ELSEVIER

Plant Science 118 (1996) 89–95

PLANT SCIENCE

Influence of Ca^{2+} and 6-benzyladenine on chestnut (*Castanea sativa* Mill.) in vitro shoot-tip necrosis

Claudia Piagnani*[a], Graziano Zocchi[b], Ilaria Mignani[a]

[a]*Istituto di Coltivazioni Arboree, Università degli Studi di Milano, Via Celoria 2, 20133 Milan, Italy*
[b]*Dipartimento di Fisiologia delle Piante Coltivate e Chimica Agraria, Università degli Studi di Milano, Via Celoria 2, 20133 Milan, Italy*

Received 19 February 1996; revised 17 April 1996; accepted 17 April 1996

Abstract

Shoot apex necrosis affects in vitro chestnut cultures particularly during the rooting stage. Lack of cytokinins or Ca^{2+} deficiency in the culture medium have been reported to be responsible for occurrence of tip necrosis. In the present research the effects of three Ca^{2+} levels (3, 9 and 18 mmol l^{-1}) were tested on *C. sativa* 'Garrone rosso' and 'Clone 46' shoots treated for rooting. Tissue Ca^{2+} content of the apical, middle and basal portion of both brown and healthy shoots was determined. In a second trial the tip necrosis related effect of the local (tip) application of $CaCl_2$ (3 mmol l^{-1}), BA (5 μmol l^{-1}) and $CaCl_2$ + BA (3 mmol l^{-1} and 5 μmol l^{-1}) was tested on Clone 46 shoots, during rooting. With regard to Ca^{2+} concentration in the rooting medium, no significant difference could be detected from the three tested Ca^{2+} levels on tip necrosis but the highest one caused a drop in rooting ability. Regardless of Ca^{2+} concentration, Garrone rosso showed a lower percentage of the disorder and a higher affinity for calcium in terms both of uptake and ion translocation. The data of the second trial showed that the local application of 5 μmol l^{-1} BA completely eliminated tip necrosis while the application of $CaCl_2$ + BA delayed the appearance of the disorder. Apex calcium content of shoots treated locally revealed that the most the healthy tissue contained higher calcium levels than the necrotic one. BA treated shoots contained the lowest ion levels, independently from their status.

Keywords: Apical necrosis; BA; Calcium; Chestnut; Rooting

1. Introduction

Shoot apex necrosis still represents a problem during in vitro rooting of chestnut with loss of a great number of propagula. The main cause appears to be the general protocol of leaving cytokinins out of the culture medium or adding them at very low concentrations, because of their antirooting activity [1,2]. The application of a drop of BA agar mixture to the shoot tip of chestnut resulted in tip necrosis [2], but the same authors bypassed the problem by decapitating the apex and by placing BA directly on the cut end. Apical necrosis of apple shoots was found to be associated with a decrease in the endogenous

* Corresponding author. Tel.: +39 2 70600165/70638134; fax: +39 2 2365302; e-mail: piagnani@imiucca.csi.unimi.it

0168-9452/96/$15.00 © 1996 Elsevier Science Ireland Ltd. All rights reserved
PII: S0168-9452(96)04423-8

hormone content [3]. IBA added to *Corilus avellana* rooting medium was responsible for the apex degeneration of the shoots [4].

Along with the hormonal hypothesis, deficiency of calcium or boron has been suggested as the most likely cause of tip necrosis [5,6]. Mason and Gutteridge [5] found that strawberry tips of healthy plants contained twice as much calcium as similar portions of tip of necrotic plants.

Calcium plays an important role in plant growth and development [7] and it has been shown to prevent or delay the onset of a range of physiological disorders in a variety of trees and horticultural crops [8,9].

Abousalim and Mantell [10] completely prevented apical necrosis of *Pistacia vera* by periodically immersing shoots in a calcium supplemented liquid medium.

Ca^{2+} concentration in the medium directly influenced shoot-tip necrosis and necrosis significantly decreased as calcium content increased on in vitro cultures of *Castanea mollissima* and *dentata* [11] and *Cydonia oblonga* [12]; on the other hand, Mullins [13] could not find any benefit in increasing the calcium level of the medium in *C. sativa,* although no details were given.

By these considerations, the aim of the present work was to contribute to the knowledge on the role of calcium in the development of shoot apical necrosis both by increasing the levels of the ion in the culture medium or applying it directly to the shoot tip of *Castanea sativa* in an attempt to control shoot tip necrosis, particularly during the rooting stage. At the same time, the effect of the local application of BA was also evaluated.

2. Materials and methods

2.1. Plant material and standard culture protocol

Experiments were performed on two genotypes: *Castanea sativa* proliferating shoots of mature and juvenile origin. Shoots (~1.5 cm) of cv. 'Garrone rosso' and 'Clone 46', derived, respectively from bud culture of mature plants and from in vitro cloning of a zygotic embryo, were grown on the low NH_4NO_3 culture medium MS-15 [14]. The organic component consisted of Bourgin and Nitsch [15] vitamins, sucrose 87 mmol l^{-1} plus IBA 5 μmol l^{-1} and BA 0.044 μmol l^{-1} during the 8-day 'root induction stage', and only BA during the following 'rooting phase'. The pH was adjusted to 5.5 before autoclaving. Shoots were incubated at 22–24°C under 16-h photoperiods delivered by cool, white fluorescent lamps, with 35 μmol m^{-2} s^{-1} of mean irradiances.

Table 1
Macroelements composition (mmol l^{-1}) of culture media adapted for three different Ca^{2+} concentrations

Ion	Source	3 mmol l^{-1} Ca^{2+}	9 mmol l^{-1} Ca^{2+}	18 mmol l^{-1} Ca^{2+}
NO_3	NH_4NO_3	3	0	0
	KNO_3	15	10	0
	$Ca(NO_3)_2 \cdot 4H_2O$	0	4	13
NH_4	NH_4NO_3	3	0	0
	$(NH_4)_2HPO_4$	0	1.5	1.5
K	KNO_3	15	10	0
	KH_2PO_4	1.25	0	1.25
	K_2SO_4	0	0	7.5
Ca	$CaCl_2 \cdot 2H_2O$	3	5	5
	$Ca(NO_3)_2 \cdot 4H_2O$	0	4	13
Mg	$MgSO_4 \cdot 7H_2O$	1.5	1.5	1.5
SO_4	K_2SO_4	0	0	7.5
	$MgSO_4 \cdot 7H_2O$	1.5	1.5	1.5
PO_4	KH_2PO_4	1.25	0	1.25
	$(NH_4)_2PO_4$	0	1.5	1.5
Cl	$CaCl_2 \cdot 2H_2O$	3	5	5

2.2. Increasing Ca^{2+} concentration in the culture medium

During the rooting stage, shoots were placed into culture tubes stoppered with metal caps and not sealed with Parafilm, containing 10 ml MS-15 medium (Table 1, first column) which was modified to obtain 9 and 18 mmol l^{-1} calcium concentrations. The three culture media differed only by the macroelement content (Table 1). After a further 26 days' incubation the number of rooted shoots and the number of shoots showing apical necrosis were recorded. All the samples (shoots) were thoroughly washed with distilled water then cut into apical, middle and basal portion, and fresh and dry weights were obtained. Calcium content was determined by flame spectrometry (Varian Spectr AA 20) at 422 nm wavelength, using La^{3+} as ionizing suppressor, after wet digestion of the tissue in a triacid mixture (HNO_3/H_2SO_4/$HClO_4$, 5:1:1 v/v/v) at 80°C until red vapour, and then at 150°C till clear residue [16]. The trial considered 30 shoots per treatment, replicated twice. Data were processed by χ^2 or Anova and Tukey's test [17] by the Statgraphic program.

2.3. Ca^{2+} and BA localized treatments

The second trial, performed on MS-15, considered only Clone 46 since data of the first experiment showed it to be particularly affected by apical necrosis. When shoots were transferred to the 'root induction' medium three different localized treatments were performed: 3 mmol l^{-1} $CaCl_2$, 5 μmol l^{-1} BA and the two combined together, respectively. The solutions were mixed with 1% w/v molten agar (Difco) and subsequently plated in multiwell dishes (Sterilin). The blocklets formed after agar solidification were removed from the wells, cut into smaller cubes ($\sim$0.5 cm^3), by using a sterile scalpel, and put on the top of the shoots. Each tip fit in the hole shaped on the bottom side of the cube by the overheated tip of a sterile forcep. Ten shoots per localized application ($CaCl_2$, BA, $CaCl_2$ + BA, agar only, and a control without the agar block) were cultured for rooting as described in the first experiment; the trial was repeated three times. The number of shoots affected by tip necrosis was recorded at the end both of the 'root induction stage' (8 days) and 'rooting phase' (26 days).

The calcium content of both green and necrotic shoot tips was spectrophotometrically determined.

3. Results

3.1. Effect of increasing Ca^{2+} concentration in the culture medium

Data reported in Fig. 1 show significant differences among rooting percentage in shoots treated with different calcium concentrations. The highest calcium concentration had a negative effect inducing a remarkable reduction of shoot rooting ability in both genotypes. On the contrary, tip necrosis was not significantly influenced at any Ca^{2+} concentration used. The data revealed, however, a strong genotypic response to the disorder. Regardless Ca^{2+} level, Clone 46 shoots were affected more than twice as much as those of Garrone rosso (Fig. 2).

Shoots of Clone 46 grown on the highest Ca^{2+} level had the highest Ca^{2+} content, while Garrone rosso did not show any significant differences among the three different treatments (Table 2).

Calcium uptake was mainly localized in the basal and middle portion of Clone 46 shoots (Table 2) while the apex had the lowest calcium content (0.41 mmol g dry $wt.^{-1}$), on the contrary,

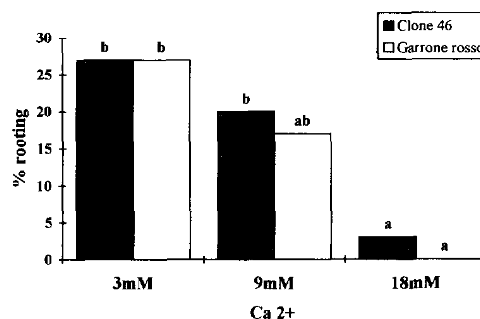

Fig. 1. Effect of Ca $^{2+}$ concentration in 0.044 μmol l^{-1} BA-supplemented rooting medium on rooting percentage in Clone 46 and Garrone rosso. For each genotype, bars with the same letter are not different at 5% level, χ^2-test.

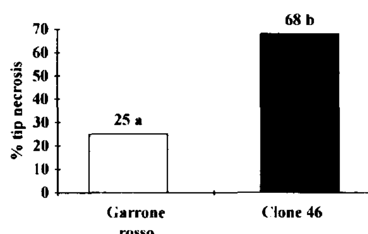

Fig. 2. Shoot tip-necrosis percentage: comparison between the two genotypes Clone 46 and Garrone rosso. Data were collected from a total of 90 shoots per genotype cultured on BA-supplemented rooting medium containing 3, 9 and 18 mmol l^{-1} Ca^{2+}. Bars with the same letter are not different at 5% level, χ^2-test.

Garrone rosso shoots showed an opposing pattern with the highest calcium content allocated in the apical portion (1.96 mmol g dry $wt.^{-1}$) (Table 2).

The two genotypes showed a different behaviour concerning the calcium content of brown tissues as well: Garrone rosso had a higher Ca^{2+} concentration in affected shoots, whilst the calcium content of Clone 46 was higher in the healthy ones (Table 2).

3.2. *Effect of Ca^{2+} and BA localized treatments*

The data obtained in the second experiment (Table 3) confirmed the results of the first one as far as tip necrosis incidence is concerned. In fact, the disorder involved 76.7% of the shoots, after 26 days. The local application of 5 μM BA completely avoided the occurrance of tip necrosis, while the application of both $CaCl_2$ + BA and agar delayed

Table 2
Clone 46 and Garrone rosso Ca^{2+} content (mmol g^{-1} dry wt.). Comparison between: shoots from three different Ca^{2+} conditions, different shoot portions, brown and green tips

Probes	Clone 46	Garrone rosso
Concentration		
Ca^{2+} 3 mM	0.35^a	1.79^a
Ca^{2+} 9 mM	0.49^a	0.99^a
Ca^{2+}18 mM	0.71^b	1.10^a
Shoot portion		
Tip	0.41^a	1.96^b
Middle	$0.51^{a,b}$	0.89^a
Basal	0.64^b	1.03^a
Tip type		
Brown tip	0.36^a	1.81^b
Green tip	0.67^b	0.78^a

In each column means followed by the same letter are not significantly different at 5% level, according to Tukey's test.

Table 3
Effect of the localized treatments (agar, BA, $CaCl_2$ and $CaCl_2$ + BA) on the percentage of Clone 46 shoots affected by tip-necrosis, after 8 days and 26 days, respectively

Treatment	%Tip necrosis (8 days)	%Tip necrosis (26 days)
Control (no agar)	23.3^b	76.7^c
Agar	10.0^a	30.0^b
BA	0.0^a	0.0^a
$CaCl_2$	10.0^b	33.3^b
$CaCl_2$ + BA	0.0^a	33.3^b

Means of three experiments with 10 replicates in each. Data were transformed to angular values before analysis and were back transformed to percentages to presentation in the table. Means with the same letter are not different at $P = 0.05$, according to Tukey's test.

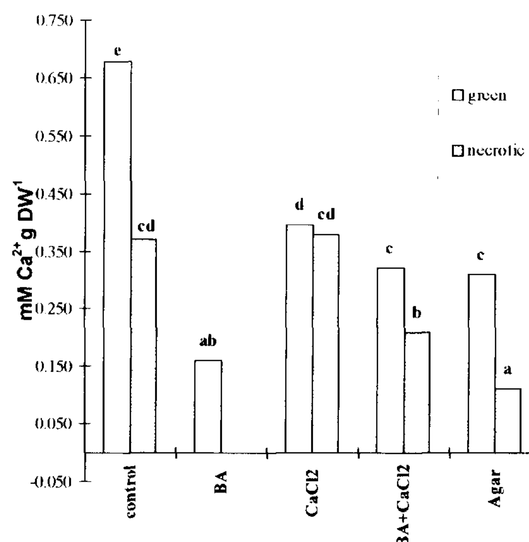

Fig. 3. Effect of the shoot-tip localized treatment on Ca^{2+} in both green and necrotic tips. Data referred to 5 μmol l^{-1} BA-treated necrotic tips are lacking as no samples were available. Tips were pooled in three samples of 0.1–0.3 g each per treatment. Bars with the same letter are not different, according to Tukey's test ($P = 0.05$).

the appearance of the symptom. In any case, the application of agar alone or agar plus $CaCl_2$ reduced the incidence of the disorder by more than 40%. Such an effect could be due to the presence of calcium in the agar itself which seems to be almost sufficient to match its requirement. In fact, calcium determination of agar blocklets revealed that it was present at the mean concentration of 0.39 ± 0.02 nmol ml agar^{-1}. When 3 mM Ca^{2+} was added to the agar its concentration became possibly too high to give additional positive effects (see the negative effect on BA treatment). BA-treated tips kept on growing vigorously up, to force their way through the agar cube. None of the local treatments was shown to influence rooting ability of the shoots (data not shown). Fig. 3 shows apex calcium contents of shoots submitted to the localized treatments. Apart from $CaCl_2$-treated tips, there was a general response that the healthy tissue contains higher calcium levels than the necrotic one.

The sharp decrease in Ca^{2+} content upon BA treatment is likely due to the major growth of the tip so leading to a dilution of the calcium concentration. This treatment seems to bring Ca^{2+} level in the tip to the right requirement avoiding the appearance of tip necrosis (Fig. 3). All the other treatments by adding Ca^{2+} keep its concentration higher enough to alter its equilibrium with the cytokinin.

4. Discussion

In vitro shoot-tip necrosis is a quite widespread disorder affecting several woody plant cultures. It was possible to remarkably reduce this disorder by increasing the calcium level in the medium of two different species of *Castanea* [11]. Similar results were also obtained in shoots of *Cydonia oblonga* grown in vitro by increasing calcium concentration in the culture medium from 3 to 18 mmol l^{-1} [12] and in potato [18]. Potato shoots growing on 3 mmol l^{-1} Ca^{2+} culture medium, were affected by tip necrosis as a consequence of uptake deficiency. High humidity inside the vials reduced transpiration and consequently ion uptake, starting about 10 days after transferring onto a new proliferation medium [18]. Working on the same genera (*Castanea*) but on different genotypes, we were not able to confirm the data previously reported [11]. A similar conclusion was offered by Mullins [13].

The data presented in this work seem to suggest that calcium does not have a direct influence on tip necrosis incidence. A more indirect role on this disorder may be suggested by the evidence that the genotype with higher affinity for calcium is also less sensitive to tip necrosis. A genotypic response was found for both of uptake and ion translocation. Clone 46 shows a lower efficiency in calcium uptake than Garrone rosso as it absorbs high calcium amounts only when the ion is present at elevated concentration in the culture medium. Calcium translocation has shown the tendency to accumulate in the basal and middle part of the shoot with a very slow movement to the apex in Clone 46. Viceversa Garrone rosso always showed a higher Ca^{2+} uptake, regardless of the calcium concentration in the culture substrate, and it also translocated more calcium than Clone 46, by a strong tendency to move it into the apex. The different responses to Ca^{2+} for the two genotypes assayed could be due to several factors: different Ca^{2+} requirements of the genotypes, differences in Ca^{2+} uptake and redistribution (translocation) and differences in Ca^{2+} transport at the tip cell membranes. Raising the calcium level to 9 and 18 mmol l^{-1} involved some obvious alterations in the ionic balance of the medium but such modifications should not interfere with shoot calcium uptake and consequently with the reliability of the results. Concerning the possible involvement of other ions on rooting, our experience (unpublished data) led us to suppose that the variations at the tested levels of NH_4^+ and K^+ did not have any significant effect. The same is true for SO_4^-.

The results from this research allow us to conclude that the cytokinins present in the culture medium, have a key role on apex necrosis control, most likely through calcium as the mediator of hormone activity [7]. A controlled Ca^{2+} concentration is important for the formation of the mitotic apparatus. In fact, submicromolar [Ca^{2+}] seems to be required for polymerization of tubulin into microtubules [19]. At higher [Ca^{2+}], on the contrary, a Ca^{2+}-activated disassembly of microtubules occurred [20]. The incidence of tip

necrosis could be the result of an inappropriate redistribution of Ca^{2+} in the tissue. Obviously enough, Ca^{2+} alone does not seem to be sufficient to avoid such an incidence, and could account for the discrepancy found by different authors (see above). Hormonal balance seems to be much more important. Lack of cytokinins should impair cell division and be responsible for necrosis of the tissue also when $[Ca^{2+}]$ is at optimal levels. On the other hand, cytokinins alone could not reverse the negative effect of Ca^{2+} on the disassembly of microtubules (Table 3). A correct ratio between $[Ca^{2+}]$ and cytokinins has to be met to obtain avoidance of necrosis.

A continuous supply of cytokinins is more likely to be necessary to sustain cellular division. In fact, administration of calcium directly to the tip did not solve this problem. One of the limits of in vitro culture is that plants do not have a, or have a reduced, capacity to synthesize cytokinins. Moreover, they are usually omitted or added at very low concentrations during rooting. In our conditions the addition of a low level of BA to the rooting medium, in contrast with the results of Vieitez et al. [2], did not prevent tip necrosis but probably, in agreement with their data, contributed to limiting the rooting rates. As regards the role of calcium on rooting, it is known that the level of exchangeable calcium in the rooting medium influences the number of roots per cutting and root length [21]; cuttings of different woody species respond in a different way to different levels of calcium in the rooting medium [21]. Moreover, there is a close interrelationship between auxin and Ca^{2+}: the ion moves in a direction opposite to that of the hormone and protoplasts from soybean cultured in auxins decreased Ca^{2+} uptake and increased efflux of the ion [7]. In consideration of this, further studies are needed to better understand the role of calcium on root induction in chestnut shoots taking into particular account the possible interaction with the auxins that are currently used to stimulate ryzogenesis.

Acknowledgement

Authors wish to thank Dr Ana M. Vieitez for her critical reading of the manuscript.

References

[1] A.M. Vieitez, M.L. Vieitez and E. Vieitez, Chestnut (*Castanea spp.*), in: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry, Vol. 1, Trees I. Springer-Verlag, Berlin, Heidelberg, 1986, pp. 393–414.

[2] A.M. Vieitez, C. Sanchez and C. San-José, Prevention of shoot-tip necrosis in shoot cultures of chestnut and oak. Sci. Hortic., 41 (1989) 151–159.

[3] N.V. Kataeva, I.G. Alexandrova, R.G. Butenko and E.V. Dragavtceva, Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured in vitro. Plant Cell, Tiss. Org. Cult., 27 (1991) 149–154.

[4] C. Pérez, R. Rodríguez and R. Tamés. In vitro filbert (*Corylus avellana* L.) micropropagation from shoots and cotyledonary segments. Plant Cell Rep., 4 (1985) 137–139.

[5] G.F. Mason and C.G. Gutteridge, The role of calcium, boron and some divalent ions in leaf tipburn of strawberry. Sci. Hortic., 2 (1974) 299–308.

[6] M. Barghchi and P.G. Alderson, Pistachio (*Pistacia vera* L.). in: Y.P.S Bajaj (Ed.), Biotechnology in Agriculture and Forestry, Vol 5, Trees II. Springer-Verlag, Berlin, 1989, pp. 68–98.

[7] P.K. Hepler and R.O. Wayne, Calcium and plant development. Annu. Rev. Plant Physiol., 36 (1985) 397–439.

[8] F. Bangerth, Calcium related physiological disorders of plants. Annu. Rev. Phytopathol., 17 (1979) 91–122.

[9] G. Zocchi and I. Mignani, Calcium requirements in fruit trees. Acta Hortic., 383 (1995) 15–23.

[10] A. Abousalim and S.H. Mantell, A pratical method for alleviating shoot-tip necrosis symptoms in in vitro shoot cultures of *Pistacia vera* cv. Mateur. J. Hortic. Sci., 69 (1994) 357–365.

[11] Q. Yang, P.E. Read, C.D. Fellman and M.A. Hosier, Effect of medium constituents and rooting regime on in vitro culture of *Castanea mollissima* and *C. dentata*. Hortic. Sci., 20 (1985) 593.

[12] S. Shinga, E.C. Towensend and G.H. Oberly, Relationship between calcium and agar on vitrification and shoot-tip necrosis of quince (*Cydonia oblonga* Mill.). Plant Cell, Tissue Org. Cult., 23 (1990) 135–142.

[13] K.V Mullins., Micropropagation of chestnut (*Castanea sativa* Mill.). Acta Hortic., 212 (1985) 525–528.

[14] C. Piagnani and T. Eccher, Factors affecting the proliferation and rooting of chestnut in vitro. Acta Hortic., 227 (1987) 384–386.

[15] J.P. Bourgin and J.P. Nitsch, Obtentions de nicotiana haploïdes à partir d'étamines cultivées in vitro. Ann. Physiol. Veg., 9 (1967) 377–382.

[16] I. Lamiani Mignani, C. Poma Treccani and S. Cocucci, Bitter pit incidence and changes in Ca^{2+} content in apples after post-harvest dip in $CaCl_2$ or GA_3. Sci. Hortic., 19 (1983) 295–299.

[17] G.W. Snedecor. Statistical Methods. The Iowa State University Press, Ames, IA, 1956, pp. 251–253.

[18] L. Sha, B.H. McCown and L.A. Peterson, Occurrence and cause of shoot-tip necrosis in shoot cultures. J. Am. Soc. Hortic. Sci., 110 (1985) 631–634.

[19] J.B. Hanson, The function of calcium in plant nutrition, in: P.B. Tinker and A. Läuchli (Eds.), Advances in Plant Nutrition I, Praeger Publishers, New York, 1984, pp. 149–208.

[20] B.E.S. Gunning and A.R. Hardham, Microtubules. Annu. Rev. Plant. Physiol., 33 (1982) 651–698.

[21] H.T. Hartmann and D.E. Kester. Plant Propagation. Prentice-Hall, Inc., Englewood Cliffs, NJ, 1983, pp. 281.