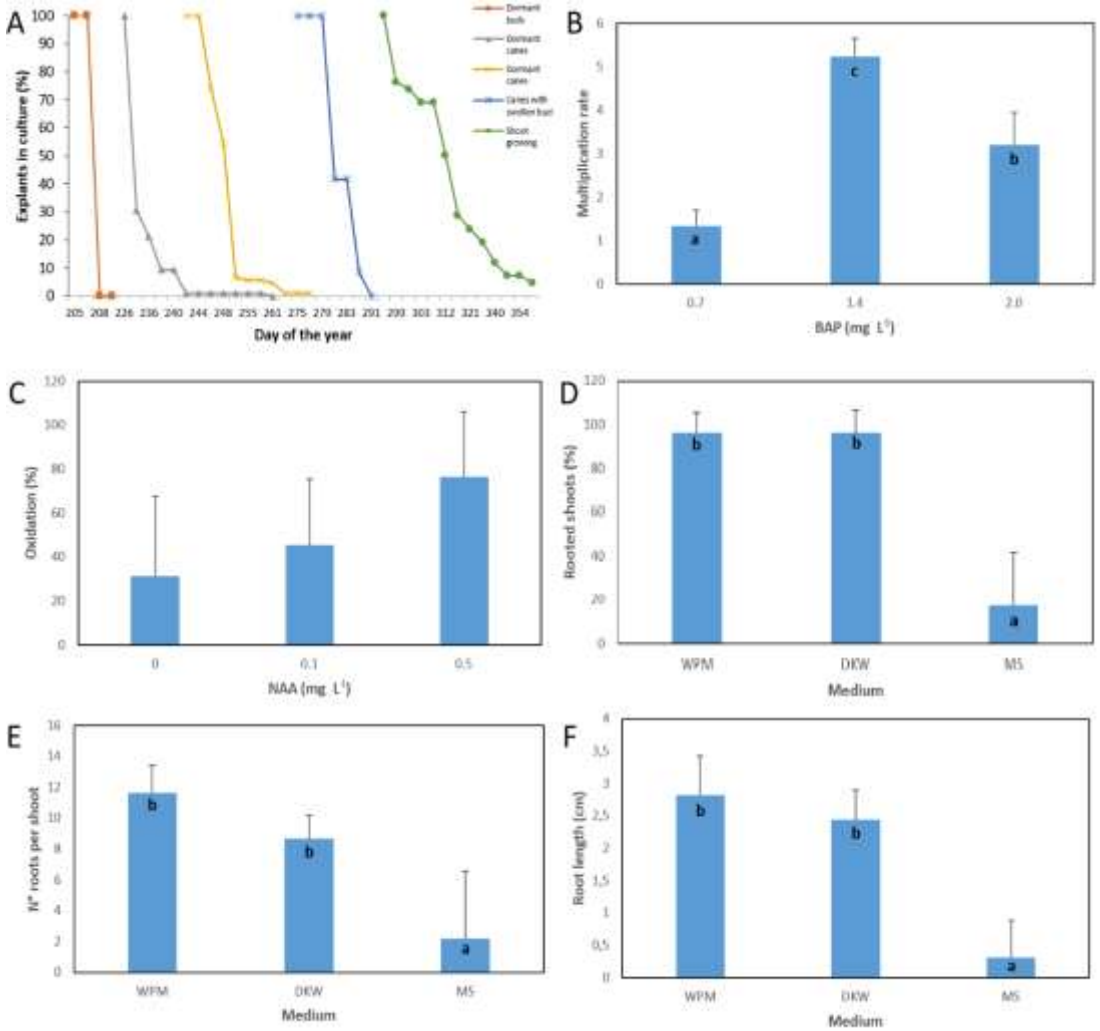

Figure 1: ***In vitro*** **responses of blackberry cv. 'Chester'. A, Explant survival from different phenological stages in axenic culture. B, Shoot multiplication rate on media with different levels of BAP. C, Shoot oxidation on media with different levels of NAA. D, Proportion of rooted shoots on different culture media. E, Number of roots produced by a shoot on different culture media. F, Root length for shoots cultured on different culture media. Bars represent the standard deviation. Letters indicate statistically significant differences**

Table 1
Type of plant material and sterilization conditions for *in vitro* tissue establishment of blackberry cv. 'Chester'.

Explant type	Benomyl		NaClO	
	Time (min)	Concentration (g L^{-1})	Time (min)	Concentration (% v/v)
Experiment 1: dormant bud	-	-	5	2.5
	-	-	5	5.0
	-	-	10	2.5
	-	-	10	5.0
	-	-	15	2.5
	-	-	15	5.0
Experiment 2: dormant cane	5	1	5	2.5
	5	2	5	2.5
	10	1	10	2.5
	10	2	10	2.5
	15	1	15	2.5
	15	2	15	2.5
	5	1	5	5.0
	5	2	5	5.0
	10	1	10	5.0
	10	2	10	5.0
	15	1	15	5.0
	15	2	15	5.0
Experiment 3: dormant cane	5	0.5	5	1
	5	1.0	5	1
	10	0.5	10	1
	10	0.1	10	1
	15	0.5	15	1
	15	0.1	15	1
	5	0.5	5	2
	5	0.1	5	2
	10	0.5	10	2
	10	0.1	10	2
	15	0.5	15	2
	15	0.1	15	2
Experiment 4: swollen bud	-	-	5	0.1
	-	-	10	0.1
	-	-	5	0.5
	-	-	10	0.5
	-	-	5	1.0
	-	-	10	1.0
Experiment 5: active shoot	-	-	5	0.5
	-	-	10	0.5
	-	-	5	1.0
	-	-	10	1.0
	-	-	5	1.5
	-	-	10	1.5

Conclusion

The use of actively growing shoots of blackberry cv. 'Chester' taken in spring time and sterilized with 1.5% NaClO in agitation for 5 min, allowed to obtain axenic material on MS medium with 2 mg L^{-1} BAP.

Dormant tissues are not recommended for establishing *in vitro* cultures since they show high incidence of contamination. Shoot multiplication showed its highest rate (5.2) on MS medium supplemented with 1.4 mg L^{-1} BAP. Simultaneous elongation and rooting were observed on WPM medium with more and longer roots than on MS or DKW medium. Plantlets were acclimatized. The protocol described permits the micropropagation of blackberry cv. 'Chester', opening a new alternative for plant production in the difficult propagation of this cultivar.

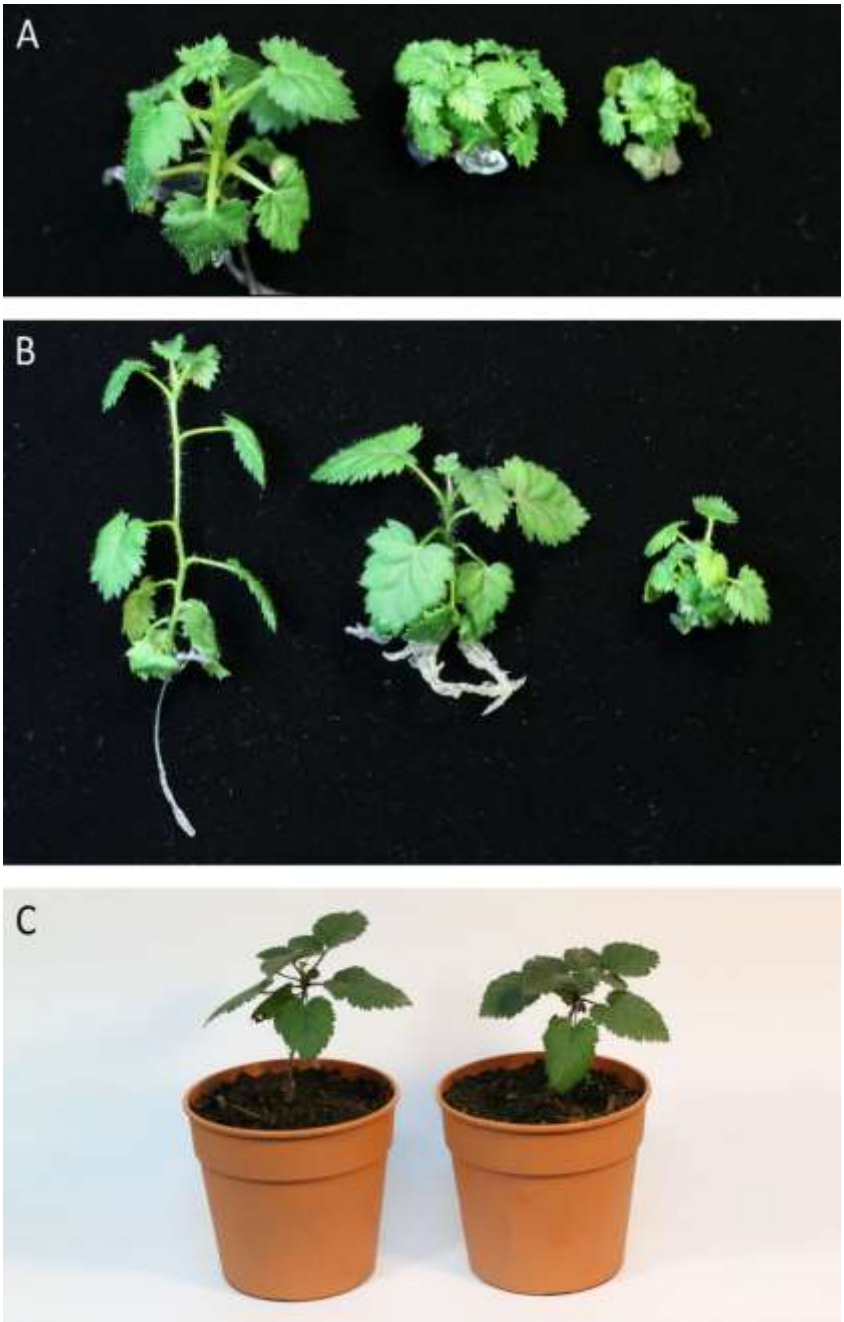

Figure 2: *In vitro* regenerated shoots and plants of blackberry cv. 'Chester'. A. Shoot multiplication on media with 0.7 (left), 1.4 (centre) and 2.0 (right) mg L^{-1} BAP, B. Rooted shoots on DKW medium (left), WPM medium (centre) and MS medium (right), C. Acclimatized plants

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