Plant Tissue Culture in Crop Improvement©

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Plant tissue culture, the art and science of growing an organelle, cell, tissue, or organ on a defined medium under controlled conditions in an aseptic environment, has come a long way since it was discovered over a century ago. The obvious advantages of extra-rapid multiplication while maintaining genetic uniformity and freedom from pathogens and pests brought this technique into the plant propagation industry after Morel successfully cultured orchids in vitro in 1960. Since then, thousands of plant species have been micropropagated and more of them, once thought recalcitrant, are being multiplied through this method. This article gives a brief history and description of the various techniques that are still relevant and beneficial, especially when used with conventional breeding and modern molecular methods.

INTRODUCTION AND BACKGROUND

At Dry Creek Labs we are involved in the business of micropropagating improved fruit and nut cultivars and their rootstocks. The list includes almonds, apples, blueberries, blackberries, citrus, olives, pomegranate, raspberries, stone fruits, pistachios, and walnuts. The recent additions to the list are avocados and pecans. We have also been selecting clones for salt tolerance and eliminating known pathogens and viruses through meristem culture, thermotherapy, and cryotherapy in order to provide "clean plants" to our customers.

Plant tissue culture is defined as the art and science of growing a plant organelle, cell, tissue or an organ in a test tube on a defined medium under controlled environmental conditions (Hartman et. al., 1990*)*. For many who have worked in the tissue culture field, it's as much an art as science that. Perhaps that is the reason why many laboratories are successful in culturing a specific plant while others cannot duplicate the process.

Following the discovery that all living beings are made of smaller compartments called cells (Schleiden, 1838; Schwann, 1839), Haberlandt was the first to try, albeit unsuccessfully, to culture plant cells in vitro in Germany (Haberlandt, 1902). It was left to Gautheret (1934) in France, to demonstrate the successful culture of an isolated plant tissue. White (1939) developed techniques to continuously grow carrot root cell cultures for prolonged periods of time. Plant tissue culture got a real boost with the development of the theory of totipotency which postulated that each living cell has all the ingredients to become a complete organism if given the right conditions (Stewart et al., 1958; Street, 1967; Vasil and Hildebrandt, 1965). The recognition of the role of plant hormones, like auxins (Went, 1928), gibberellins (Kurosawa, 1926) and cytokinins (Miller et al., 1955) in plant growth and their availability enabled the plant tissue culturist to grow plant tissues into unorganized masses of cells called callus or to induce the formation of roots, shoots, or whole plants. How these hormones actually function in plants is still being worked out more than a century after they were discovered. Some excellent reviews have recently appeared that describe the history and development of plant tissue culture (Vasil, 2008; Sussex, 2008).

The applications of plant tissue culture got a real boost in the early sixties after Morel (1960) grew orchids and other plants in vitro. Following the development of basic growth media like MS (Murashige and Skoog, 1962) and Woody Plant Medium (Lloyd and McCown, 1980) several commercial tissue culture labs sprang up all over the globe during the seventies. Prominent ones were Twyford in England doing date palm, Oglesby in Florida culturing bananas, and Oki Nurseries in California doing ornamentals amongst other crops. The 1980s saw the proliferation of many labs doing orchids, foliage crops,

and other ornamentals in Florida, California and other states. However, commercial tissue culture, being a labor intensive operation, invited stiff competition from developing countries where labor costs are relatively low. As a result, many of the labs in the USA closed down in the 1990s. Only those laboratories that were producing high-value crops or had adjoining nurseries coupled with good business acumen survived. During the past two decades, the advances in molecular biology have shifted the emphasis and funding of research away from plant tissue culture. The recent recognition of the existence of endophytes in many tissue cultured plantlets and the imposition of stricter quarantine controls has restricted plant movement across borders and revived the local tissue culture micropropagation industry. The development of new cultivars in perennial horticultural crops through various breeding programs and their demand in the market place has further encouraged their micropropagation so they are available to growers in a much shorter span of time.

TECHNIQUES

There are several tissue culture techniques that have been developed and utilized to improve crop plants. They have found useful applications in improving crops and the work done during the last 50 years is beginning to show up in the horticultural and forestry enterprises. Some of the techniques that have found favor are listed below:

Somatic Embryogenesis

Somatic embryogenesis refers to the in vitro conversion of vegetative cells into viable embryos which are later induced to become complete plantlets. The conversion of callus and cell suspension cultures into somatic embryos was first achieved in 1958 (Reinert, 1958; Stewart et al., 1958). In general, the procedure involved pulsing the tissue with a high dosage of an auxin like 2,4-D for a brief period followed by growing on a hormone-free medium. Most of the genetically transformed varieties of crops, forest trees and several vegetable, fruit and ornamental plants are being multiplied by this technique. Figure 1 depicts somatic embryos of Chandler walnut regenerating shoots.

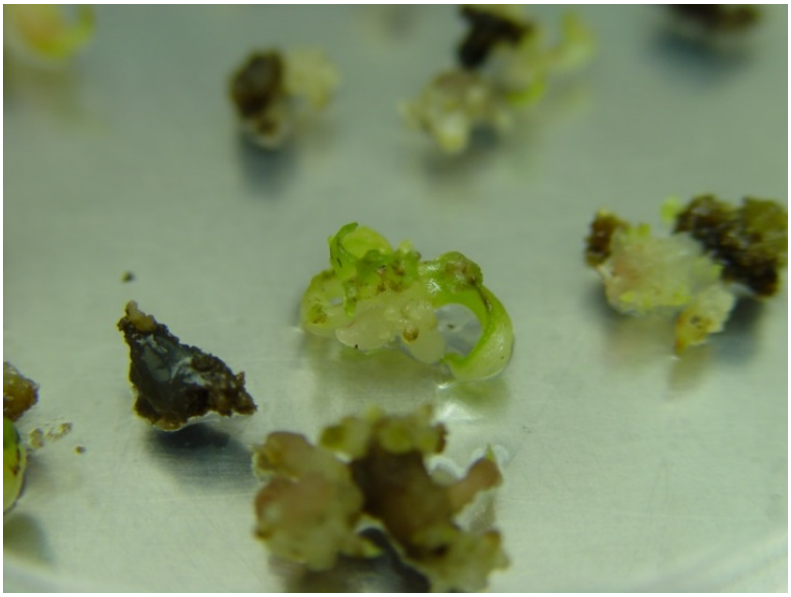


Fig. 1. Somatic embryos of walnut regenerating into shoots.

Anther Culture and Embryo Rescue

The pollen grains or anthers, the organ containing these male spores, when successfully cultured on a defined medium and conducive environment, generate into haploid plants that contain only half the normal number of chromosomes. They are very useful in

breeding programs to develop genetically uniform homozygous-haploids. The technique was developed by Guha and Maheshwari (1966) and has since been successfully employed in several plant species. Some improvements like selecting the proper stage of anther development for successful culture, double-layer medium, and determining ploidy levels through flow cytometry in plants further enhanced the reliability and application of this technique (Sharma et al., 1983). The haploid plantlets germinating from *Nicotiana paniculata* pollen grains are depicted in Figure 2.

Somewhat similar methodology was employed to develop whole plants from unfertilized or fertilized embryos in vitro. Intergeneric hybrids like plumquats, apriots, apriums, and peachquats have been developed by inter-crossing peach, plum and apricot through "embryo rescue" technology which would not be possible otherwise.

Fig. 2. Germinating pollen grains from *Nicotiana paniculata* anthers.

Protoplast Culture and Fusion

Plant protoplasts were isolated for the first time in 1960 by treating cells with enzymes like cellulase, pectolyase, and hemicelluloses that would dissolve cell wall (Cocking, 1960). These protoplasts coming from diverse cultivars or species could be fused together under specific conditions and grown and regenerated into a new plant (Fig. 3). Alternately, a gene of interest could be engineered into a vector like the Ti (tumor inducing) plasmid and either be physically injected into the nucleus of the protoplast by "microinjection," by applying electric current to open up the pores for easy introduction (electroporation) or being briefly co-cultured for incorporation with protoplast nuclear DNA. The resulting fused or transformed protoplast products could be sorted out from the rest of the population by flow cytometry and regenerated into whole plants (Galbraith and Harkins, 1982). New improved citrus cultivars and rootstocks have recently been released that were developed through protoplast fusion at the USDA at Citrus Research and Experiment station in Florida (Grosser, 2012).

Transformation

It was discovered that the whole tissue, like an epicotyl segment from a germinated seed, can also be transformed by co-culturing briefly with *Agrobacterium tumefaciens* carrying the Ti plasmid with the gene of interest. It is later transferred to an antibiotic-containing medium to kill the bacterium and the transformed tissue is recovered and regenerated into a whole plant (Sharma et al., 2006). The latest arsenal in biotechnology is the Gene Gun

(Sanford, 2000) where the microprojectiles coated with genes of interest are bombarded at high velocity to successfully transform a range of plant species.

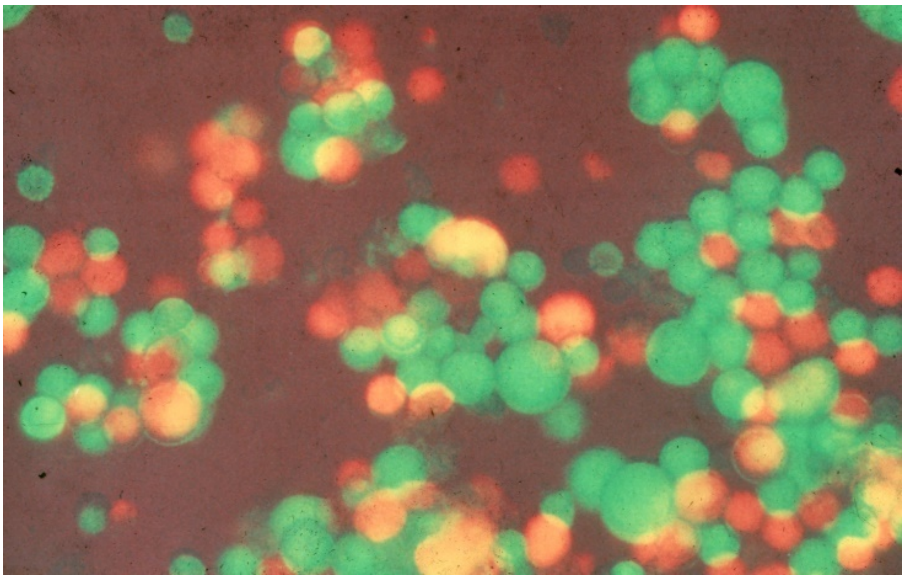


Fig. 3. Fusing protoplasts of *Nicotiana sylvestris* (stained with Fluoresceinisothiocyanate, light gray) and of *N. paniculata* (stained with Rhodamine, dark gray). The fused protoplasts turn orange in color and can be sorted out.

Developing Stress-Tolerant Plants

Various types of abiotic (air, water, salts, chemicals, temperature, etc.) and biotic (insects, pathogens, viruses, etc.) stressors are known to adversely affect the quality and productivity of crop plants. At Duarte Nursery we have an interest in developing rootstocks for fruits, nuts, and vines that can withstand saline soils and brackish irrigation water. An aspect which is becoming more important as good quality fresh water is becoming limited in supply in the central valley of California. A relatively simple procedure was employed for the situation where salts equal to or twice the concentrations that existed in a representative sample of brackish San Joaquin Valley well water (Sanden et al., 2009) were added to the plant growth medium. The tissue-cultured shoots of various cultivars of fig and rootstocks of *Prunus*, grape, and the selected in vitro lines from the hybrid seed of pistachio rootstock UCB-1 (*Pistacia atlantica* × *P. integerrima*) developed at the University of California, Davis through controlled crosses, were placed on media containing different salt concentrations and evaluated for growth. In general, figs could withstand saline conditions better than pistachio, *Prunus* and grape rootstocks (Fig. 4). The plants that are already known for their tolerance or susceptibility to salts like Salt Creek grape and 'Lovell' peach showed similar behavior in this in vitro experiment and proved the fidelity of the technique (Figs. 5-7). The experiment also provided information on rootstocks for which salinity tolerance data is still not available. The results of these experiments shall be further tested in field trials.

The high concentrations of chlorides, sulfates, carbonates, and bicarbonates of calcium, magnesium and sodium individually or in combination are the main causes of saline and alkaline soils. In addition, high boron also adversely affects plant growth. Different concentrations of these individual salts ranging from zero to 10,000 $mg \cdot L^{-1}$ were added to the growing medium and the influence on the growth of 7 fig cultivars, 20 grape, 25 *Prunus* rootstocks, and 35 UCB-1 pistachio seedling lines was studied. The results are summarized in Table 1. The plants showing high level of tolerance to salts are being evaluated in the field trails.

Table 1. Tolerance limits of plant cultivars/rootstocks to individual salt concentrations in the medium.

Salt	Maximum survival limit ($mg \cdot L^{-1}$)	Fig cultivars	Growth[+]	Grapes cultivars	Growth	*Prunus* cultivars	Growth
$CaCl_2$	10,000	Calimyrna	4.0	Salt Creek GRN-4*	4.75	Hansen	1.5
$MgCl_2$	10,000	Sierra	7.25	NA		Nemaguard	7.00
NaCl	10,000	Calimyrna Sequoia*	3.75	Salt Creek	1.00	Krymsk-1*	2.0
$CaSO_4$	10,000	Adriatic	6.0	Salt Creek	8.00	Hansen	5.75
$MgSO_4$	10,000	Calimyrna	7.0	GRN-5*	8.00	Br.Hy.5-7*	8.00
Na_2SO_4	10,000	Calimyrna	5.25	Salt Creek	0.50	Hansen	1.00
$CaCO_3$[++]	10,000	Sequoia*	7.75	RS 3-1*	6.75	Hansen	5.25
$MgCO_3$	10,000	Brown Turkey	3.5	GRN-5*	2.5	Br. Hy. 5-7*	1.75
Na_2CO_3	5,000	Brown Turkey	2.00	RS3-1*	3.25	Br.Hy 5-7*	1.00
$NaHCO_3$	1,000	Brown Turkey	4.00	GRN-4*	4.00	Nemaguard	1.75
HBO_3	100	Sequoia*	3.00	Salt Creek	3.00	Nemaguard HBOK-1*	3.5

+ Growth based on visual rating from 0 (no growth) to 10 (shoot filling the 4" tube).

++ Because of low solubility, observations are based on medium saturated with $CaCO_3$.

* Patented clones. Details in Table 2.

Table 2. Details of the patented clones evaluated in the trial for their salt tolerance.

	Clone	Patent#	Patent holder	Notes
FIG	SEQUOIA13-4	PP20038 P3	The Regents of the University of California	
PRUNUS	ADARA 12-20	Not Filed	P2G™ Progressive Genetics Group	
	BB 106	Patent Pending	Bright's Nursery Inc.	
	BRIGHT'S HYBRID DCL5-3	PP18,782	P2G™ Progressive Genetics Group	Brights Hybrid 5
	BRIGHT'S HYBRID DCL5-7	PP18,782	P2G™ Progressive Genetics Group	Brights Hybrid 5
	5C		The Regents of the University of California	Controller 5
	EMPEREAN 1	usppaf	P2G™ Progressive Genetics Group	Empyrean©1
	HBOK 1-4		The Regents of the University of California	Under Testing
	HBOK 10	PP22505	The Regents of the University of California	AKA Controller 8
	HBOK 28		The Regents of the University of California	Under Testing
	HBOK 32	PP22845 P3	The Regents of the University of California	AKA Controller 7
	HBOK 50	PP22208 P3	The Regents of the University of California	AKA Controller 9.5
	KRYMSK 1	PP15,995	P2G™ Progressive Genetics Group	
	KRYMSK 2	PP15,957	P2G™ Progressive Genetics Group	
	KRYMSK 5	PP15,723	P2G™ Progressive Genetics Group	
	KRYMSK 6	PP16,114	P2G™ Progressive Genetics Group	
	KRYMSK 7	PP16,353	P2G™ Progressive Genetics Group	
	KRYMSK 9	PP20,847	P2G™ Progressive Genetics Group	
	KRYMSK 86	PP16,272	P2G™ Progressive Genetics Group	
	PENTA	usppaf	P2G™ Progressive Genetics Group Empyrean"	
	SAM 1		Varieties lnternationai	Under testing
	WIEROOT 13		P2G™ Progressive Genetics Group	No patent information
GRAPES	MCKENRY RS3-1	PP16291 P3	The Regents of the University of California	
	MCKENRY RS9-1	PP16115 P3	The Regents of the University of California	
	8909-05 WALKER UCD GRN 1	PP19981 P2	The Regents of the University of California	
	9363-16 WALKER UCD GRN2	PP199993 P2	The Regents of the University of California	
	9365-43 WALKER UCD GRN3	PP20051 P2	The Regents of the University of California	
	9365-85 WALKER UCD GRN4	PP21358 P3	The Regents of the University of California	
	9407-14 WALKER UCD GRN5	PP23532 P3	The Regents of the University of California	

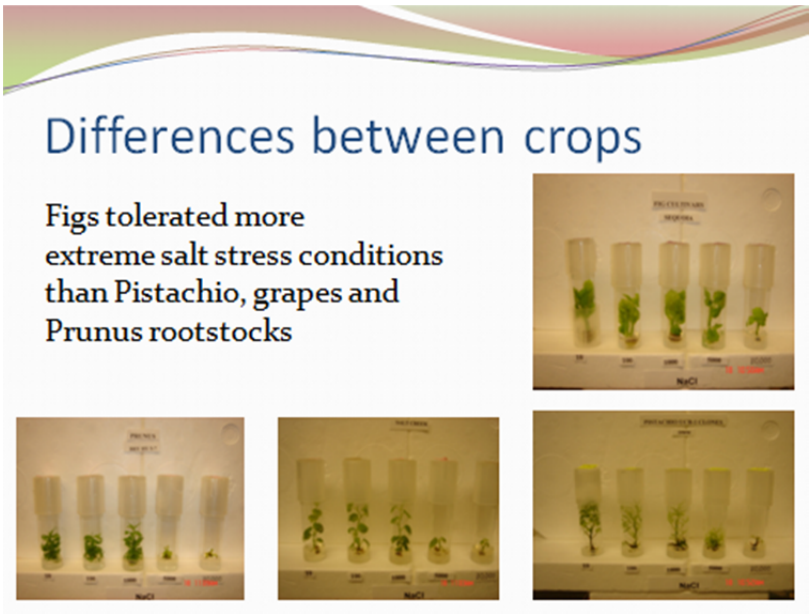


Fig. 4. Differences in the tolerance levels of crops to salt stress.

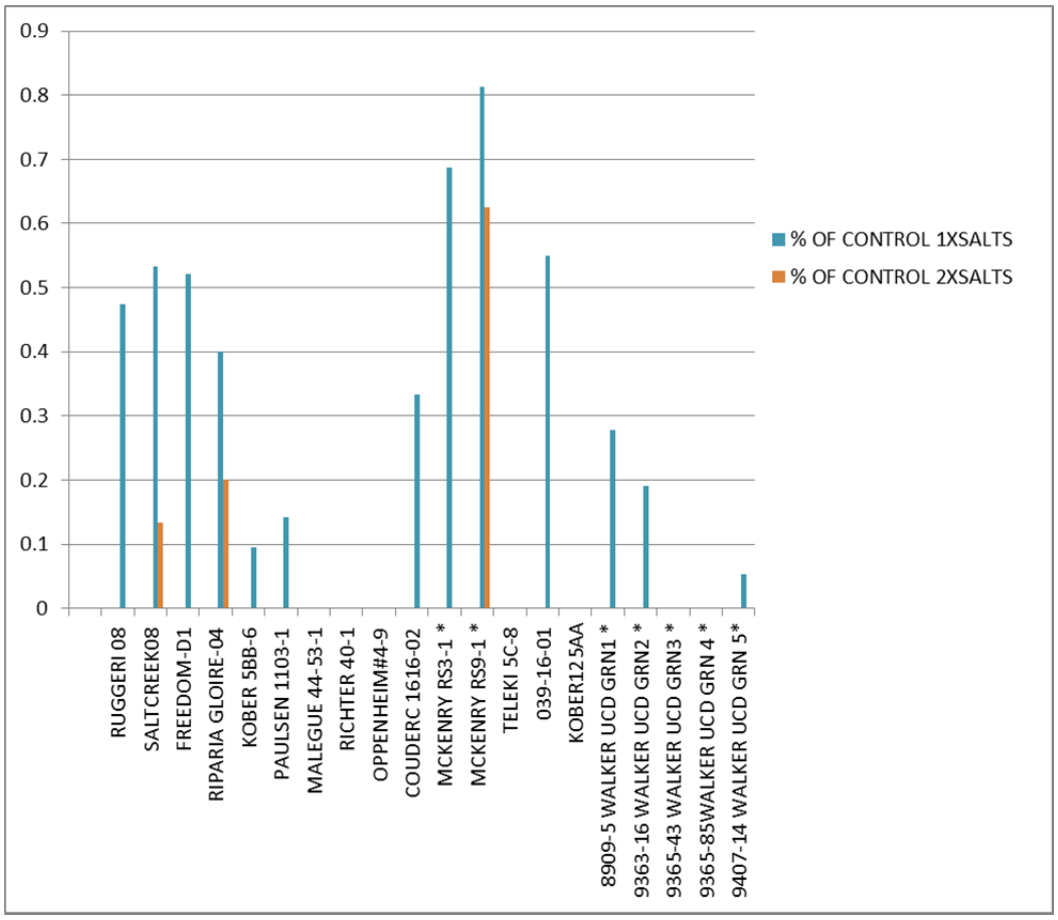


Fig. 5. Influence of salt concentrations equal to (1X) or twice the amount (2X) in San Joaquin well water (Sanden et al., 2009) on different grape rootstocks cultured in vitro. The names marked with * hold patents and are detailed in Table 2.

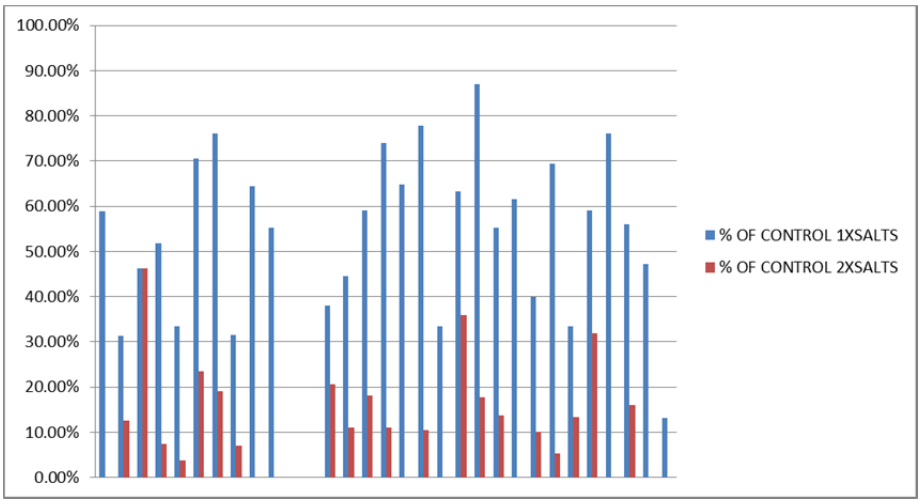


Fig. 6. Influence of salt concentrations equal to (1X) or twice the amount (2X) in San Joaquin well water (Sanden et al., 2009) on different UCB-1 pistachio seedling lines cultured in vitro. The code names for the clonal lines on X-axis are excluded here being proprietary in nature.

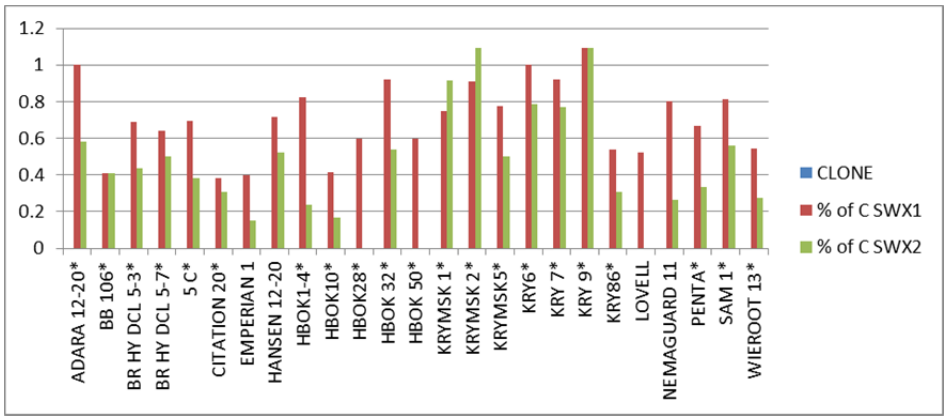


Fig. 7. Influence of salt concentrations equal to (1X) or twice the amount (2X) in San Joaquin well water (Sanden et al., 2009) on different *Prunus* rootstocks cultured in vitro. The names marked with * hold patents and are detailed in Table 2.

Removing Microbes and Viruses from Plant Tissues

Various studies have confirmed the presence of viruses and other microorganisms in tissue-cultured plants that can withstand surface sterilization and stay in the tissue without showing up until conditions become favorable. This can result in serious losses and the spread of diseases if not controlled in the initial stages of micropropagation. Normally, an apical meristem, less than 0.2 mm tall, excised from the actively growing shoot apex should be relatively free from most of the pathogens. However, some still remain for which the tissues are subjected to high temperatures for a given period that can kill pathogens but not the plant tissue. We were able to get rid of fig mosaic virus from several fig cultivars by combining these two procedures (Sharma, 2010). Some obstinate endophytes, like *Badnavirus* complex in figs (Laney et al., 2012), leaf roll virus in grapes (Pathirana et al., 2013) and bushy dwarf virus in raspberry (Wang and Valkonen, 2009) which are tightly embedded within the apical dome of the meristematic tissue still stay and can be eliminated only by combining apical meristem culture and heat therapy with cryotherapy as the last step. This three-step procedure can be very challenging to the

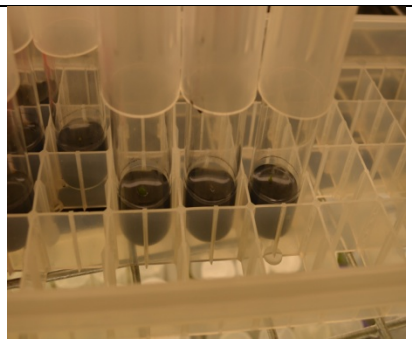
survival of the tissue and certain specific cultural supplements are required to keep it alive (Wang et al., 2009). Figure 8 shows 'Calimyrna' fig that underwent and survived this three-step procedure to eliminate *Badnavirus* complex.

Fig. 8. Cleaned fig (*Ficus carica* 'Sierra') that survived the three-step cleaning process: meristemming, thermotherapy and cryotherapy.

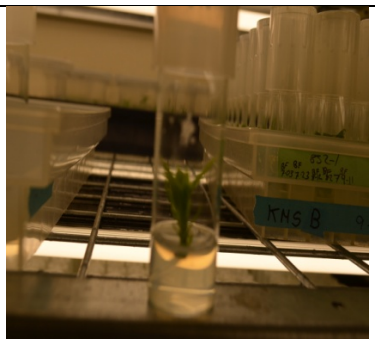
Micropropagating New Crops

The procedures for the micropropagation of several crops have been established, but still some remain that are recalcitrant or too slow in culture to be commercially viable. There are so many unknown variables that are simultaneously at play and have not been defined. So every new plant species or cultivar becomes a challenge to put into culture. Sometimes the rooting of some species like walnut has posed challenges that have been partially solved by understanding the physiology and modifying the growing media (Sharma et al., 2006).

Dry Creek Labs has recently achieved success in commercially micropropagating avocado rootstocks and the finished trees should be available in the 2015 growing season (Fig. 9). The micropropagated rootstocks shall save at least 2 years in growing time. The conventional procedure for avocados employs growing of seedlings in the first season and grafting the rootstock on it the following year. The graft point is covered with soil or other medium to encourage the rootstock portion to develop roots. Finally, the scion cultivar is grafted onto the rootstock and the seedling part severed to finish the tree in the following season. Figure 9 illustrates the steps being followed for micropropagating avocados after endophytes have been eliminated from the explants by the procedures described earlier.



A. Excised meristems.



B. Growing shoot.

C. To rooting medium.

D. Acclimation.

E. To pots.

F. Ready for grafting.

Fig. 9. Different steps followed in micropropagating avocado rootstock.

ACKNOWLEDGEMENTS

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QUESTIONS AND ANSWERS

Douglas Justice: How was it discovered that plants could withstand the very low temperatures involved in the cryotherapy treatment?

Dharam Sharma: The technique was developed by Bart Panis in Copenhagen within the last 5 years or do. It's a very harsh technique which requires that the tissue be infused with dehydrating solutions that will protect it.

Jim Conner: What kind of work have you done on avocado scion cultivars?

Dharam Sharma: We are trying new procedures for micropropagating scions also.