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Shortcut to long-distance developing of a tissue culture medium: micropropagation of mature almond cultivars as a case study

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Abstract: Culture medium development methods are time-consuming, laborious, and require a large amount of experimental materials. The Nas hypothesis for the development of a micropropagation medium offers a less time-consuming and a less laborious approach. According to this hypothesis, the composition of a culture medium for a particular species should resemble the seed composition. In accordance with this hypothesis, first, an almond culture medium [Nas Almond Medium (NAM)] was developed based on almond kernel composition. Then, using mature tissues of almond cultivars, the growth of cultures on NAM was compared to that on Murashige and Skoog medium (MS), Woody Plant Medium (WPM), Driver and Kuniyuki medium (DKW), and Nas and Read medium (NRM). Medium composition had profound effects on the 3 growth parameters of number of shoots per explant, mean shoot length, and the productivity. With respect to mean number of shoots and mean shoot length, NAM was the best medium. NAM was also the most productive medium. NAM was up to 35%, 49%, 68%, and 69% more productive than NRM, MS, DKW, and WPM, respectively. The results suggest that formulating the composition of a culture medium based on the seed content can be a straightforward universal method of medium development for micropropagation.

Key words: Almond, culture medium, micropropagation, *Prunus dulcis*

1. Introduction

Evidently the development and the use of a defined tissue culture medium are among the most important factors that affect explant morphogenic reactions and the success of in vitro studies. The growth of cultures (Ramage and Williams, 2002; Niedz and Evens, 2007; Ashrafi et al., 2010), rooting of microshoots (Goncalves et al., 2005), initiation and growth of bulblets (Staikidou et al., 2006), somatic embryogenesis (Pinto et al., 2008), plant regeneration (Kothari-Chajer et al., 2008), and seed germination and protocorm formation (Bektaş et al., 2013) were affected by the culture medium. Morphological and physiological disorders may become serious problems and the cultures may not be maintained everlastingly if a suboptimal culture medium is used (Monteiro et al., 2000; Staikidou et al., 2006).

Essential mineral elements and other medium components, namely vitamins, organics, and plant growth regulators, are required for in vitro growth of cultures. The interactions between these medium components make the optimization of the culture medium a highly complex and time-consuming process. Historically, various time-consuming tissue culture medium development methods have been used. For instance, to optimize White's (1942) basal medium, Hildebrandt et al. (1946) applied the 'triangulation' method and used more than 16,000 tobacco and sunflower explants. It took 5 years to develop a defined medium [Murashige and Skoog (MS) medium] for tissue culture of the model plant *Nicotiana tabacum* (Murashige and Skoog, 1962). In order to decrease large factorial combinations, in a 'broad spectrum experiment' De Fossard et al. (1974) grouped all medium components into 4 categories. They tested each category at 3 levels (low, medium, and high) and used $3^4 = 81$ culture media combinations. These time-consuming and laborious methods are not feasible when the species being tested is recalcitrant, or when for various reasons the number of explants is limited (Nas and Read, 2004a).

Two main strategies have frequently been used to optimize composition of the culture medium. The first approach is an empirical, time-consuming process that relies on trial and error. Using this approach, modifications

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of a few basic media formulations have extensively been used for the culture of divergent species. However, especially recalcitrant species are highly discriminating for composition and ionic strength of the medium. Additionally, a medium optimized for a particular species may not be favorable for the culture of unrelated ones (Nas and Read, 2004a). A variation of the above-mentioned method was used to regulate callus growth of *Citrus sinensis* L. Niedz and Evens (2007) divided medium salts into 5 groups and tested 48 treatment combinations by applying an '*n*-dimensional experimental design space model' to precisely characterize the salt levels of the culture medium.

The second medium development method composes the level of medium components based on nutrient analysis of various plant tissues. Nutrient analyses of whole plants (Morard and Henry, 1998), leaves (Monteiro et al., 2000; Goncalves et al., 2005), in vitro-grown shoots (Ashrafi et al., 2010), developing seeds (Litvay et al., 1981), bulbs (Staikidou et al., 2006), and mature seeds (Nas and Read, 2004a) have been used for the formulation of the media.

Other methods that use uncommon experimental designs (Nas et al., 2005) or special computer technologies (Gago et al., 2011) have also been employed. These approaches require reasonable statistical experience to properly design the unusual experiments and/or the skill to use special software. Thus, they may be useful, but it is unlikely that they will be practiced worldwide.

To limit the number of repeated attempts and avoid the use of time-consuming factorial treatments, it was suggested that the amounts of mineral and organic components in the culture medium for micropropagation of a given species should be similar to their relative amounts naturally found in mature seeds of that species. Micropropagation of mature hazelnut, a recalcitrant species, provided convincing results for this hypothesis (Nas and Read, 2004a). However, in order for other species to be applicable, the need for more substantiating evidence for this approach was also highlighted.

The objectives of this research were to redemonstrate the reliability of the Nas medium development hypothesis (Nas and Read, 2004a) for other important tree nuts, and, in accordance with this hypothesis, to develop a culture medium suitable for micropropagation of almond (*Prunus dulcis* (Mill.) D.A.Webb).

2. Materials and methods

2.1. Plant materials and culture establishment

Almond stock plants were established by micrografting (splice graft) semihardwood scions taken from mature trees on almond seedlings of 1.5–2 months old growing in a greenhouse. Cultures were initiated using shoot tips and nodal explants taken from actively growing 6-month-old stock plants of almond cultivars (Christo Morto, Ferraduel, Ferragnes, and Nonpareil) and 1-year-old peach × almond hybrid rootstock GF677.

Explants were surface disinfested with 15% sodium hypochlorite solution (1% active chlorine) + 10 drops of Tween-20 for 15 min and rinsed 3 times with sterile, distilled water. Nas and Read (2004a) medium containing 0.05 µM indole-3-butyric acid (IBA), 4.44 µM benzyladenine (BA), and 30 g L^{-1} sucrose was used as the culture medium. The pH was adjusted to 5.5, and then 6.0 g L^{-1} agar (Merck microbiological) was added and the medium was autoclaved at 121 °C and 1.2 kgf cm^{-2} for 15 min. Individual shoot tip and nodal explants were cultured in 15 × 250-mm glass test tubes containing 14 mL of medium and kept in the dark for 1 week. Cultures were then subjected to a 16/8-h (light/dark) photoperiod under cool-white fluorescent light at 80 µmol m^{-2} s^{-1} in a growth chamber (23–25 °C) for 3 weeks. One month after culture establishment, apparent microbial contamination-free explants were subcultured on fresh medium in Magenta GA7 vessels (Magenta Corp., Chicago, IL, USA), kept in a growth chamber (23–25 °C) for 1 month with a 16/8-h (light/dark) photoperiod under cool-white fluorescent light at 80 µmol m^{-2} s^{-1}.

2.2. Formulation of the new medium

The new medium was formulated according to the Nas medium development hypothesis (Nas and Read, 2004a). Minerals and vitamins were estimated based on presented composition of raw almond kernels (Kelly, 1997; USDA, 2002, 2005, 2007). The US Department of Agriculture (USDA) database does not list molybdenum content in almonds (or any other foods) and there are only a few web sites that present approximate amount of molybdenum in almond seeds (www.diet.com). As the starting point, except for nitrogen, the amounts of minerals and vitamins found in 100 g of raw almond kernels (Kelly, 1997; USDA, 2002, 2005, 2007) were chosen as ingredients for 1 L of medium. To avoid the complexity of medium formulation, amino acids (except glycine), carbohydrates, and oil found in the kernels were not included in the medium. The amount of Mo was estimated based on Mo content in 100 g of almond seeds presented on a web site (www.diet.com). To determine the amount of nitrogen, the amount of protein found in 100 g of seeds was divided by a conversion factor of 5.18 for almonds (Greenfield and Southgate, 2003). Roughly 15% of the nitrogen found in 100 g of seeds was chosen as the amount of nitrogen to be used in 1 L of medium (Nas and Read, 2004a). Assuming that phytic acid represents 70% of total phosphorus (Raboy, 1997) in almond seeds, the amount of phytic acid was estimated based on the amount of phosphorus found in 100 g of almond seeds, and the amount of myo-inositol was estimated based on that of the phytic acid.

2.3. Determining the best strength of the new medium

Shoot tip explants were taken from stabilized cultures that were subcultured 4 times on Nas and Read medium (NRM). Preliminary experiments were conducted with 3 strengths of the new medium composition:

1) 0.5× = minerals and vitamins found in 50 g of almond seeds,
2) 0.75× = minerals and vitamins found in 75 g of almond seeds, and
3) 1× = minerals and vitamins found in 100 g of almond seeds.

The 0.75× strength of the new medium was found superior for the growth of cultures and it was chosen for comparison of culture media. Thus, except for nitrogen, the amounts of minerals and vitamins found in approximately 75 g of almond seeds were chosen as ingredients for 1 L of the new medium. The amount of nitrogen used in the new medium was roughly about 15% of that found in 75 g of raw almond seeds.

2.4. Comparison of culture media

Two independent experiments were conducted to find out whether the new medium, which was formulated according to the medium development hypothesis, would be more suitable for micropropagation of almond than the other commonly used culture media.

2.4.1. Experiment I

Cultures that were subcultured 6 times on NRM containing 4.44 μM BA, 0.05 μM IBA, and 30 g L^{-1} sucrose were used as the explant source. Using approximately 0.5-cm-long shoot tips, the growth of cultures on new medium was compared to that on tobacco medium (MS; Murashige and Skoog, 1962), Woody Plant Medium (WPM; Lloyd and McCown, 1980), Driver and Kuniyuki (1984) walnut medium (DKW), and Nas and Read (2004a) hazelnut medium (NRM). All media were supplemented with 4.44 μM BA, 0.05 μM IBA, and 30 g L^{-1} sucrose. After adjustment of pH (5.5), 5.5.0 g L^{-1} agar (Merck microbiological) was added to the media. The media were autoclaved at 121 °C and 1.2 kgf cm^{-2} for 15 min. After autoclaving, filter-sterilized vitamins of each medium were added to a corresponding medium and 80 mL of medium was distributed into Magenta GA7 vessels. The whole experiment was conducted 3 times by using explants randomly taken from shoots grown on each medium being tested and subculturing explants on the same fresh media for another 35-day subculture period. The cultures were subjected to a 16/8-h (light/dark) photoperiod under cool-white fluorescent light at 80 μmol m^{-2} s^{-1} in a growth chamber (23–25 °C).

2.4.2. Experiment II

In Experiment I, cultures of some cultivars could not be sustained on MS and WPM. When apparent contamination-free explants of some cultivars were subcultured, bacterial contamination became evident. Cultures were reinitiated but the problem persisted, and each time after the second or third subcultures, microbial contaminations emerged. Because of too many missing data, MS and WPM had to be excluded from statistical analysis (see Section 3). However, for an unquestionable comparison of media an experiment with no missing data would be desired, and thus Experiment II was performed.

After obtaining the results of Experiment I, some components of the new medium were slightly revised (with decimals rounded to the nearest value), and the finalized new medium was referred as Nas Almond Medium (NAM) (Table 1). Explants were taken from cultures that had been subcultured 10 times. These cultures were growing on NAM for the last 3 subcultures. Unless mentioned otherwise, explants, culture conditions, and experimental design were the same as in Experiment I. The whole experiment was conducted 3 times. To avoid potential carry-over effect of NAM on culture growth, data obtained from the first subculture on media being tested were ignored. For statistical analysis, data obtained from the last 2 subcultures were used.

2.5. Rooting of microshoots and acclimatization of plantlets

Half-strength (0.5×) NAM was used as the root induction medium. Microshoots (≥1.5 cm) were transferred to the root induction medium containing 2.5, 4.9, 7.4, or 9.8 μM IBA for 3 weeks. The rooting experiment was conducted 4 times, and each time at least 2 replications (Magenta GA7 vessels containing 4–9 shoots) of each genotype were used. The rooting and acclimatization procedures reported by Nas et al. (2012) were applied.

2.6. Experimental design and analysis of data

Four shoot tips of each cultivar taken from shoots grown on NRM (Experiment I) or on NAM (Experiment II) were cultured in separate Magenta GA7 vessels containing 80 mL of the medium being tested. The micropropagation experiments (Experiment I and Experiment II) were completely randomized designs. Five cultivars (Christo Morto, Ferraduel, Ferragnes, Nonpareil, and GF677) were used and 3 replications of each cultivar (Magenta GA7 vessels each containing 4 shoot tips) were randomly applied to each medium, and culture vessels were randomly placed on the growth shelves. The cultures were subjected to a 16/8-h (light/dark) photoperiod under cool-white fluorescent light at 80 μmol m^{-2} s^{-1} in a growth chamber (23–25 °C).

At the end of 35-day culture periods, shoot lengths and shoot numbers were recorded, and the appearance of cultures was visually evaluated. Data for explants in a culture vessel were divided by the number of explants, and the mean shoot lengths and mean shoot numbers were used

Table 1. The composition of Nas Almond Medium (NAM) in comparison with DKW, MS, NRM, and WPM.

Chemical composition	NAM	NRM	MS	DKW	WPM
Macrominerals (mg L^{-1})					
NH_4NO_3	900	530	1650	1416	400
$Ca(NO_3)_2 \cdot 4H_2O$	1050	700	-	1960	556
$CaCl_2 \cdot 2H_2O$	45	90	440	147	96
$MgSO_4 \cdot 7H_2O$	2050	1650	370	740	370
KNO_3	250	550	1900	-	-
KH_2PO_4	1550	1300	170	259	170
$KHSO_4$	-	-	-	1560	990
Microminerals (mg L^{-1})					
H_3BO_3	11	6.5	6.5	4.8	6.2
$CuSO_4 \cdot 5H_2O$	3.2	2.5	0.025	0.25	0.25
$MnSO_4 \cdot H_2O$	6	20	16.9	33.5	22.3
$Na_2MoO_4 \cdot 2H_2O$	0.1	2.5	0.25	0.39	0.25
$ZnSO_4 \cdot 7H_2O$	11	8.8	8.6	-	8.6
$Zn(NO_3)_2 \cdot 6H_2O$		-	-	17	-
Sequestrene 138 Fe / or Sequestrene 330 Fe	100 / 50	100	-	-	-
$FeSO_4 \cdot 7H_2O$	-	-	27.8	33.4	27.8
$Na_2 \cdot EDTA$	-	-	37.3	44.7	37.3
KI	-	-	0.83	-	-
$CoCl_2 \cdot 6H_2O$	-	-	0.025	-	-
Vitamins (mg L^{-1})					
Thiamine-HCl (B_1-HCl)	0.2	0.6	0.1	2.0	1.0
Riboflavin (B_2)	0.65	0.21	-	-	-
Nicotinic acid (B_3)	2.8	1.15	0.5	2.0	0.5
Pyridoxine-HCl (B_6-HCl)	0.13	0.60	0.5	-	0.5
α-Tocopherol (E)	19.5	20	-	-	-
Ascorbic acid (vitamin C)	0.75	1.0	-	-	-
Glycine	1.13	0.85	2	2.0	2.0
myo-Inositol	250	200	100	1000	100

for statistical analysis. Productivity, another important growth parameter (a measurement of total shoot length), was estimated as the mean number of shoots per cultured explant × mean shoot length (Gago et al., 2011), and it was also subjected to statistical analysis. The analysis of variance was completed using the SAS PROC GLM (SAS Institute Inc., Cary, NC, USA). Separation of means was done by Fisher's least significant difference test at $P \leq 0.05$.

3. Results

3.1. Comparison of culture media

3.1.1. Experiment I

When in vitro cultures of woody plants are initiated from mature tissues, hyperhydricity, browning, and phenolic oxidation of explants usually become obstacles. In the current study, no browning or phenolic oxidation of explants was observed and in vitro cultures of all cultivars

were easily established. The only problem encountered during establishment of almond cultures was microbial contamination. When apparent contamination-free explants of some cultivars were subcultured, bacterial contamination became evident. To alleviate the contamination problem, cultures were reinitiated. The problem persisted and each time after the second or third subcultures, microbial contaminations emerged. Apparently, these contaminants were endogenous, and in the presence of these contaminants the cultures grew well on NRM. The contaminants gradually disappeared when shoot tip explants were continuously subcultured on copper-rich NRM or NAM (data not shown).

Interestingly, in Experiment I, growth of microbial contaminants on different culture media varied. On MS and WPM, microbial contaminants became prevalent. Even if only shoot-tip explants were used, subculturing of these contaminated cultures could not be sustained. Compared to MS and WPM, the growth of microbial contaminants on DKW was relatively low. The least contaminant growths were observed on NRM and the new medium, both of which contained high amounts of $CuSO_4 \cdot 5H_2O$ (data not shown). Because of high contamination, there were too many missing data on MS and WPM and some missing data on DKW, NRM, and the new medium. The culture growth on MS and WPM could not properly be analyzed. Thus, the results of Experiment I will not further be discussed here.

3.1.2. Experiment II

For convincing results, data should be complete or the number of missing data should be minimal. Therefore, to ensure an unquestionable media comparison, Experiment II was performed. It was feasible to gradually minimize and eventually eliminate contamination by subculturing only shoot-tip explants. Using Cu-rich NAM and repeatedly subculturing only shoot-tip explants, apparent contamination-free cultures of all tested cultivars were obtained at the end of the 10th subculture (at the beginning of Experiment II).

Medium ($P = 0.001$) was the most important factor that affected the number of shoots per cultured explant and the highest mean shoot numbers were obtained when the explant were cultured on NAM. The effects of cultivar ($P = 0.53$) and cultivar × medium interaction ($P = 0.08$) on the mean shoot numbers were nonsignificant. Considering all genotypes, per cultured explant 3–10 shoots were obtained. Mean shoot numbers were 9.5 for NAM, 7.5 for NRM, 6.3 for WPM, 5.3 for MS, and 2.8 for DKW. A 'perfect medium' is expected to produce similar and reasonable numbers of shoots for the majority of genotypes. When NAM was used, mean shoot numbers were always ≥8.0 for all cultivars. On 2 cultivars in both NRM and WPM and all but 1 on MS, ≤6.0 shoots were obtained per cultured explant. With respect to mean shoot numbers, the orders of cultivars cultured on different media were generally similar. However, the difference between the highest and the lowest shoot numbers of cultivars obtained on a particular medium varied. The difference between the highest and the lowest shoot numbers obtained on NAM was 2.1 (10.3 – 8.2), on NRM was 4.6 (9.8 – 5.2), on MS was 5.3 (8.6 – 3.3), and on WPM was 5.9 (8.2 – 2.3). The least mean shoot number differences among cultivars were obtained on DKW, but mean shoot number never exceeded 4 when this medium was used (Figure 1A).

Medium ($P = 0.001$), cultivar ($P = 0.001$), and their interaction ($P = 0.042$) significantly affected mean shoot length. As in shoot number, NAM was the best medium for production of longer shoots. Considering all cultivars, the mean shoot length was 2.3 cm for NAM, 2.1 cm for DKW, 2.0 cm for NRM, 1.8 cm for MS, and 1.2 cm for WPM. Shoot lengths of GF677 observed on all media were usually ≤1.5 cm. Most of the cultivars produced the longest shoots when NAM was used. Mean shoot lengths of some cultivars on DKW were similar to that on NAM, but this can be ascribed to the low number of shoots produced on DKW. The cultivar × medium interaction indicated that when an inappropriate culture medium (i.e. WPM) was used, even the shoots of the cultivar that produced the longest shoots on the other media (Ferragnes) did not elongate and none of the cultivars developed long shoots. On a suboptimal medium (i.e. MS) only some cultivars developed long shoots. However, when the optimized culture medium (NAM) was used, all cultivars (except hybrid GF677) developed long shoots (Figure 1B). Thus, NAM was the only medium that provided high numbers of long shoots for all cultivars.

Both medium ($P = 0.001$) and cultivar ($P = 0.003$), but not their interaction ($P = 0.3097$), had great effects on productivity. As the result of being the only medium that provided a high number of longer shoots for all cultivars, NAM was by far the most productive medium. Considering all genotypes, the mean productivity was 21.8 for NAM, 14.2 for NRM, 11.1 for MS, 6.9 for DKW, and 6.7 for WPM. When NAM was used, all 4 almond cultivars were much more productive compared to the other media. Interestingly, the productivity of almond × peach hybrid rootstock GF677 on NAM was significantly lower than that of almond cultivars cultured on the same medium. However, even this low productivity of GF677 on NAM was higher than those observed on the other media, except on NRM. On MS, only the most productive cultivar, Ferragnes, was able to exhibit its growth performance. In general, MS, DKW, and WPM were nonproductive for the almond cultivars tested. NRM, the medium that was developed based on seed composition of hazelnut, was less productive than NAM but more productive than MS, DKW, and WPM (Figure 1C).

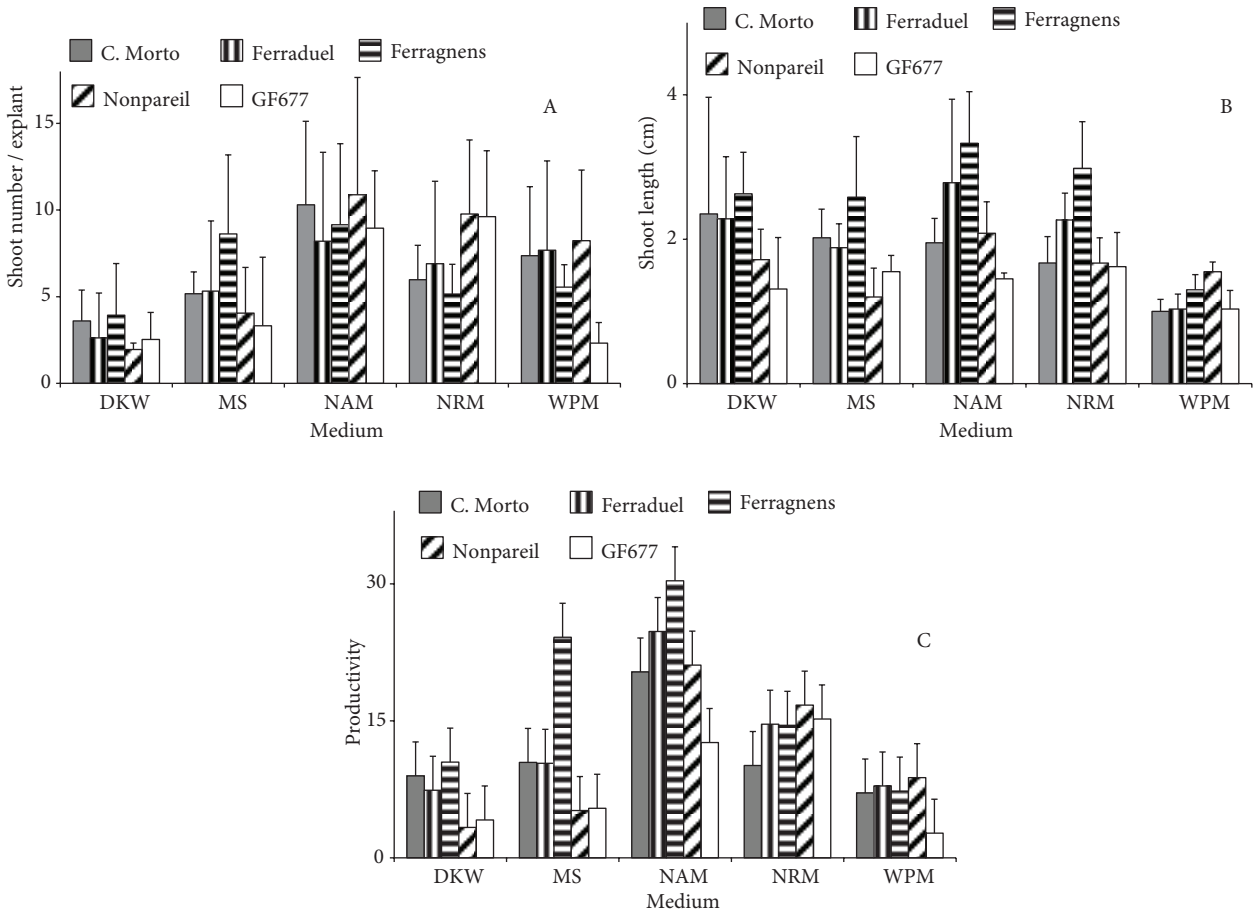

Figure 1. (A) Mean numbers of shoots obtained per cultured explant, (B) mean shoot lengths, and (C) mean productivity of almond cultivars cultured on NAM, NRM, MS, DKW, and WPM. Bars represent standard deviations (A and B) and standard errors (C).

3.2. Rooting of microshoots and acclimatization of plantlets

The rooting rate of GF677 microshoots was usually over 80%. However, rooting rates of almond microshoots were unsatisfactory. Only a few (about 2%) microshoots of Nonpareil rooted. The highest rooting rates of microshoots were 33% for Christo Morto and Ferraduel, 23% for Tuano, and 13% for Ferragnes, respectively (data not shown). Rooted microshoots were successfully acclimatized to the greenhouse conditions and then they were transplanted to the field. The performance of own-rooted (micropropagated) almond cultivars in comparison with their grafted counterparts are currently under further investigation in the field.

4. Discussion

The use of an optimized culture medium is one of the most important factors that greatly influence the success of micropropagation. Unfortunately, because of interaction between medium components, it is very difficult to determine the optimum levels of minerals and organics used in the culture medium. Moreover, total ionic strength of the medium affects explant morphogenic reactions (callogenesis, somatic embryogenesis, regeneration, and micropropagation), and this makes the medium optimization process more complex and more difficult. The failure of early tissue culture studies up to early 1960s was to a large extent due to the lack of a suitable tissue culture medium. Historically, time-consuming and laborious methods have been applied to optimize the culture medium (White, 1942; Hildebrandt et al., 1946; Murashige and Skoog, 1962; De Fossard et al., 1974). Nevertheless, we do not have a universal approach that can be used either to develop or to modify a micropropagation medium for a large number of species. As a result, to optimize a culture medium numerous studies have been repeated.

Some researchers have suggested deciding the level of medium components based on composition of growing whole plants or developing tissues (Litvay et al., 1981; Morard and Henry, 1998; Monteiro et al., 2000; Goncalves et al., 2005; Staikidou et al., 2006; Ashrafi et al., 2010). However, the nutrient contents of growing plants and

developing tissues are much influenced by growing conditions (Marschner, 1998). Thus, the composition of the medium components based on the analysis of growing plants or developing tissues may not always be appropriate. The method described here offers several benefits over the others:

- Seed compositions of many important fruit and crops are already known. In such cases there is no need for seed nutrient analysis (for the rationale of the hypothesis, see Nas and Read, 2004a).
- It is a short-cut to a long-distance approach. It considers all nutrients as a single group and tests only several strengths of the whole group.
- It avoids time-consuming factorial treatments, reduces the size of experiments, and requires fewer explants and less labor.
- Unlike the other developing tissues, mature seeds are much less affected by growing conditions.

To decrease micropropagation cost, usually high mean numbers of shoots per cultured explant are desired. Additionally, if node explants are going to be used, longer shoots containing more axillary buds (nodes) are preferred. Longer shoots are also more suitable for rooting and handling. Eventually, the potential multiplication rates are determined by the total number of axillary buds and shoot tips used as the explants. On the other hand, the total number of axillary buds usually depends on the productivity (total shoot length). Therefore, a superior micropropagation medium should provide a higher mean number of shoots per cultured explant, produce longer shoots, and be more productive than any commonly used 'standard' medium. With respect to the 3 growth parameters (shoot length, shoot number per cultured explant and productivity), NAM was superior to NRM, MS, DKW, and WPM. Combined mean shoot number for all cultivars observed on NAM was up to 21%, 35%, 44%, and 71% higher than those observed on NRM, WPM, MS, and DKW, respectively.

A significant cultivar × medium interaction effect on mean shoot length deserves a closer look at individual cultivars cultured on different media. NAM was the best medium for production of longer shoots. Mean shoot lengths of all cultivars observed on NAM were always greater than or similar to those observed on the other media. Some cultivars (i.e. GF 677) did not develop long shoots on any media tested and mean shoot length of some cultivars on DKW and NRM were similar to those observed on NAM, but this was primarily due to the lower number of shoots produced on DKW and NRM compared to that on NAM. The interaction indicated that when an unsuitable culture medium (i.e. WPM) was used, none of the cultivars developed long shoots, and on a suboptimal medium (i.e. MS), only some cultivars developed long shoots. However, when the optimized culture medium NAM was used, all cultivars (except hybrid GF677) developed long shoots (Figure 1B). Thus, NAM was the only medium that provided high numbers of long shoots for all cultivars. Considering all cultivars, shoots developed on NAM were up to 11%, 12%, 20%, and 48% longer than those on DKW, NRM, MS, and WPM, respectively.

A 'perfect medium' is expected to produce high numbers of long shoots for the majority of genotypes. When some cultivars do not develop long shoots on any media and some cultivars develop reasonably long but few shoots on only some media being tested, it becomes more difficult to choose the 'right medium'. In such cases, productivity may become a helpful growth parameter to decide the best medium. Higher productivity indicates a high number of long shoots that contain more axillary buds and that are more suitable for handling. As the result of being the only medium that provided a high number of long shoots for all cultivars, NAM was by far the most productive medium. The differences between productivities observed on different media clearly showed that NAM was the most suitable medium for almond micropropagation. Combined mean productivity for all cultivars observed on NAM was up to 35%, 49%, 68%, and 69% higher than those observed NRM, MS, DKW, and WPM, respectively.

The proportions of medium components may be more important than their absolute concentrations (Pinto et al., 2008) and interactions could considerably be minimized when an optimized culture medium is used (Nas and Read, 2004a). A strong species × medium interaction effect on bulblet production was related to important differences between mineral compositions of *Galanthus* species; this difference suggested that it might be better to calculate separate formulae for individual species (Staikidou et al., 2006). In the current study, the differences in growth among almond cultivars on NAM could be a result of their ability to utilize nutrients. It might also indicate the need to slightly change the ionic strength of the medium for individual cultivars. Changing the ionic strength of the medium will not change the proportions of nutrients in the medium and it is in agreement with the hypothesis upon which NAM was developed.

The absolute concentrations of nutrients in media tested and their proportions to those found in the almond seeds are shown in Table 2 and Figure 2. The amount of nitrogen in NAM is 15.5% of that found in 75 g of almond kernels, and this is consistent with the medium development hypothesis (Nas and Read, 2004a). Excluding Fe and Mo, the amounts of all the other components in NAM are proportional to nearly 100% of those found in the almond seeds (Table 2). Since Fe is an immobile element in plants, its deficiency appears in young leaves and newly formed

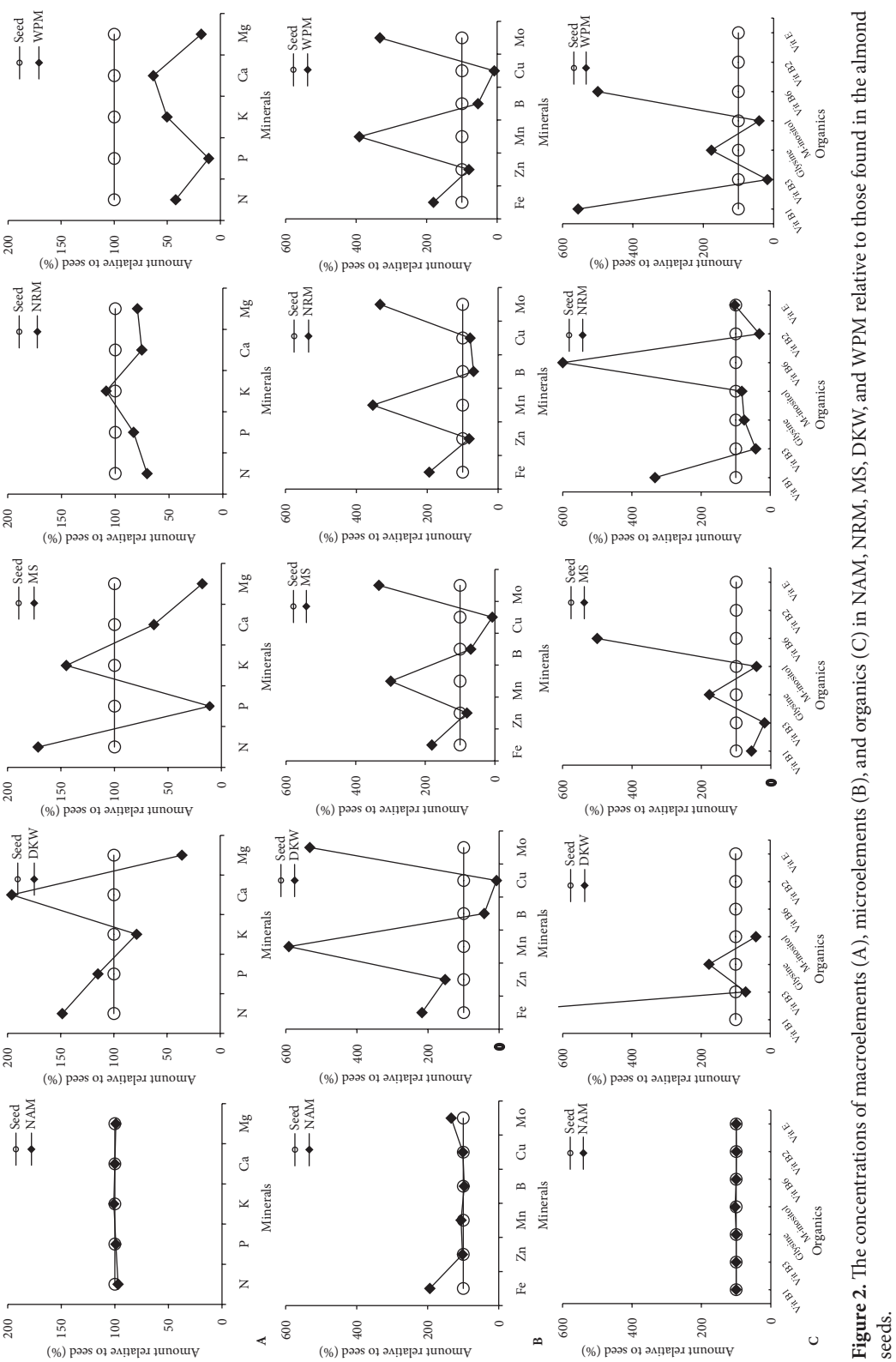

Figure 2. The concentrations of macroelements (A), microelements (B), and organics (C) in NAM, NRM, MS, DKW, and WPM relative to those found in the almond seeds.

Table 2. Comparison of NAM, NRM, MS, DKW, and WPM nutrient compositions with the nutrient contents of almond seeds.

Nutrient	Mean concentrations (mg 75 g^{-1} seed)	Nutrient concentrations in the media (mg L^{-1}) and [their proportion (%) to those found in the 75 g of almond seeds]				
		NAM	NRM	MS	DKW	WPM
N	3064	474 [15.5]	345 [11.3]	840 [27.4]	728 [24]	206 [6.72]
Ca	190	190 [100]	143 [75.3]	120 [63.2]	372 [196]	120 [63.2]
Fe	3.09	6 [194]	6 [194]	5.6 [181]	6.71 [217]	5.6 [181]
Mg	205	202 [99]	163 [79.5]	36.5 [18]	73 [36]	36.5 [18]
P	358	353 [99]	296 [83]	38.7 [11]	412 [115]	38.7 [11]
K	540	542 [101]	586 [108.5]	784 [145]	424.4 [78.6]	271 [50.2]
Na	0.75					
Zn	2.46	2.502 [102]	2.0 [81.3]	1.96 [80]	3.74 [152]	1.96 [80]
Cu	0.81	0.814 [101]	0.64 [79]	0.06 [7.4]	0.064 [7.9]	0.064 [7.9]
Mn	1.84	1.95 [106]	6.5 [353]	5.5 [299]	10.9 [592]	7.2 [391]
B	1.98	1.92 [97]	1.37 [69.2]	1.37 [69.2]	0.84 [42.4]	1.08 [54.5]
Mo	0.03	0.04 [134]	0.99 [3300]	0.1 [333]	0.16 [533]	0.1 [333]
Thiamine	0.18	0.18 [100]	0.6 [333]	0.1 [55.6]	2.0 [1111]	1.0 [556]
Riboflavin	0.66	0.65 [99]	0.21 [32]	-	-	-
Niacin	2.81	2.8 [100]	1.15 [42]	0.5 [17.8]	2.0 [71.2]	0.5 [17.8]
Pyridoxine	0.1	0.1 [100]	0.6 [600]	0.5 [500]	-	0.5 [500]
α-Tocopherol	19.5	19.5 [100]	20 [103]	-	-	-
Ascorbic acid	-	0.75 [-]	1.0 [-]	-	-	-
Glycine	1.13	1.13 [100]	0.85 [75.2]	2.0 [177]	2.0 [177]	2.0 [177]
myo-Inositol	243	250 [103]	200 [82.3]	100 [41.2]	1000 [412]	100 [41.2]

shoots. High numbers of long shoots were produced on NAM, and towards the end of subcultures, the shoots became chlorotic if Fe was used at 3.09 mg L^{-1} (in proportion to that found in almond seeds). The chlorotic shoot formation can be explained by the fact that Fe in the medium was mostly removed, or that physical (i.e. light; Papathanasiou et al., 1996) and chemical (i.e. high P; Shibli et al., 2001; Ramage and Williams, 2002) environments in the tissue culture may have caused Fe to become less available. To avoid shoot chlorosis, the amount of Fe in the NAM was raised to 6 mg L^{-1}. This amount of iron was close to that used in the other media tested. The amount of Mo used in the NAM was considerably lower than that used in the other media. However, it should be noted that the Mo content in almond seeds was unknown and the amount of Mo used in NAM was based on the approximate amount presented on a web site (www.diet.com), not from a scientific database.

With respect to shoot number, shoot length, and productivity, best results were obtained on NAM that contained nutrients in proportions similar to those found in the almond seeds. When all medium components are analyzed, obviously NRM is the only medium that has a composition somewhat closely related to that of NAM (Figure 2), and more or less it granted better results compared to MS, DKW, and WPM. WPM was the medium least suitable for almond micropropagation and the amounts of its components are considerably different from those of NAM. The 4 tested media contained a higher amount of Mn but a lower amount of Mg than NAM. Additionally, along with NRM, NAM contains much more Cu than MS, DKW, and WPM. The benefits of using higher amounts of Cu in the culture medium have been presented for both herbaceous and woody plants (Sahrawat et al., 1999; Nas and Read, 2004a; Staikidou et al., 2006). On the other hand, microbial contaminants may survive in vitro for years and they can hold back or promote culture growth. Furthermore, the composition and concentration of contaminants in vitro may vary over time depending on the plant species cultured (Ulrich et al., 2008) and perhaps on the culture medium used. The results of this study also indicated that at least some microbial contaminants could be eradicated if culture media contained high amounts of $CuSO_4$. No consistent patterns can be seen when components of MS and DKW are analyzed in comparison with that of NAM. These results support the hypothesis that the nutrients in proportions similar to those found in the seed composition could provide an optimum micropropagation medium.

Almond is a species that is difficult to root and even the rooting of microshoots is low (Rugini and Verma, 1982; Channuntapipat et al., 2003). Increasing the number of subcultures usually improves rooting of microshoots, but depending on species and cultivars, the required numbers of subcultures vary (Economou and Read, 1986; Nas and Read, 2004b). In the current study, subculturing up to 13 times did not significantly increase rooting and the low rooting ratio remains a problem that limits micropropagation of mature almond cultivars. The low rooting ratios previously reported (Rugini and Verma, 1982; Channuntapipat et al., 2003) and presented here indicate that almond is genetically a species that is difficult to root. To induce rooting, usually microshoots are cultured on a medium containing auxins such as IAA, IBA, or NAA. It might be useful to investigate other methods that may promote rooting of microshoots.

In conclusion, the optimization of culture medium is one of the most important factors that greatly affect the micropropagation success. Since medium optimization is a laborious, time-consuming, complex, and difficult process, numerous studies have been repeated to optimize the culture medium. There are many economically, environmentally, and pharmaceutically important species that need to be micropropagated in vast numbers. Thus, it is necessary to develop a new medium or modify a known one for the culture of almost each individual species. Previous results (Nas and Read, 2004a) and the results presented here substantiate the hypothesis that formulating the composition of a culture medium based on the seed content could become a straightforward universal method of medium development for micropropagation of higher plants.

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