IN VITRO PROPAGATION OF THORNY BLACKBERRIES

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ABSTRACT

Tissue culture of 3 newly released thorny blackberry cultivars, 'Womack', 'Rosborough', and 'Brison' was studied for sterilization procedure, shoot and root development, and plantlet acclimatization. Best sterilization procedure was found to be a 10-15 sec dip in 30% ethanol, then rinsed in double distilled water. Under the laminar flow hood, the explants were placed in a 5 min dip in 0.25% sodium hypochlorite plus Tween-20 and rinsed 3 times in double distilled sterile water. For shoot development media, 4 types of explants were cultured in a basic Murashige and Skoog salt mixture plus 0.4 mg/l myo-inositol, 0.1 mg/l GA_3, 30 g/l sucrose, 8 g/l agar, 0.1 mg/l BA, and 0.1 mg/l IBA. Stem cuttings had less % contamination and longer shoot length. Shoots developed after 4 days in culture and once reached a size of 1 cm, they were transferred to a rooting media of 0, 1, 3, 6, 10 mg/l IBA. Optimum concentration to induce rooting was 3 mg/l IBA. Immediately after root initiation, plantlets were transferred to a medium with no growth regulators. Plantlets 5 cm in length were transplanted to small plastic pots containing 1 peat : 1 perlite (v/v). Acclimatization procedure followed a weekly exchange of a series of polyethylene covers with 0, 15, 50, and 98 perforations. Following this procedure, 100% of plantlets survived.

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I wish to thank my parents, Mr. and Mrs. A.H. Ringhoffer, for their love, prayers, encouragement, moral support and for raising me in a beautiful Christian home.

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INTRODUCTION

The Texas blackberry industry began at Lindale in 1890 with less than 50 acres of berries and a small canning plant. By 1910, acreage had increased to 1,000 acres and two canning plants were operating. Fresh berries also were shipped in refrigerated cars to distant markets. By 1950, Texas was the leading U.S. producer with approximately 6,000 acres, 45% of crop being canned, 45% frozen, and 10% going to fresh market.

Presently, in 1981, the acreage has been reduced to an estimated 200 acres of commercial operations. The total acreage including small 1 to 5 acre plots is about 300 - 350 acres, with 95% of the fruits produced being processed by the cannery.

The continual reduction in acreage seems to be a result of two problems: labor and marketing. Since the cannery pays 17¢ - 25¢ per pound of fruit, growers have a difficult time finding crews for harvesting at 7¢ - 10¢ per pound.

Mechanical harvesters have been developed and might aid in solving the harvesting problem. Breeding programs are emphasizing the selection of cultivars with more erect canes for easier adaptation to mechanical harvesters, higher sugar content, better flavor and firmness of the fruit. In 1966, selection from an F_2 progeny of Brainard x Brazos were back-crossed to Brazos by E.T. Graham of the Texas Agricultural Experiment Station. Approximately 1,000 seedlings were established in the field from this cross, and from these, 30 selections were made, by H.H. Bowen. Screening in 1971, reduced the number of selections in trial plots to 7.

This thesis follows the style of the Journal of the American Society for Horticultural Science.

In 1977-1978, three thorny blackberry cultivars were released. These cultivars are the 'Brison', 'Womack', and 'Rosborough', and they exceeded all others in erect growth habit, flavor, and sugar content. However, there were limitations to increase the number of clones per cultivar. Besides the few number of root cuttings available, initiation and development of roots and shoots occurred at a low percentage.

A technique that could improve the propagation of these newly released cultivars is tissue culture. It was for this reason that this study was initiated with the following objectives in mind:

1. to establish sterilization techniques
2. to establish shoot development media
3. to establish root initiation media
4. to study different explant sources
5. to establish best method for plantlet acclimatization.

LITERATURE REVIEW

Rubus ursinus, commonly named thorny blackberries are a member of the family Rosaceae, subgenus Eubatus; they have a ploidy level from 2n=14 to 2n=84. There are many species and hybrid forms of blackberry, including the cultivated boysenberry, loganberry, youngberry, and dewberry. The main producing states of blackberries are Oregon, California, Arkansas, and Washington, with the Pacific Coast states accounting for about 80% of the U.S. total (11, 12).

Blackberries are categorized into two types of growth habits-- erect and trailing. Erect blackberries have arched self-supporting canes. Trailing blackberries, also called dewberries, ground blackberries, or running blackberries, have canes that must be tied to poles in cultivation (4).

Fruit characteristics differ in these two types of blackberries. Fruit clusters of the trailing blackberry are more open than those of the erect blackberry. Trailing blackberries generally ripen earlier and are often larger and sweeter than the erect type (4).

A third difference occurring between erect and trailing blackberries is the method by which they are propagated. Trailing blackberries, which include thornless blackberries, are commercially propagated by tip layering or sometimes by stem cuttings (4, 11); whereas, erect blackberries, which include thorny blackberries, are propagated by root cuttings (4, 11). To clarify the terms, thorny refers to thorns being present on the canes and thornless means that the thorns are absent.

In early 1977, H.H. Bowen (1) of the Texas Agricultural Experiment Station of the Texas A&M University System released three new thorny black-

berries for Texas homeowners and commercial blackberry producers. Problems occurred due to the insufficient number of root cuttings available to the public and the failure to propagate them by root cuttings. Due to this low number of plants, tissue culture was initiated to increase the number of blackberry clones.

Tissue culturing of woody species has recently been successful in apples (6), plums (7), blueberries (9), roses (10), cherries (7), and thornless blackberries (2).

Thornless blackberries were tissue cultured by Broome (2), using shoot tips with a length of 4 to 5 cm. The shoot development media consisted of a Murashige and Skoog salt mixture (8), and 1.0 mg/l BA (benzyladenine) plus 1.0 mg/l IBA (indolebutyric acid). Rooting occurred in solid medium minus BA and GA_3. Harper (3), reported abnormal growth when stems sections with a lateral bud were cultured in 1.0 mg/l BA plus 0.1 mg/l IBA. Later addition of 1.0 mg/l IBA was used for rooting.

MATERIALS AND METHODS

"Stock" plants were grown under greenhouse conditions for the area of College Station, Texas. Plants were grown in 1 gallon plastic pots in a medium composed of 1 peat : 1 perlite (v/v). Plant sections (explants) consisted of shoot tips, lateral buds, stem cuttings (piece of stem and lateral bud) and leaf bud cuttings (leaf, stem piece and lateral bud) according to experiments.

Explants were placed in 10 ml of sterile media in 15 x 2.5 cm test tubes. Cultures were grown on growth shelves at $26^{o} \pm 1^{o}$ C, 16 hours photoperiod, and light intensity ranging from 130 - 150 f.c.

Objectives were accomplished through a series of 7 experiments. All experiments were in a completely randomized block design with 3 replications per treatment. Data was analyzed by ANOVA and means compared by Duncan's Multiple Range Test.

STERILIZATION TECHNIQUE

Two sterilization methods were used:

1. 0.1% sodium hypochlorite for 15 min with addition of 2 drops per 100 ml Tween-20 as a surface surfactant.
2. A quick dip (10-15 sec) in 30% ethanol, administered in the greenhouse, then rinsed in double distilled water. Under the laminar flow hood, the explants were placed in a 5 min dip in 0.25% sodium hypochlorite plus Tween-20. Explants were then rinsed three times in double distilled sterile water.

Two types of explants were used in these initial experiments: lateral

buds and shoot tips. Explants were cultured in a basic Murashige and Skoog media. Data was collected as % contamination.

SHOOT DEVELOPMENT MEDIA

The type of explants initially used to determine the optimum media for shoot development were:

1. lateral buds 0.1-0.2 cm in length
2. shoot tips 0.4-0.5 cm long, with 2-4 small leaves enclosing the apex.

Explants were collected from cultivars 'Brison', 'Rosborough', and 'Womack'. Later experiments included stem cuttings and leaf bud cuttings from 'Rosborough' and 'Womack'.

Explants were cultured in the basic Murashige and Skoog salt mixture plus 0.4 mg/l myo-inositol, 0.1 mg/l GA_3, 30 g/l sucrose, and 8 g/l agar. Treatments consisted of the following combination of BA and IBA concentrations:

	BA (mg/l)	IBA (mg/l)	BA (mg/l) 15 sec dip
1.	0	0	0
2.	0.1	0.1	0
3.	0.1	1.0	0
4.	1.0	0.1	0
5.	1.0	1.0	0
6.	1.0	10.0	0
7.	10.0	1.0	0
8.	0	0	1000
9.	0	10.0	1000

The media was adjusted to a ph of 5.7 with drops of 0.1N KOH. The IBA was filter-sterilized using a millipore-syringe filter and added to the autoclaved medium. Data was collected as shoot length.

ROOT INITIATION MEDIA

Lateral shoots greater than 1 cm, which developed from stem cuttings cultured in a basic shoot development media plus 0.1 mg/l BA and 0.1 mg/l IBA, were excised and transferred to a medium containing a basic shoot development media; however, IBA at 0, 1, 3, 6, and 10 mg/l was the only growth regulator added. Data was collected as root number, root length and % callus.

Once the explants showed signs of root initiation, they were immediately transferred to a plantlet development media which was composed of the same media as shoot development media except that no growth regulators were added.

PLANTLET ACCLIMATIZATION

Once the plantlets obtained a size of 5 cm, they were transplanted to a sterilized mixture of 1 peat : 1 perlite (v/v) in 5x5 cm plastic pots. Acclimatization involved a series of polyethylene covers with 0, 15, 50 and 98 holes (0.8 cm in diameter). The number of holes were increased every 7 days. Plantlets were sprayed with distilled water while transplanted and watered with half strength Murashige and Skoog salts and 1.2 g/l Banrot, and covered immediately with a whole polyethylene cover. Flats were placed under growth shelves with similar conditions as for cultures. Data was collected as % survival.

RESULTS

STERILIZATION TECHNIQUE

The first method of sterilization using 0.1% sodium hypochlorite for 15 min plus Tween-20 resulted in a 90-100% contamination.

The second method using 30% ethanol for 10-15 sec plus the 0.25% sodium hypochlorite for 5 min resulted in 100% sterilization in lateral buds and 90% in shoot tips.

SHOOT DEVELOPMENT MEDIA

Lateral buds

The survival rate of BA and IBA treated blackberry lateral buds are shown in Table 1. Data seems to indicate that a concentration of 1.0 mg/l BA plus 0.1 mg/l IBA give the highest survival rates for 'Brison' and 'Rosborough' as compared to other concentrations. 'Womack' performed similarly at 1.0 mg/l BA plus 0.1 mg/l IBA and 1.0 mg/l BA plus 1.0 mg/l IBA. No explants survived when concentrations of 0 mg/l BA plus 0 mg/l IBA, or 0.1 mg/l BA plus 1.0 mg/l IBA were incorporated into the medium.

Explants grown in 1.0 mg/l BA plus 0.1 mg/l IBA had initially better growth than any other concentration, (Fig. 1).

Comparison of cultivars after 4 weeks showed that 'Rosborough' had better growth, with 'Womack' second, and 'Brison' last, (Fig. 2).

Shoot tips

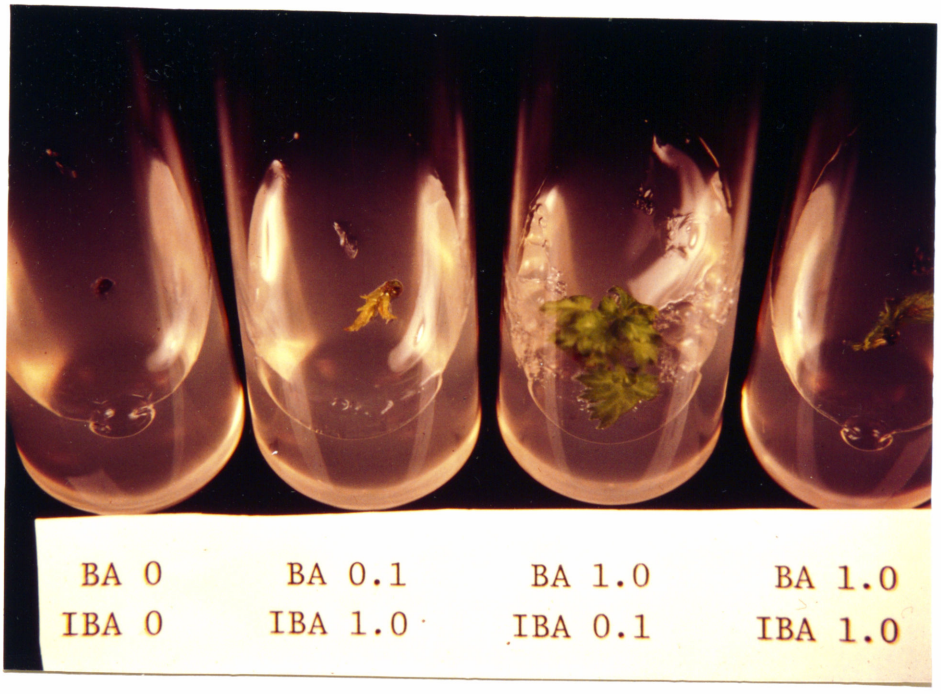
Explants had better length, uniformity, and were undistorted at 0.1 mg/l BA plus 0.1 mg/l IBA, (Fig. 3). Treatments with 1.0 mg/l BA plus 0.1 mg/l IBA induced some distortion of leaves, (Table 2). The 1000 mg/l BA

Table 1. Survival rate of BA and IBA treated blackberry lateral buds

Cultivar	BA mg/l	IBA mg/l	Survival %
'Brison'	1.0	0.1	11%
'Rosborough'	1.0	0.1	22%
'Womack'	1.0	0.1	16%
'Brison'	1.0	1.0	0%
'Rosborough'	1.0	1.0	5%
'Womack'	1.0	1.0	16%
all 3	0	0	0%
all 3	0.1	1.0	0%

Figure 1. Lateral buds of thorny blackberries after 4 weeks of culture in shoot developing media.

Figure 2. Lateral buds of three thorny blackberry cultivars after 4 weeks of culture in shoot developing media.


BA 0
IBA 0
BA 0.1
IBA 1.0
BA 1.0
IBA 0.1
BA 1.0
IBA 1.0


BRISON
ROSBOROUGH
WOMACK

Figure 3. Lateral shoots of thorny blackberries cultured in media containing 0.1 mg/l BA and 0.1 mg/l IBA.

Figure 4. Plantlet of thorny blackberries cultured after a 1000 mg/l (15 sec dip) of IBA and adventituous buds induced by this treatment.

Figure 5. Explants of thorny blackberries: a, stem cutting before culture; b, stem cutting after culture; arrow indicates lateral shoot; c, leaf bud cutting after culture; arrow indicates undeveloped lateral shoot.

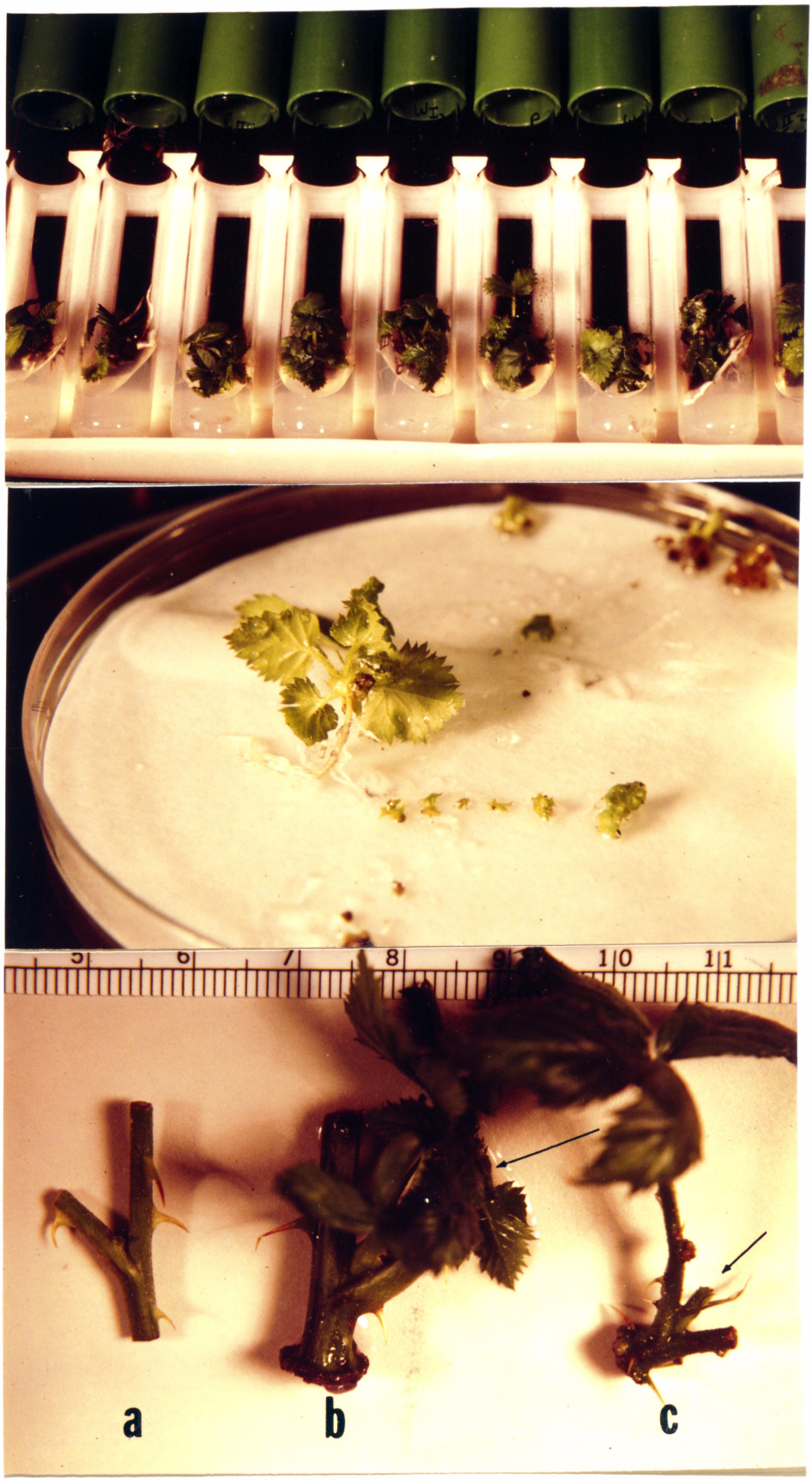

5
6
7
8
9
10
11
a
b
c

Table 2. Effect of BA and IBA on Blackberry Shoot Tips

Cultivar	BA mg/l	IBA	BA 15s dip mg/l	Survival %	Root No/Exp	Explant Length (cm)
Brison	1.0	10.0	0	50% (2)	0	1.00
Rosborough	1.0	10.0	0	100% (5)	0	.96
Womack	1.0	10.0	0	50% (4)	0	.85
Brison	10.0	1.0	0	50% (2)	0	1.00
Rosborough	10.0	1.0	0	100% (5)	2.0	.96
Womack	10.0	1.0	0	75% (4)	0	.80
Brison	1.0	0.1	0	50% (2)	0	1.00
Rosborough	1.0	0.1	0	100% (5)	.4	1.54
Womack	1.0	0.1	0	50% (4)	0	.60
Brison	0	0	1000	33.3% (3)	0	1.5
Rosborough	0	0	1000	100% (6)	.83	2.0
Womack	0	0	1000	80% (5)	0	1.08
Brison	0.1	0.1	0	67% (3)	3.5	4.0
Rosborough	0.1	0.1	0	83% (6)	2.0	3.0
Womack	0.1	0.1	0	60% (5)	0	1.83
Brison	0	10.0	1000	0% (3)	0	0
Rosborough	0	10.0	1000	83% (6)	0	1.22
Womack	0	10.0	1000	60% (5)	0	.67

dip yielded few adventious shoots, (Fig. 4). With 10 mg/l BA or IBA, a distortion and twisting of leaves were noted.

Stem cuttings and leaf bud cuttings

Stem cuttings had less contamination, better shoot development, and length, than leaf bud cuttings. Ninety-five percent of the shoot developed had a shoot length between 0.5-1.0 cm and 53% of these were transferred to a rooting media, (Table 3). Figure 5 illustrates stem cutting before culture (a), and after 4 weeks of culture (b) where lateral shoot has greater than 1 cm length. The lateral shoot did not develop in leaf bud cuttings after 4 weeks of culture (c). Percent contamination was higher in leaf bud cuttings and in 'Womack', (Fig. 6). Percent shoot development and shoot quality was higher in stem cuttings than leaf bud cuttings. There were no differences among cultivars 'Womack' and 'Rosborough', (Fig. 7, 8).

Most of these shoots developed between 2 and 3 weeks and contamination level was maximum by the second week, (Fig. 9).

ROOT INITIATION MEDIA

Concentrations of IBA at 3 and 6 mg/l induced higher number of roots than at 1.0 mg/l IBA. Though there was no difference in root length, the 3 mg/l IBA did appear to have longer roots and seemed to be the optimum concentration for rooting without causing any inhibitory affects, (Table 4). Figure 10 shows cuttings cultured in rooting media for two weeks. Note the limited number of root at 1.0 mg/l IBA. At 3 mg/l IBA, numerous, long roots are initiated with little callus formation. A concentration of 6 mg/l shows more root than in 1.0 mg/l, but less than 3 mg/l. Also the roots were shorter than the explants in 3 mg/l and callus size increased

Table 3. Influence of cutting type on thorny blackberries *in vitro*

Cutting	Contamination (%)	Shoot Developed (%)	Shoot Length	Transfer (%)
Leaf Bud	63**[y]	33	0.67[z]	11
Stem	38	95**	2.38**	53**

[z]Shoot scale ranged from 0 to 3 with, 0 = no shoot development, 1 = shoot length 0.5 cm, 2 = shoot length 0.5 - 1 cm, 3 = shoot length 1 cm

[y](**) significant at 1% by F-test

Figure 6. Influence of cutting type and cultivar on percent contamination of thorny blackberries *in vitro*.

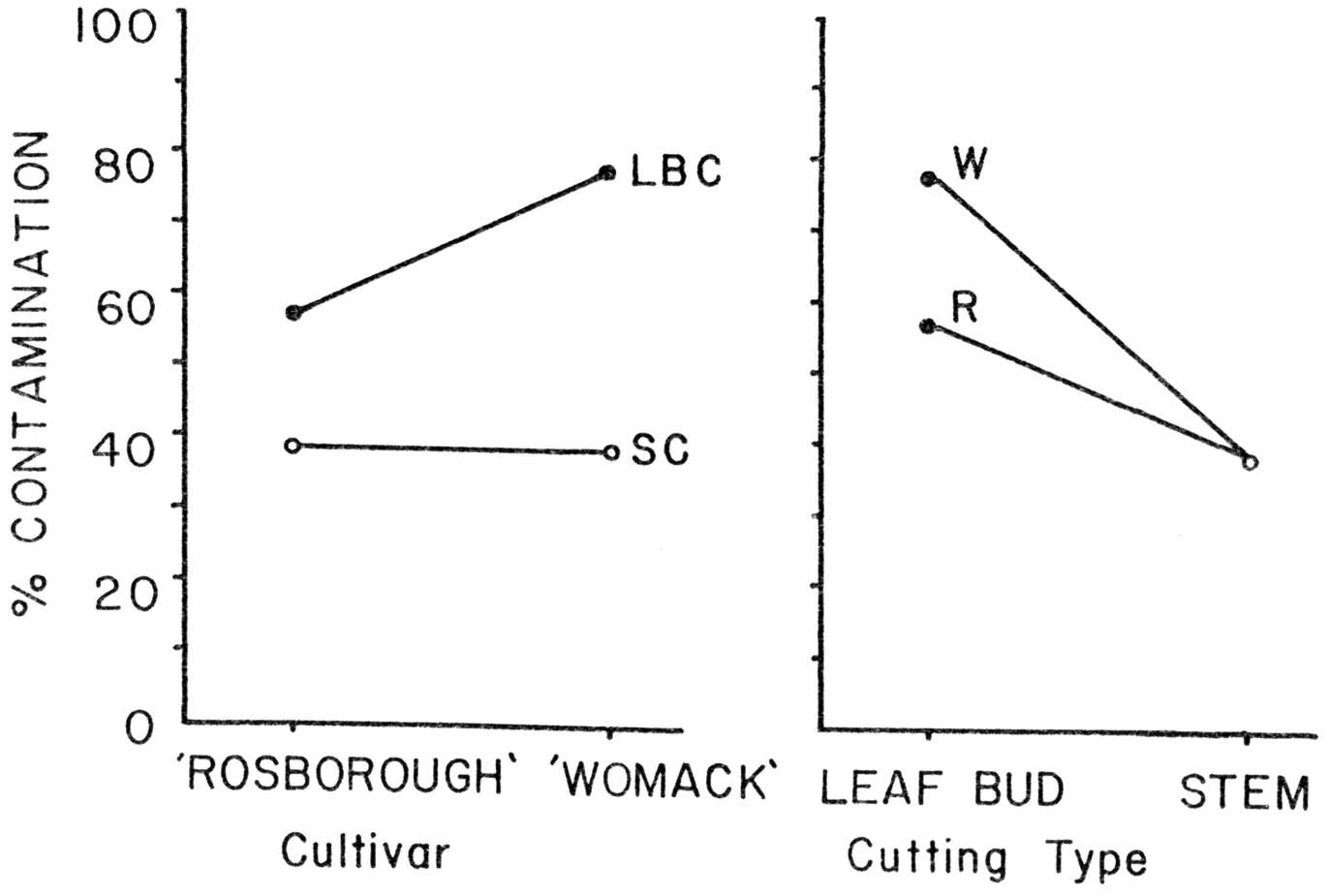

100
80
60
40
20
0
% CONTAMINATION
LBC
SC
'ROSBOROUGH'
'WOMACK'
Cultivar
W
R
LEAF BUD
STEM
Cutting Type

Figure 7. Influence of cutting type and cultivar on shoot development of thorny blackberries *in vitro*.

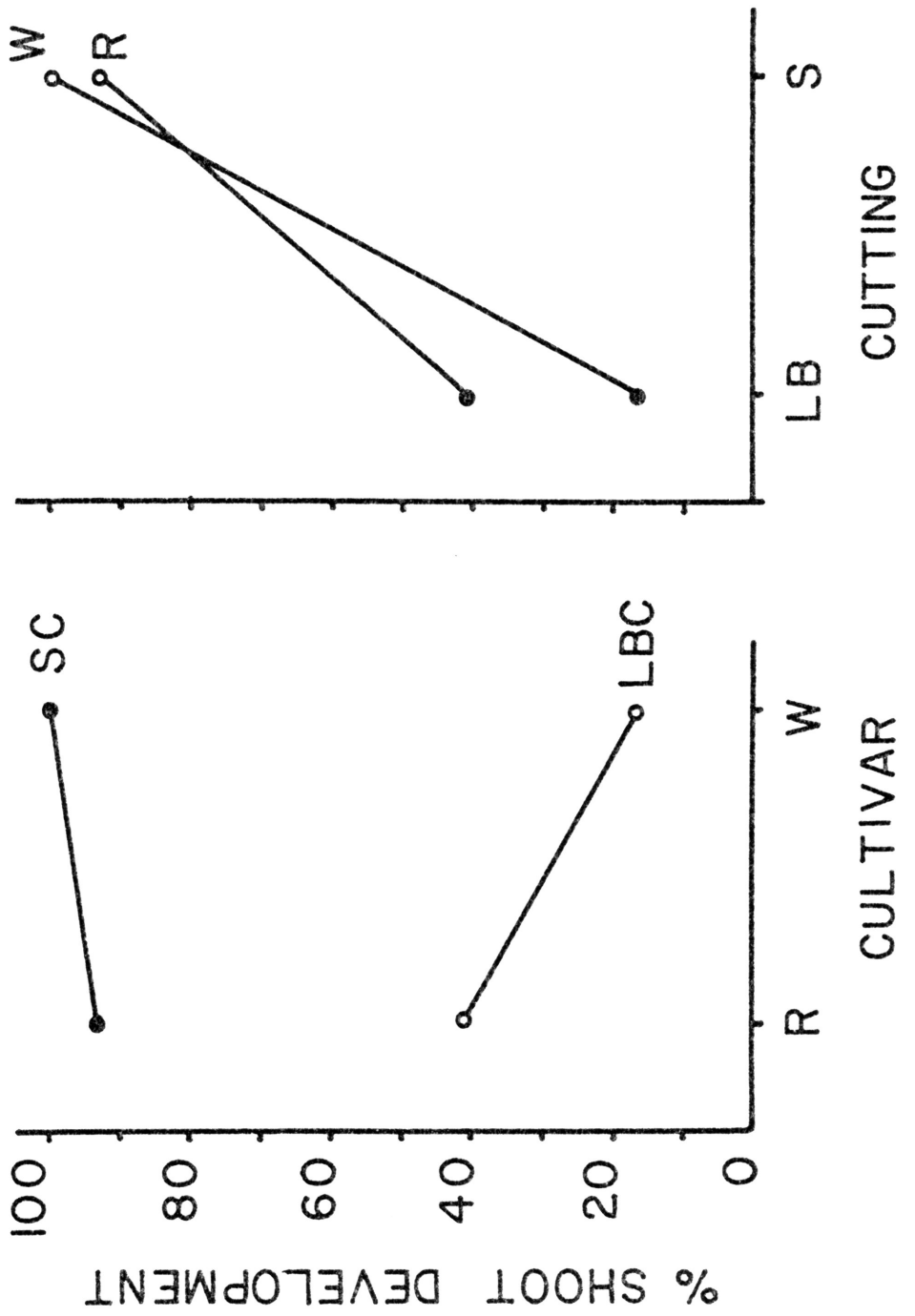
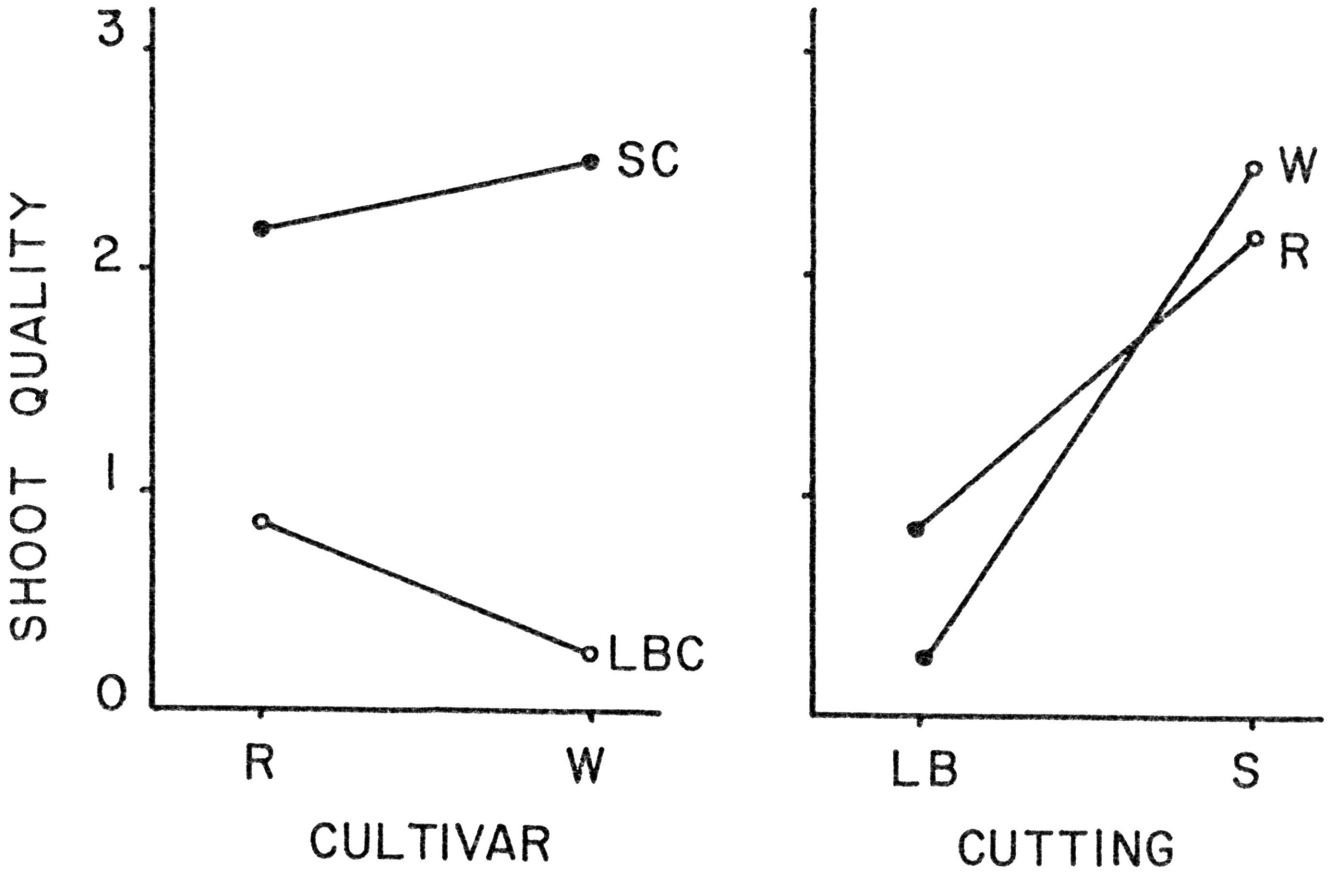

% SHOOT DEVELOPMENT
100
80
60
40
20
0
SC
LBC
R
W
CULTIVAR
W
R
LB
S
CUTTING

Figure 8. Influence of cutting type and cultivar on shoot quality on thorny blackberries *in vitro*.



0= none
1 = <0.5 cm
2 = 0.5 - 1
3 = >1

Figure 9. Influence of time on thorny blackberries *in vitro*.

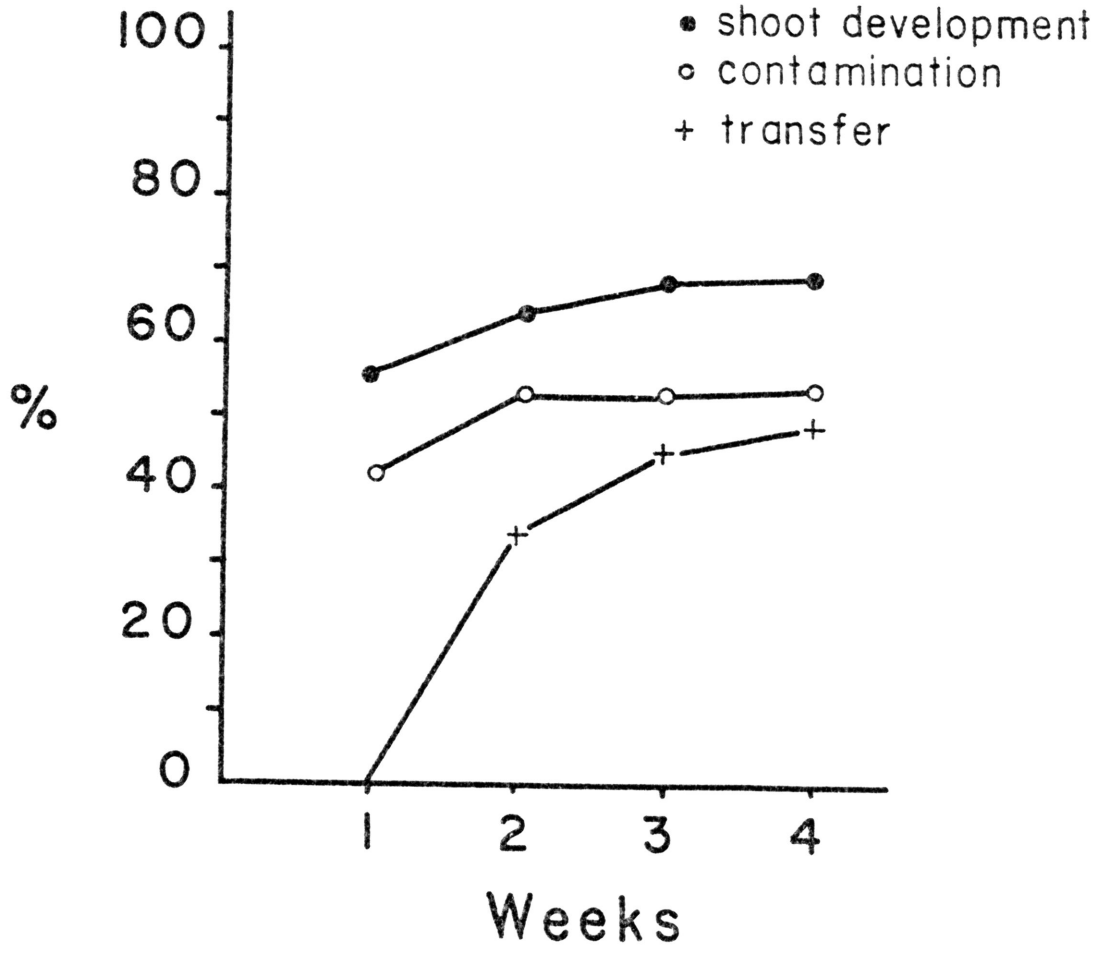

shoot development
contamination
transfer
100
80
60
40
20
0
%
1
2
3
4
Weeks

Table 4. Effect of IBA rooting of thorny blackberry shoots *in vitro*

IBA (mg/l)	Root Number	Root Length (cm)	Callus (%)
1	1.72b[z]	0.31a	63b
3	3.27a	0.32a	91a
6	3.27a	0.22a	87a

[z]Mean separation in columns by Duncan's multiple range test, 5% level.

Figure 10. Root development in thorny blackberries after 2 weeks of culture in media containing 3 levels of IBA in mg/l.

Figure 11. Root development in thorny blackberries after 2 weeks of culture in media containing 10 mg/l IBA.


1
3
6

with concentration. Explants at 10 mg/l IBA developed abundant callus and short, stubby roots, (Fig. 11). The majority of explants rooted within two weeks and were immediately transferred to the plantlet development media which had no growth regulators, to allow for normal development of roots and shoots, (Fig. 12).

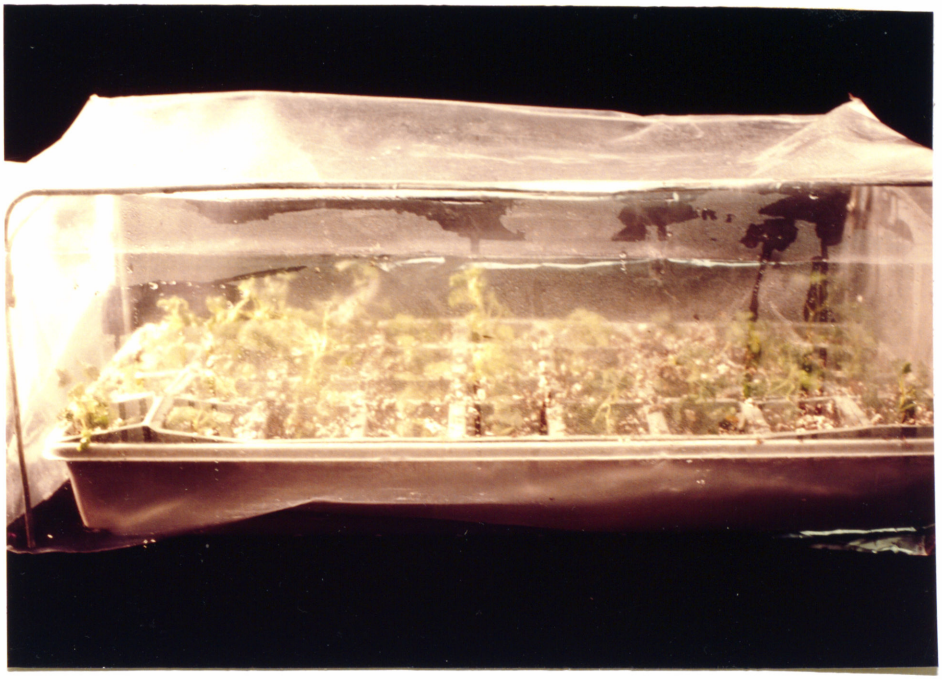
PLANTLET ACCLIMATIZATION

Potted plantlets had 100% survival rate with the use of polyethylene covers, (Fig. 13). Plantlets were completely acclimatized and placed under 50% shade in a greenhouse after 4 weeks, (Fig. 14). Plantlets were moved to 10% shade after 1 month. This procedure avoided the desication and wilting of the plantlets.

Figure 12. Plantlet of thorny blackberries cultured in media containing no growth regulators.

Figure 13. Acclimatization of plantlets of thorny blackberries using a polyethylene cover.

Figure 14. Acclimatized plantlets of thorny blackberries.



DISCUSSION AND CONCLUSIONS

Sterilization procedures were more effective when explants were dipped immediately in the 30% ethanol, and then in 0.25% sodium hypochlorite. The ethanol dip seemed to clean the material and thus lower % contamination without damaging or browning the explants.

Shoot tips had a higher survival rate than the lateral buds probably because shoot tips had longer internodes permitting a better disinfestation. Both explants when initially placed on culture leached, within 2 days, some compounds into the medium. Incorporating phloroglucinol into the medium as suggested by Jones (5) at 162 mg/l did not control the leaching. However, by relocating the explant within the same test tube, any inhibitory action by the leachates were prevented and the leaching stopped.

All of the lateral buds eventually turned brown and died. The explants may have just been too small. A few of the shoot tips survived, but proliferation of the shoots did not occur until dipped in sterile concentration of BA at 1000 mg/l. This production of adventitious buds is highly desirable for rapid clonal multiplication in ornamental crops. But since there is a possibility of mutations occurring because of calli development and dedifferentiation processes, this method proved unreliable to rapidly propagate certified blackberry cultivars. Since there is a limited number of shoot tips on blackberry plants and numerous lateral buds are present on a cane, stem cuttings and leaf bud cuttings were the obvious explant to culture and not shoot tips as used by Broome (2).

The buds in the stem cuttings began growth within 4 days; whereas, some buds in the leaf bud cuttings didn't grow until after 2 weeks, with the majority of them never growing at all. This growth inhibition might be

related to inhibitors produced in the leaf. The leaf bud cuttings showed to have more contamination because of the leaf presence. Leaves are difficult to disinfest. Cuttings taken from the most actively growing part of the "stock" plant seemed to have less contamination than cuttings taken from older portions of the cane.

From all of the results it appears that stem cuttings are the best method of clonal propagation for thorny blackberries since it offers such a large number of explants. Stem cuttings produce a very uniform growth and thus production cycles could be scheduled for industry. A high survival rate occurs using stem cuttings.

The shoot development media of 0.1 mg/l BA and 0.1 mg/l IBA seemed optimum for culture and this concentration differed from Broome (2) who used concentration of 1.0 mg/l BA plus 1.0 mg/l IBA. From the 3 cultivars cultured, 'Rosborough', and 'Womack' grew well under this treatment; however, 'Brison' produced smaller and weak shoots. This difference is in accordance with many studies which show different requirements between cultivars and species.

Preliminary experiments on root initiation indicated that cuttings placed in media containing 0 mg/l IBA did not develop roots, and cuttings placed at 10.0 mg/l IBA developed a high number of stunted roots and abundant callus. From the 3 IBA treatments, 1 mg/l induced few small roots; this observation was in contrast to optimum rooting concentration of 1 mg/l reported by Broome (2) and Harper (3). Treatments with 3 and 6 mg/l induced similar number of roots; however, 6 mg/l induced shorter and stubier roots and more calli. Inhibition of root growth and larger amounts of calli increased with higher IBA concentrations. Once roots were initiated, they were immediately transferred to a plantlet development

medium where they grew larger shoots and roots. The acclimatization procedure proved ideal for 100% survival.

The procedure to tissue culture blackberries will aid not only in the clonal propagation of the cultivars 'Womack' and 'Rosborough', but also to produce plantlets for future research on mutation breeding and studies on growth inhibitors.

SUMMARY

1. Best sterilization procedure involved a 10-15 sec dip in 30% ethanol, administered in the greenhouse, then rinsed in double distilled water. Under the laminar flow hood, explants were placed in a 5 min dip in 0.25% sodium hypochlorite plus Tween-20. Then explants were rinsed three times in double distilled sterile water.
2. Optimum shoot development media was 0.1 mg/l BA and 0.1 mg/l IBA.
3. Optimum rooting was induced by 3 mg/l IBA.
4. Stem cuttings had low contamination percentage, a higher percentage of shoot development, and longer shoots.
5. Once explants were rooted, they were called plantlets, and placed in a medium similar to the shoot development medium, except no growth regulators were added. The purpose of this medium was increasing the size of shoots and roots. Then after plantlets reached a size of about 5 cm, they were acclimatized.
6. Plantlets were transplanted to a sterile mixture of 1:1 peat-perlite (v/v) in 5x5 cm plastic pots for plantlet acclimatization. The method was 100% successful when using a series of polyethylene pot covers. Polyethylene covers having perforations from 0 to 98 holes were changed weekly and finally removed when plants were placed under 50% shade.

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