Hindawi
International Journal of Agronomy
Volume 2023, Article ID 3581881, 7 pages
https://doi.org/10.1155/2023/3581881

Research Article

Optimization of Hormonal Compositions of Media in In Vitro Propagation of Orange Cultivars from Shoot Tip Nodal Segments

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Received 14 April 2022; Revised 7 September 2022; Accepted 19 June 2023; Published 28 June 2023

Academic Editor: Maria Serrano

Orange is one of the most horticulturally important and widely cultivated *Citrus* species. *Citrus* cultivar improvement via conventional breeding strategies is normally impeded by factors related to its reproductive biology. The present study was undertaken to investigate the optimization of growth regulators' composition of media in *in vitro* propagation of orange cultivar from nodal segment explants. The nodal segment explants were collected from sweet orange cultivars. The MS medium supplemented with sucrose and different concentrations of growth regulators were used for shoot proliferation and root induction. The optimum compositions of growth regulators in MS medium were assessed. The result indicated that the highest shoot response was recorded for Washington naval orange with maximum shoot proliferation rate (99.75%), shoot number per explant (3.10), shoot length (10.70 cm), leaf number per explants (12.50) after three weeks of culture. In all experiments, no growth was observed for the basal MS medium. Phytohormones combinations of indole-3-acetic acid, IAA (1.2 mg/L), and kinetin (2.0 mg/L) were found to be the best for shoot proliferation. Among the cultivars, significantly, the highest rooting rate (81.25%), root number (4.95), and root length (2.95 cm) were recorded for Washington naval orange cultivar. The least rooting rate (48.45%), root number (3.55), and root length (2.26 cm) were observed from the Valencia cultivar. Significantly, the highest rooting rate (84.90%), root number per microshoot (5.20), and root length (3.05 cm) for MS medium supplemented with 1.5 mg/L 1-naphthalene acetic acid (NAA). The comparison of different concentrations of IAA and NAA on root induction of microshoots from nodal segments of sweet orange cultivars demonstrated NAA as the more effective hormone than IAA.

1. Introduction

The *Citrus* can be propagated by conventional methods like budding and grafting. Therefore, there is a possibility of virus transmission from the mother plant to the propagated plant. Despite substantial genetic diversity and interspecific fertility, the genus *Citrus* includes some of the most difficult species to breed [1]. This is often due to several obstacles to conventional breeding including large plant size, self- and cross-incompatibility and pollen and/or ovule sterility, extended juvenility, and especially to nucellar embryony, and high polyembryony since sweet oranges generally contain one to several adventive nucellar embryos, and most species are highly heterozygous and produce progeny that segregates widely for several characters when crosses are made. Genetic transformation is an alternative to overcome these difficulties. For a successful transformation, regeneration of whole plants from the transformed cells may be a prerequisite [2].

Sweet orange (*Citrus sinensis* (L.) Osbeck) is the most grown *Citrus* species [3]. Zygotic sweet orange hybrids are difficult to obtain, are often weak, and do not produce fruit that resembles sweet orange. It is generally accepted that commonly grown sweet orange cultivars probably originated from the selection of a chance seedling well-adapted to a particular area or a mutation in a particular cultivar or seedling [2, 4]. Mutations visible as bud or limb sport or sectors on chimeric fruits occur frequently in citrus [5].

A great number of viruses and other graft transmissible infectious agents were known to affect and endanger the citrus industry worldwide [6]. Obtaining pathogen-free citrus plants is one of the most important steps in the

citrus breeding program. Numerous methods are developed to recover virus-free plants. Shoot-tip grafting is the most effective technique for the elimination of all major viruses and virus-like pathogens, including those not eliminated by thermotherapy. Plants obtained by shoot tip grafting are true-to-type and do not have juvenile characters. Thus, these plants might be used for budwood production after they are indexed [7].

Sustainable development of the citrus industry is especially dependent on the continuous supply of new and improved cultivars. The genetic improvements of perennial woody fruit crop plants often take a few years using traditional plant-breeding methods [8]. Plant tissue culture made it easy to enhance citrus against different abiotic stresses, low yield, and conserve important citrus genotypes through exploiting somaclonal variations, vegetative cell hybridization [9, 10], and the transformation of high-yielding cultivars and disease-free plants [11].

Micropropagation of citrus offers rapid propagation of fruit crops in limited space and time under controlled conditions around the year [12]. Multiple shoot induction and regeneration are potentially useful for the genetic improvement of fruit crops [7]. It is known that the tissues obtained from young plant parts have relatively more regenerative capacity than old tissues. The basis for bud formation in tissue culture is dependent on a specific equilibrium between the auxins and the cytokinins, gibberellins, and cytokinins ratio, which controls the shoot and leaf development [8, 13]. Sweet orange and mandarin are polyembryonic, whereas lemon is monoembryonic. These species are generally cross-pollinated. They are highly heterozygous, and zygotic embryos, whether or not produced by selfing, would differ from the maternal parents. The plant regenerated from seeds usually exhibits prolonged juvenility in citrus fruits. Although sweet orange widely grows, some introduced cultivars are being found in limited farm areas in Ethiopia. There are no well-established citrus breeding programs and seed production found in the country despite huge consumption and market demand. Therefore, this study was undertaken to investigate the optimization of growth regulators' composition of media in *in vitro* propagation of orange cultivar from nodal segment explants.

2. Materials and Methods

2.1. Sample Collection. The nodal segment explants samples were collected from three orange cultivars including Washington Naval, Valencia, and Tangelo from Tony Farm in Dire Dewa City, Ethiopia. The nodal segments close to the shoot tip were cut with scissors at about 30 cm. Then, the explant samples were brought to Plant Biotechnology Lab, in the School of Plant Sciences, Haramaya University.

2.2. Media Preparation. The MS Murashiege and Skoog [14] medium was manually prepared as per the standard procedure. The pH of the media was adjusted to 5.5–5.7 using 0.1 N HCl and 0.1 N NaOH, and finally, 0.8% (w/v) agar-agar was added as the gelling agent. Then, the agar was allowed to dissolve with the medium in microwave. Forty (40) ml of molten MS media was dispensed into each flask plugged with aluminum foil and autoclaved at 121°C and 15 lb/in^2 pressure for 15–20 min in 150 ml Erlenmeyer flasks. The sterilized medium was kept for 1-2 days before inoculations to screen for inherent contamination. The vitamins and hormone supplements were filter sterilized and poured onto the prepared MS media.

2.3. Explant Preparation and Surface Sterilization. The shoot tip nodal segments of adult orange cultivars were used as explants sources. Following the removal of leaves with sterile scissors, the nodal segments (15–20 cm in length) were washed with distilled water in 1000 ml beakers for 5 min. Then, the explants were successively surface sterilized in 75% (v/v) alcohol (30 sec), 0.1% (w/v) mercuric chloride plus 2 drops of Tween-20 per 100 ml disinfectant solution (10 min) in a laminar hood. To remove all traces of detergents and Tween-20 from the surface, explants were rinsed in sterile double distilled water 3-4 times. The exposed cut ends of explants were trimmed off with sharp secateurs to eliminate all toxic effects of mercuric chloride. All experiments were conducted in two replications.

2.4. Shoot Initiation and Proliferation. The culture media for *in vitro* shoot initiation consisted of the basic salts and vitamins of Murashige and Skoog (MS) culture medium at full strength (basal medium) supplemented with kinetin at 1.50, 2.00, and 2.50 mg/L, IAA at 1.00 and 2.00 mg/L. The media was also supplemented with 3.5 percent (w/v) sucrose (as carbon and energy source) and bactoagar at 0.8 percent (w/v) as a solidification medium. Two g/L ascorbic acids were used to minimize explant browning by adsorbing phenolics, inactivating polyphenol oxidases, and peroxidases. About 15 cm nodal segment explants were inoculated into a culture jar containing 100 ml MS growth medium with three explants per jar. The cultured jars were plugged with non-absorbent cotton and placed in a growth chamber room at 26 ± 2°C and under fluorescent light receiving 16 hrs illumination followed by 8 hrs dark period. A similar procedure was used for shoot proliferation with half-strength MS medium. Data were recorded for shoot response, the average number of shoots/explants, the average length of proliferated shoots/explants, average and number of leaves/explants after about three weeks.

2.5. Rooting of Regenerated Shoots. The basic salts and vitamins of MS at half strength were used for rooting media. During the growing period, healthy regenerated shoots were excised and transferred individually under aseptic conditions and cultured vertically in glass jars (9×4.5 cm) each containing 30 ml basal medium supplemented with 0.30, 0.50, 1.00, and 1.50 mg/L 1-naphthalene acetic acid (NAA), and 0.30, 0.50, 1.00, and 1.50 mg/L indole-3-butyric acid (IBA) were added solely or in combinations, amended with sucrose at 30 g/L and 7 g/L bactoagar. The rooting media pH was adjusted to 5.8 before the addition of agar. The

cultured jars were capped with aluminum foil and autoclaved at 121°C for 20 min, then left to cool, and harden for five days before being used. The cultured jars were incubated at 25 ± 1°C and exposed to photoperiod high light intensity for 16 hrs (1500 lux) and 8 hrs darkness.

Young shoots, when subjected to root initiating treatment, invariably callused at the base, and over some time callus mass increased and root initials were barely visible. A two-step strategy for *in vitro* rooting was adopted. In the first step, microshoots were implanted in basal MS medium supplemented with various IBA concentrations. Maximum root initiation and least callusing were observed on full-strength MS medium supplemented with 14.70 μM IBA. Exposure to root initiation medium was not allowed for more than 11 d since microshoots were overwhelmed by callus mass, and root development was arrested. Indeed, root development was apparent in course of as brief as 5 days of exposure of microshoots to the rooting medium. In the second step, immediately after root initiation, the microshoots were reimplanted on a PGR-free half-strength MS medium for root development. The root formation was maximal when organics were supplied at full strength. Data were recorded for rooting rate, root number per plantlet, and root length per plantlet after 5 weeks of culturing.

2.6. Data Analysis. The data in Tables 1–4 (supplementary materials file) about shoot regeneration and rooting were subjected to a one-way analysis of variance (ANOVA), and the differences among means were compared based on the least significant difference (LSD) *t*-test using SAS software version 9.2.

3. Results and Discussion

3.1. Optimization of MS Medium for Shoot Proliferation of Orange Cultivars. The effects of MS medium supplemented with various concentrations of indole-3-acetic acid (IAA) and kinetin on shoot proliferation rate, shoot number per explants, shoot length, and leaf and leaf number are indicated in Table 1 and Figure 1. Significantly, the highest shoot response was recorded for Washington naval orange with maximum shoot proliferation rate (99.75%), shoot number per explant (3.10), shoot length (10.70 cm), and leaf number per explants (12.50) after three weeks of culture. The last shoot responses were recorded for the Valencia cultivar with shoot proliferation rate (68.75%), shoot number per explant (1.85), shoot length (6.85 cm), and leaf number per explant (7.90).

The basal MS mediums without any supplements were used as a control. In all experiments, no growth was observed for the basal MS medium. Phytohormones combinations of IAA (1.2 mg/L) and kinetin (2.0 mg/L) were found to be the best for shoot proliferation. The shoot responses were found to be increasing with an increase in kinetin concentration with IAA concentration at 1.20 mg/L. Al-Teha et al. [15] suggested that somatic embryogenesis and plantlet regeneration were achieved in callus cultures of nucellus tissues derived from undeveloped ovules of immature fruits of local orange *C. sinensis* using half-strength MS medium supplemented with BAP and 2,4-D. The highest (70%) shoot formation was obtained from BARI Malta-1 (*Citrus sinensis*) seeds without seed coat treated with MS basal media + BAP 1.0 mg/L while kinetin showed no response for shoot formation in any supplemented concentration.

3.2. Optimization of MS Medium for Root Initiation of Orange Cultivars. The root inductions of microshoots generated for the three sweet orange cultivars were conducted by supplementing basal MS medium with phytohormones including IAA and NAA as in Tables 2 and 3. Root induction was observed after 15 days of culturing. Significant rooting rate, root number, and root length were observed for all treatments. Among the cultivars, significantly, the highest rooting rate (81.255), root number (4.95), and root length (2.95 cm) were recorded for Washington naval orange cultivar while the least rooting rate (48.45%), root number (3.55), and root length (2.26 cm) were observed for Valencia cultivar. As the concentration of IAA within the medium increases, the root responses including root initiation rate, root number, and root length also increase with maximum rooting observed at 1.5 mg/L. No root growth was observed in the control medium (MS basal medium).

Several factors have been observed to be associated with the rooting of the microshoot that includes the nature of cuttings, rooting cofactor, the synergistic role of exogenously applied growth hormone and endogenously present cofactors in the rooting, the relative efficiency of different auxins, their combination, and methods of application [16–19]. High light intensity also induces better rooting and causes hardening of the plant which renders them more tolerant to moisture stress and diseases. A low salt medium is found satisfactory for rooting of shoots in a large number of plant species [20]. The addition of activated charcoal in the root expression medium improved the overall rooting capacity in *Pinus panaster* [21].

The effect of MS medium supplemented with NAA on root response rate, root number, and root length of microshoot generated from nodal segment explants of three sweet orange cultivars is presented in Table 3. The microshoots from Washington naval orange cultivar have demonstrated significantly the highest rooting rate (84.90%), root number per microshoot (5.20), and root length (3.05 cm) for MS medium supplemented with 1.5 mg/L NAA. On the other hand, the least root response in terms of rooting rate (58.95%), root number per microshoot (4.86), and root length (2.68 cm) was recorded for the Valencia cultivar.

It was observed from the result of the present study that root response was increased as the concentration of the growth regulator increased from 0.3 to 1.5 mg/L. The comparison of different concentrations of IAA and NAA on root induction of microshoots from nodal segments of sweet orange cultivars as in Figure 2 and Table 5 (in Supplementary material) demonstrated significantly higher effects of NAA than IAA. Significant differences were observed between rooting rates due to NAA and IAA for all three

Table 1: The effects of a different combination of IAA and kinetin on shoot proliferation from nodal segment explants of three orange cultivars.

Cultivars	Half strength MS + IAA + Kn (mg/L)	Shoot proliferation rate (%)	Shoot number/explants	Microshoot length (cm)	Leaf number/explants	Initiation date
Washington naval	MS basal medium	0.00i	0.00f	0.00e	0.00h	0.00f
	1.00 + 0.00	41.50f	1.40de	5.95c	7.89def	12.00 d
	1.00 + 1.50	95.70b	2.10b	8.35b	9.15cd	9.50e
	1.20 + 2.00	99.75a	3.05a	10.70a	12.50a	8.50e
	1.20 + 2.50	97.80ab	3.10a	9.90a	10.90b	9.00e
Valencia	0.00 + 0.00	0.00i	0.00f	0.00e	0.00h	0.00f
	1.00 + 0.00	15.75h	1.15e	3.10d	5.55g	19.00a
	1.00 + 1.50	60.65e	1.20e	3.75d	6.85f	16.00b
	1.20 + 2.00	67.60d	1.85cbd	6.85c	7.90def	14.50bc
	1.20 + 2.50	68.75d	1.05e	6.55c	7.60ef	16.00b
Tangelo	0.00 + 0.00	0.00i	0.00f	0.00	0.00h	0.00f
	1.00 + 0.00	34.20g	1.25e	5.90c	7.70ef	19.00a
	1.00 + 1.50	85.70c	2.05bc	6.75c	8.60de	15.50b
	1.20 + 2.00	89.10c	2.20b	9.95a	10.60b	13.50cd
	1.20 + 2.50	87.80c	1.50cde	8.60b	10.30bc	15.55b

Means followed by the same letter within a column were not significantly different at a 0.05 probability level based on the least significance difference (LSD) test. Small letters: significance within a column.

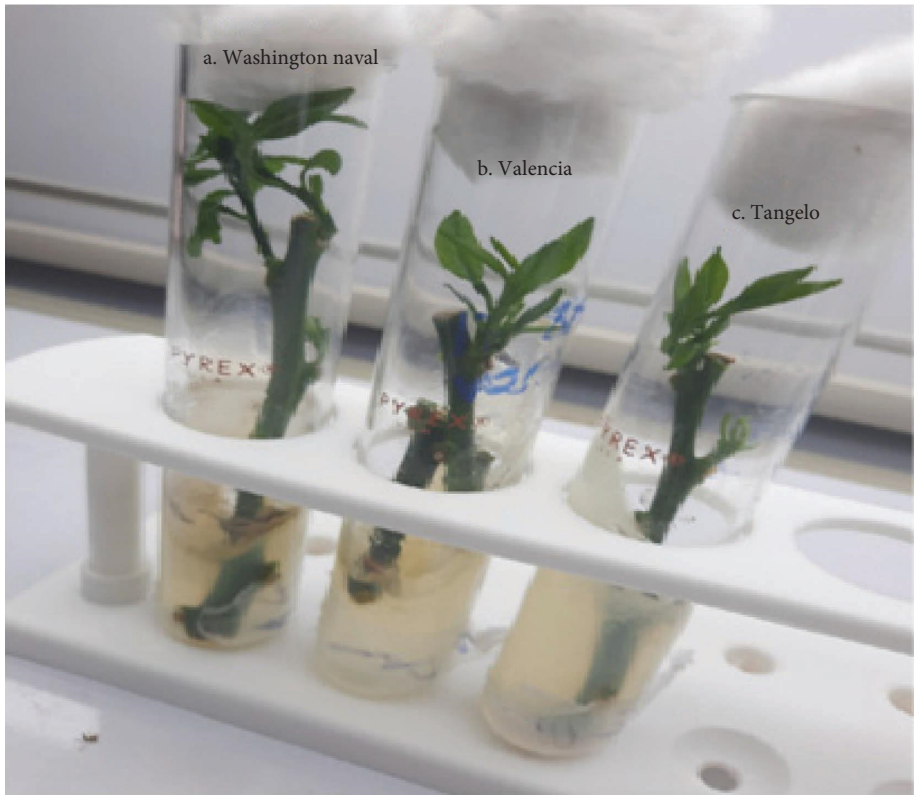

Figure 1: Regenerated microshoots after two weeks. a. Washington naval. b. Valencia. c. Tangelo.

Table 2: The effects of MS medium supplemented with IAA on root response rate, root number, and root length of microshoot generated from nodal segment explants of three orange cultivars.

Cultivars	Half strength MS + IAA (mg/L)	Rooting rate (%)	Root number per macroshoot	Root length (cm)
Washington naval	MS basal medium	0.00j	0.00h	0.00e
	0.3	51.20d	3.24e	1.78c
	0.5	68.16b	4.05c	2.25b
	1.0	69.55b	4.15c	2.70a
	1.5	81.25a	4.95a	2.95a
Valencia	0.0	0.00j	0.00h	0.00e
	0.3	20.00i	2.18g	1.17d
	0.5	44.70f	2.67f	1.80c
	1.0	41.55g	3.26e	2.29b
	1.5	48.45e	3.55d	2.26b
Tangelo	0.0	0.00j	0.00h	0.00e
	0.3	26.00h	2.54f	1.40d
	0.5	47.85e	3.26e	2.01bc
	1.0	49.75de	4.00c	2.06bc
	1.5	56.00c	4.56b	2.15bc

Means followed by the same letter within a column were not significantly different at a 0.05 probability level based on the least significance difference (LSD) test. Small letters: significance within a column.

tested cultivars. NAA has presented a significantly higher rooting rate (84.90%) than the rooting rate due to IAA (81.25%) at the maximum concentration of the growth regulators. However, for other parameters including root number and root length, no significant differences were observed between both phytohormones.

Table 3: The effects of MS medium supplemented with NAA on root response rate, root number, and root length.

Cultivar	NAA (mg/L)	Rooting rate (%)	Root number per macroshoot	Root length (cm)
Washington naval	MS basal medium	0.00j	0.00h	0.00g
	0.30	57.35ef	3.69e	2.22d
	0.50	69.66c	4.15d	2.43c
	1.00	72.60b	4.50c	2.70b
	1.50	84.90a	5.20a	3.05a
Valencia	0.00	0.00j	0.00h	0.00g
	0.30	25.95i	2.63g	1.72f
	0.50	49.20g	3.20f	2.05e
	1.00	55.05f	4.21cd	2.29cd
	1.50	58.95e	4.86b	2.68b
Tangelo	0.00	0.00j	0.00h	0.00g
	0.30	32.65h	2.84g	1.74f
	0.50	51.05g	3.48ef	2.19de
	1.00	50.75g	4.15d	2.26d
	1.50	64.15d	5.51a	2.440c

Means followed by the same letter within a column were not significantly different at a 0.05 probability level based on the least significance difference (LSD) test. Small letters: significance within a column.

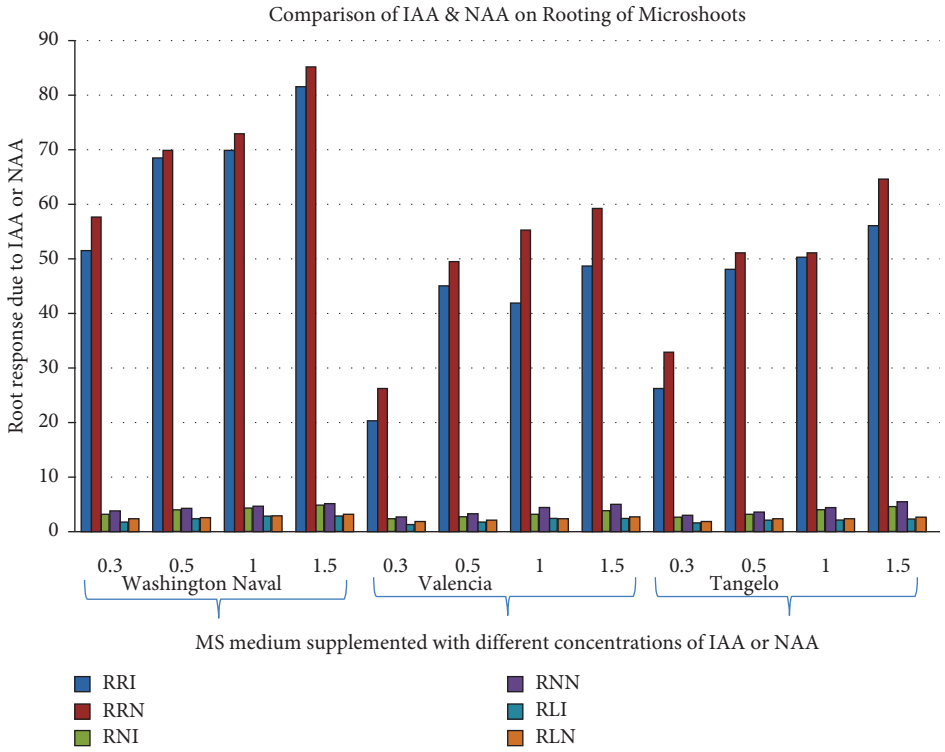

Figure 2: Comparison of the effects of MS medium supplemented with IAA and NAA on rooting of microshoots from nodal segment explants of three orange cultivars. Where RRI: rooting rate due to IAA supplement; RRN: rooting rate due to NAA supplement; RNA: root number due to NAA supplement; RNA: rooting number due to NAA supplement; RLI: rooting length due to IAA supplement; RLA: root length due to NAA supplement.

4. Conclusion

In all experiments, no growth was observed for the basal MS medium. Phytohormones combinations of IAA (1.2 mg/L) and kinetin (2.0 mg/L) were found to be the best for shoot proliferation. The shoot responses were found to be increasing with an increase in kinetin concentration with IAA concentration at 1.20 mg/L. As the concentration of IAA within the medium increases, the root responses including root initiation rate, root number, and root length also increase with maximum rooting observed at 1.5 mg/L. No root growth was observed in the control medium (MS basal medium). It was observed from the result of the present study that root response was increased as the concentration

of the growth regulator increased from 0.3 to 1.5 mg/L. The comparison of different concentrations of IAA and NAA on root induction of microshoots from nodal segments of sweet orange cultivars demonstrated NAA as a more effective hormone than IAA.

Data Availability

The data used to support the findings of this study are included within the supplementary material file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Zekeria Yusuf contributed initiation and design of the study, lab experiment, and data analysis; Fuad Abdi did the lab experiment, collected the data, and wrote the document; Yohannes Petros contributed analysis and interpretation of data. All authors contributed to drafting the article and revising it critically for important intellectual content.

Acknowledgments

The authors are grateful to Haramaya University Research Office for their financial support and laboratory facility. This project was funded by Haramaya University Research grant, under project code: HUIF_2020_06_01_97.

Supplementary Materials

This section includes the data on MS medium preparation from basal salts, the effect of MS supplemented with IAA and kinetin on shoot regeneration of sweet orange cultivars, the effect of MS supplemented with IAA on rooting response microshoots from nodal. (*Supplementary Materials*)

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