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Preliminary Evaluation of a New Polymeric Biocide as a Preservative in Plant in vitro Culture

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Abstract

Akacid-plus is a novel polymeric biocide, which is evaluated in this research project for possible application to plant in-vitro culture. Due to the specific mode of action of Akacid, it seems that an initiative treatment with bleach or similar fast-acting substances is mandatory but it could be incorporated in low levels in culture medium in order to control the latent contaminations. Among different tested concentrations and application methods, the plant tissue tolerated low concentrations of Akacid-plus, ranging between 72 mg l^{-1} to 174 mg l^{-1}, which indicates possibility of considering it as a preservative agent in plant in-vitro culture.

Keywords: Preservative; contamination control; polymeric biocide

Abbreviations: BA - 6-benzylaminopurine; NAA - naphthaleneacetic acid; Akacid-plus - Akacid-Plus® (3:1 mixture of poly-(hexamethylen-guanidinium-chloride) and poly-[2-(2-ethoxy)-ethoxyethyl)-guanidinium-chloride])

INTRODUCTION

In-vitro contamination by fungi, bacteria, or yeast is one of the most serious problems of plant tissue culture [4,5]. Already a combination of biocides is available commercially as PPM™ (Plant Preservative Mixture), which is a broad-spectrum preservative/biocide. This substance could be applied in-vitro to prevent or reduce microbial contamination in plant in-vitro culture although limitations as negative effects on explant regeneration are increasing [7,2]. In this research, another promising substance named Akacid-plus is tested as an in-vitro preservative/biocide. Akacid-plus is a macromolecular, cationic polymer, which is produced in a polymerization process. It is claimed to be non-toxic for higher life forms (mammals and intact plants because of specific mode of action) while having a biocidal effect on contaminating prokaryotes. Akacid-plus is efficient against all types of bacteria and fungi and currently is used for the disinfection of clinical areas and even as a plant protectant for hazelnut plantations [3].

This study was conducted to evaluate Akacid-plus implementation as a preservative/biocide in plant in-vitro culture and the effective concentration of this substance, in which it could act in a discriminating manner among in-vitro plant cells and the contaminating microorganisms.

MATERIALS and METHODS

Carrot taproots were used as the source of explants. Surface sterilization was done either with 20% (v/v) commercial bleach containing 1% (v/v) active chlorine for 20 minutes or Akacid-plus solution with different concentrations and exposure periods, which is mentioned in each experiment separately. 2-3 drops of Tween-20 was added to all surface sterilization solutions. After sterilization and rinsing, 3-6 millimeters thick complete disc shaped slices were made and one initial explant was transferred in each culture plate. Culture vessels were 9 cm petri dishes, each containing ~12 ml of culture medium. A single explant was plated in each petri dish.

The culture medium used for all experiments was based on MS medium [6] to which 30 gl^{-1} sucrose and 8 gl^{-1} agar was added. Medium pH was adjusted to 5.6 before adding the agar. BA at 4 mg l^{-1} and 1 mg l^{-1} NAA was added to all media and sterilized by autoclaving at 121C° and 1.1 K Pa for 30 min.

Cultures were incubated at 24±2C° in complete darkness. The observation of contaminated explants was undertaken 10 days after culture initiation for pre-experiments 1 to 4 and 7 days for experiment 1 and 2. Observation of least visually detectable contamination was used as contamination index.

The available commercial stock solution of Akacid-plus was a 25% (v/v) aqueous solution of 3:1 mixture of poly-(hexamethylen-guanidinium-chloride) and poly-[2-(2-ethoxy)-ethoxyethyl)-guanidinium-chloride]. This stock was diluted with sterile distilled water to make the desired concentrations. In some experiments Akacid-plus was added to culture medium before autoclaving which caused slight turbidity.

The data were analyzed using SPSS software and Significant differences between means were determined using the Duncan's multiple range test (DMRT) at the $P \leq 0.01$ level and displayed by alphabet codes in tables

Callus proliferation was applied as an index for tolerance and survival of plant tissue to applied concentration of Akacid-plus.

As there was no literature available on in-vitro implementation of Akacid-plus, a series of consequent pre-experiments and experiments were carried out to reach a conclusion on the possible concentration(s) and application method(s);

Experiments specific procedure

Pre-Experiment 1: Carrot discs were dipped in nine different concentrations of Akacid-plus from 725 to 23200 mg l^{-1} for 20 minutes and 9 explants were cultured in each treatment.

Pre-Experiment 2: Carrot roots were sliced and 10 discs were dipped in each of 5 concentrations of Akacid-plus for 20 minutes. Comparison was made with control treatment which was sterilized with 1% (v/v) active chlorine solution. 10 days after cultures the results recorded and the explants were rinsed 3 times with sterile water to remove possible residual effects of Akacid-plus then sub-cultured again. After another 10 days, the results were recorded and compared with the earlier one to test out possible release from observed inhibitory effects of Akacid-plus in pre-experiment 1.

Pre-Experiment 3: Intact taproots were dipped in 4350 mg l^{-1} Akacid-plus for 4, 10 and 20 hours and then cut, eight explants were cultured in each treatment. The cut surfaces of whole taproots were sealed by hot paraffin before submerging in Akacid-plus solution to avoid penetration of Akacid-plus inside the tissue.

Pre-Experiment 4: Surface sterilizing was done by with 1% (v/v) active chlorine solution. After 3 times rinsing with sterile water, the roots were sliced. The resultant discs were placed either on medium containing Akacid-plus in concentrations of 290, 87 and 29 mg l^{-1} or dipped in concentrations of 290, 232, 174, 116, 58 and 29 mg l^{-1} of Akacid-plus with 10 explant in each treatment.

Experiment 1: Surface sterilizing was done by 1% (v/v) active chlorine solution. The explants were cultured in four treatments; control (no dip treatment), 145 mg l^{-1} in medium, and 72 and 174 mg l^{-1} Akacid-plus as dip treatments before culturing in Petri-dishes. In each treatment, 40 explants were cultured, one explant per each Petri-dish.

Experiment 2: Surface sterilizing was done by with 1% (v/v) active chlorine solution. The carrot discs were dipped in Akacid-plus solutions of 72 and 174 mg l-1, respectively and compared with control. For each treatment, there were 42 replications with one explant for each replication. The used Akacid-plus solutions were pre-autoclaved.

RESULTS

Pre-Experiment 1: Effect of high concentrations of Akacid-plus

Surprisingly by increase of Akacid-plus concentration from 725 to 23200 mg l^{-1}, the occurrence and magnitude of contamination increased dramatically. Lack of any cell proliferation in uncontaminated samples implies that there are negative effects on tissue and cell in these application ranges of Akacid-plus (Table 1).

Pre-Experiment 2: Comparison of medium concentrations of Akacid-plus with 1% chlorineperformance

After subculture, callus formation was only notable in explants that were surface sterilized by 1% chlorine. Even thought the contamination was controlled effectively but callus proliferation was not seen. Akacid-plus in 4350 mg l^{-1} controlled the contamination in par with 1% chlorine until 10 days of culture initiation. After rinsing and subsequent subculture, these were contaminated indicating that residual Akacid-plus has been effective in reduction of contamination in first 10 days (Table 2).

Pre-Experiment 3: Applying intermediate concentrations of Akacid-plus in prolonged exposure time

T achieve surface disinfection comparable to 1% chlorine, intact whole taproots were placed in Akacid solution to preventing penetration of Akacid plus to inside of tissue from cut surfaces. Among intact taproots, dipped in 4350 mg l^{-1} Akacid-plus for 4, 10 and 20 hours, respectively 4, 6 and 8 out of 8 explants remained uncontaminated after 10 days while still no callus formation observed. It appeared that this substance had penetrated deeply to carrot taproots thus inhibiting callus formation along with complete sterilization in 20 hours exposure time.

Pre-Experiment 4: Effect of low Concentrations of Akacid-plus

Based on above results, the Akacid-plus application as a fast surface sterilizer for living tissues was considered impractical. Thus in next experiments we considered finding the possible *in vitro* Akacid-plus concentrations in which callus proliferation, as a sign of plant tissue survival goes on while the anti-bacterial activity of Akacid-plus could be exerted.

In this experiment the plant tissue survived and proliferated callus in 87 and 145 mg l^{-1} of Akacid-plus in dip and medium applications, respectively (Tables 3 and 4).

Table 1. Effect of increased concentrations of Akacid-plus on explant contamination (n=9)

Akacid-plus concentration (mg l^{-1})	725	1450	2170	2900	4350	5800	8700	11600	23200
Number of uncontaminated explants	8^{a*}	6^{ab}	5^{ab}	6^{ab}	0^{c}	3^{bc}	0^{c}	0^{c}	0^{c}

Grouping $\alpha \leq 0.01$

Experiment 1: Comparing Application methods of Akacid-plus in lower concentrations

Dipping of explants in 174 mg l^{-1} Akacid-plus after initial disinfection by 1% chlorine resulted a substantial decrease in contamination level compared to explants which were just disinfected with 1% chlorine. Explants which were only disinfected with 1% chlorine and those placed later in medium with 145 mg l^{-1} Akacid-plus or dipped in 87 mg l^{-1} Akacid-plus were in same statistical category. In both dip treatments explants tended to become whiter (Table 5).

Experiment 2: Conclusion on application concentration in dip method for Akacid-plus

In this experiment, the dip treatment of explants in 174 mg l^{-1} Akacid-plus after initial disinfection with 1% chlorine showed combination of callusing and reduced contamination comparing those which were only disinfected by 1% chlorine (Table 6). It seemed that autoclaving the Akacid-plus solution had enhanced its compatibility with plant tissue, as no explant discoloration was observed by dip treatment in Akacid-Plus.

Table 2. Combined effect of Akacid-plus concentration and application method on visible contamination and callus formation (n=10)

Akacid-plus concentration in primary dip treatment (mg l-1)							
		Chlorine (Control)	1450	2900	4350	5800	8700
Medium without Akacid-plus	-10 days after culture initiation	7	2	2	2	2	2
	-10 days after 1'st subculture	3cal	2	1	2	1	2
Medium with 0.25 mg l-1 Akacid-plus	-10 days after culture initiation	9	4	5	7	3	3
	-10 days after 1'st subculture	9	1	2	2	2	2

cal =callus formation observed

Table 3. Effect of low concentrations of Akacid-plus in culture medium on tissue viability and contamination (n=10)

	Akacid-plus concentration in culture medium mg l^{-1}			
	290	145	87	29
Number of uncontaminated explants	1	4 *cal*	4 *cal*	4 *cal*

cal =callus formation observed

Table 4. Effect of low concentrations of Akacid-plus in dip treatment on tissue viability and contamination (n=10)

	Akacid-plus concentration in dip treatment mg l^{-1}						
	290	232	174	116	87	58	29
Number of uncontaminated explants	1	3	0	4	4 *cal*	1 *cal*	2 *cal*

cal =callus formation observed

Table 5. Dip treatment versus incorporating Akacid-plus in medium (n=40)

	Control	145 mg l^{-1} in medium	87 mg l^{-1} dip	174 mg l^{-1} dip
Number of uncontaminated explants	3_a	3_a	11_a	27_b

Grouping $\alpha \leq 0.01$

Table 6. Dip treatment by autoclaved Akacid-plus (n=42)

	Control (no dip treatment)	87 mg l^{-1} dip	174 mg l^{-1} dip
Number of uncontaminated explants	7_a	19_b *cal*	33_b *cal*

cal =callus formation observed.

Grouping $\alpha \leq 0.01$

DISCUSSION

Contamination increase by high Akacid-plus concentration could be considered unusual and related to specific mode of action for this biocide. Even though Akacid alone didn't gave a initial comparable disinfection performance to routinely used 1% chlorine solutions, it could be beneficial in control of contaminations in *in vitro* environment in lower application levels. This could be because Akacid-plus possibly affects microorganisms when they are in active phase and it could not readily destroy more tolerant dehydrated forms of bacteria and fungi which are affected by chlorine solutions. Relative tolerance of plant tissue to lower concentrations of Akacid-plus makes it a promising agent for control of latent contaminations in *in vitro* culture in application ranges of 72 mg l^{-1} to 174 mg l^{-1} applied as dip treatment (equivalent to %0.03 to %0.06 of commercial solution). Since the bactericidal activities of this substance for many bacterial and fungal strains is previously proved in far less concentrations of 0.25 to 32 mg l^{-1} [1], we could expect a reasonable control of contamination in our application range while lower concentrations still may be beneficial in cases dealing with known contaminants for which the minimum inhibitory concentration (MIC) could be determined before initiation of plant in-vitro culture. Lack of observation of any bacterial resistance induction by Akacid-plus is another property which could regarded as an advantage for in-vitro application [1]

Ordinary autoclaving of diluted Akacid-plus solution appeared to enhance its compatibility with in-vitro cell proliferation of carrot tissue.

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