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THE COUNTERACTING EFFECT OF POTASSIUM CYANIDE IN SODIUM AZIDE-INHIBITED GERMINATION OF *PAULOWNIA TOMENTOSA* STEUD. SEEDS

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Abstract - The effect of some respiratory inhibitors on light-induced *Paulownia tomentosa* Steud. seed germination was studied. Millimolar solution of sodium azide was sufficient to completely prevent germination induced by a 5-min red light pulse. The inhibitory effect of azide was absent if seeds were rinsed before phytochrome activation by light. Sodium azide was effective only if present in the period of P_{fr} activity. The escape time from azide inhibition, compared to the escape from far-red light action, was delayed for about 24 hours. When azide was applied after phytochrome activation, its effect depended on how long it was present in the incubation medium. The removal of azide allowed full restoration of germination by another red light pulse and the far-red escape time did not differ from the escape of untreated, i.e., water-imbibed seeds. Potassium cyanide alone did not produce any effect in light-stimulated germination of these seeds. However, it counteracted the inhibitory effect of azide in light-stimulated germination, if applied simultaneously at a concentration three times higher.

Key words: Germination, light, *Paulownia tomentosa*, potassium cyanide, reversible inhibition, sodium azide.

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INTRODUCTION

The germination of empress tree (*Paulownia tomentosa* Steud.) seeds is phytochrome-controlled. Depending on seed maturation conditions, the light requirement for maximum germination varies from a very brief exposure to several hours of irradiation (in some cases up to 18 h) (Borthwick *et al.* 1964; Grubišić *et. al.* 1985). Prolonged imbibition in darkness or in heavy water leads to an increase in the light requirement for maximum germination (Grubišić and Konjević, 1986, 1990). In seeds with natural and induced long-term light requirements, two short (5-min) red light pulses, separated by a certain period of darkness, could substitute for this requirement. Moreover, the long light requirement can be reduced to a single 5-min red light pulse by the application of inorganic nitrates and nitrites (Grubišić and Konjević, 1990), substances with electron-accepting properties (Giba *et al*. 1994), or nitric oxide-releasing compounds (Giba *et al*. 1998).

Azide is a well known dormancy-breaking agent for a variety of seeds (Bewley and Black, 1982). Seeds of pigweed (Taylorson and Hendricks, 1973), apple (Dziewanowska *et al.* 1979), wild oat (Adkins *et al.* 1984), dormant rice (Cohn and Hughes, 1986) and oat (Côme *et al.* 1988) can be stimulated to germinate by NaN_3. The same is true of some other respiratory inhibitors (cyanide, carbon monoxide, sulfide). On the other hand, azide and cyanide do not substitute for the light requirement for breaking dormancy in empress tree seeds (Grubišić, 1980). The opposing effect of these inhibitors in germination is not uncommon. Hendricks and Taylorson (1972) showed that azide inhibited, but cyanide stimulated germination of seeds of *Lactuca sativa* and *Amaranthus albus.*

In the study presented here, we investigated the effects of sodium azide on light-induced seed germination, focusing on the period of P_{fr} activity. It is demonstrated that azide reversibly inhibits germination of *P. tomentosa* seeds. In addition, cyanide was found to counteract the inhibitory effect of azide in this species.

MATERIALS AND METHODS

Plant material and seed manipulation

Seeds of empress tree (*Paulownia tomentosa* Steud.) were collected in the Botanical Garden of the University of Belgrade and stored at room temperature until use. Lots of 100 seeds were placed in 6-cm diameter Petri dishes, with 2 ml of distilled water or aqueous solution of the substance to be tested. Seeds were rinsed three times with 3 ml of distilled water before replacing the test solutions. The pH values of the solutions were recorded at the start of imbibition and before removal of the seeds. Diluted HCl and NaOH were used to adjust the pH of the test solutions to the appropriate pH value. Germination was performed at 25±1°C, in darkness. Specific experimental protocols and irradiation regimes are described in the Figure Legends and Tables. Only for one subset of experiments (Table 1) was a batch of freshly harvested seeds used. These seeds could not be induced to germinate with a 5-min red light pulse, but required several hours of long red light irradiation.

Germination was scored seven days after the inductive red-light treatments or ten days after the start of imbibition. All experiments were repeated three times, with 3-5 replicates each. The data points represent means of pooled results; standard errors are not shown since they never exceeded 3%.

Light sources and pH measurement

A weak green safe light was used for seed manipulation in the darkroom. The light sources were as follows: red light - Philips TL 20/15 fluorescent tubes (Philips, Hamburg, FRG) with 3-mm plastic Röhm & Haas (Darmstadt, Germany) No. 501 filters, fluence rate of 3.54 µmol m^{-2} s^{-1}; far-red light - Osram Linestra 120/235 incandescent tubes (Osram, Munich, Germany) and Röhm & Haas No. 501 red and No. 627 blue, 3-mm plastic filters, fluence rate of 4.85 µmol m^{-2} s^{-1}; green light - Philips TL 20/17 green fluorescent tubes (Philips, Hamburg, FRG) with two No. 700 3-mm plastic Röhm & Haas (Darmstadt, Germany) filters, fluence rate of 0.8 µmol m^{-2} s^{-1}. Light was measured using a Li-180 B integrating quantum radiometer/photometer (Licor, Lincoln, Neb., USA) or a Tektronix J16 digital photometer (Tektronix, Beaverton, Ore., USA).

The pH value of the medium was recorded using a laboratory pH-meter (InoLab, pH Level 1, WTW, Weilheim, Germany).

Chemicals

Sodium azide (NaN_3) was purchased from Sigma Chemical Co., St. Louis, Mo., USA; potassium cyanide (KCN) was obtained from Merck, Darmstadt, Germany.

RESULTS

Inhibition by azide

The effects of different concentrations of sodium azide and potassium cyanide on *P. tomentosa* seed germination are shown in Fig. 1. Azide, up to 10^{-4} M, applied at the onset of imbibition, was ineffective in inhibiting red light-induced germination, while addition of 10^{-3} M azide completely suppressed it. On the other hand, cyanide concentrations as high as 10^{-2} M, applied in the same way, failed to affect germination. For further experiments sodium azide was used in the inhibitory concentration (10^{-3} M) and applied at the beginning of each of the three germination phases, *i.e.* imbibition (3 days), the phase of P_{fr} activity (3 days after the red light pulse), and the phase of radicle elongation (4 days). At the end of each phase, the seeds were washed out and the incubation medium replaced by distilled water. The inhibitory effect of azide

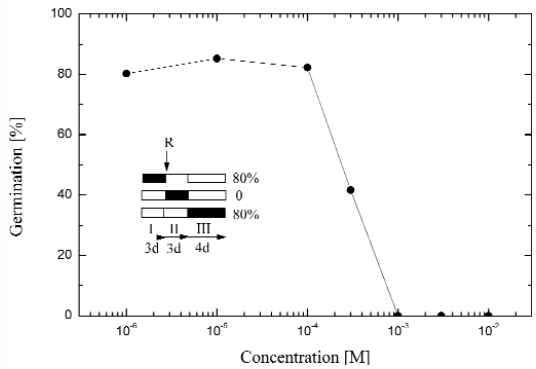


Fig. 1. Effect of sodium azide and potassium cyanide on light-induced germination of *P. tomentosa* seeds.
Seeds were imbibed either in water or in increasing concentrations (x-axis) of test solutions for 3 days at 25°C, irradiated with 5-min red light, and left in darkness. Germination was scored 7 days after the red light treatment. ˜-sodium azide; £-potassium cyanide. Insert: Effect of sodium azide applied in different phases of *P. tomentosa* seed germination. Seeds were supplied with distilled water or 10^{-3} M solution of sodium azide at different germination phases. Following these phases, the seeds were rinsed. In all treatments, seeds were irradiated 3 days after the onset of imbibition with 5-min red light. Germination was scored 7 days after the light treatment. £-distilled water; ¢-sodium azide; I-imbibition; II-P_{fr} activity; III-radicle elongation; R-5-min red light.

was evident only if it was administered during the period of P_{fr} activity (insert in Fig. 1).

In addition to this, the escape from azide inhibition was followed. A comparison of azide-inhibition escape and escape from the inhibitory effect of far-red light is shown in Fig. 2. In both experiments escape was completed 72 h after the red light pulse. However, while the inhibition to 50% of red light-induced germination by 5-min of far-red light was estimated to be at 31 h, the same effect of azide occurred about 20 h later. That was confirmed by probit analyses (insert in Fig. 2). Accordingly, there is an obvious 24-h shift of the escape from azide inhibition. If the seeds were supplied with 10^{-3} M sodium azide after the inductive 5-min red light pulse, the percent of germination decreased with the duration of sodium azide treatment. Subsequent stimulation of seed germination by an additional red light pulse, after rinsing, revealed that the azide inhibitory effect in the P_{fr} activity phase is reversible (Fig. 3).

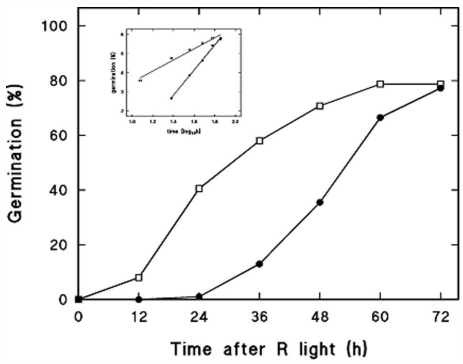


Fig. 2. Escape time for far-red light and inhibitory effect of sodium azide in light-induced germination of *P. tomentosa* seeds.
After imbibition in water for 3 days in darkness at 25°C, seeds were irradiated with 5- min red light. After that, they were either irradiated with 5-min far red light at the indicated time intervals and returned to darkness (£) or transferred after rinsing to 10^{-3} M sodium azide solution and returned to darkness (˜). Insert: Probit analysis of escape time from azide inhibition and far-red light inhibitory action.

In a similar experimental approach (seeds treated by azide, then rinsed), the far-red light escape time was rechecked and compared to the escape time for the far-red inhibitory effect in seeds imbibed only in water. Pretreatment with 10^{-3} M azide did not affect the escape time (Fig. 4), indicating full restoration of the phytochrome pigment system.

A subsequent set of experiments was performed in an attempt to understand the azide inhibitory effect in light-induced germination. A batch of *P. tomentosa* seeds requiring long light irradiation for maximum germination was used. As was shown earlier (Grubišić and Konjević, 1990), two red light pulses of 5-min separated by a 12 hours-long period of darkness can substitute for the continuous light requirement of these seeds. The application of a 10^{-3} M concentration of azide inhibited germination if applied after the second red light pulse only (Table 1).

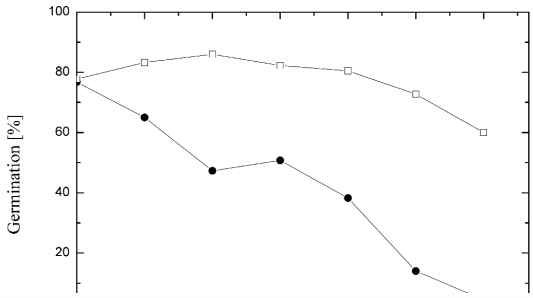


Fig.3. Effect of the length of incubation in sodium azide during the period of phytochrome activity on light-induced germination of *P. tomentosa* seeds.
Seeds imbibed in water for 3 days in darkness at 25°C were induced to germinate by 5-min red light and returned to darkness. At corresponding (12 h) intervals after that, they were transferred to 10^{-3} M solution of sodium azide and either returned to darkness (˜) or rinsed and again irradiated with 5-min red light (£). Germination was scored 7 days after the first red light treatment.

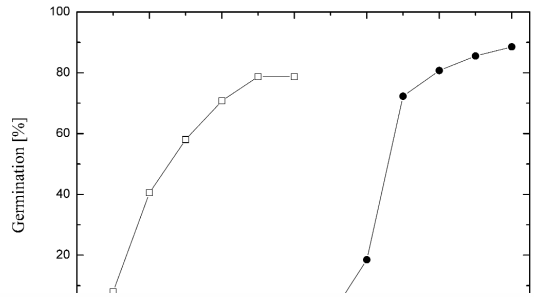
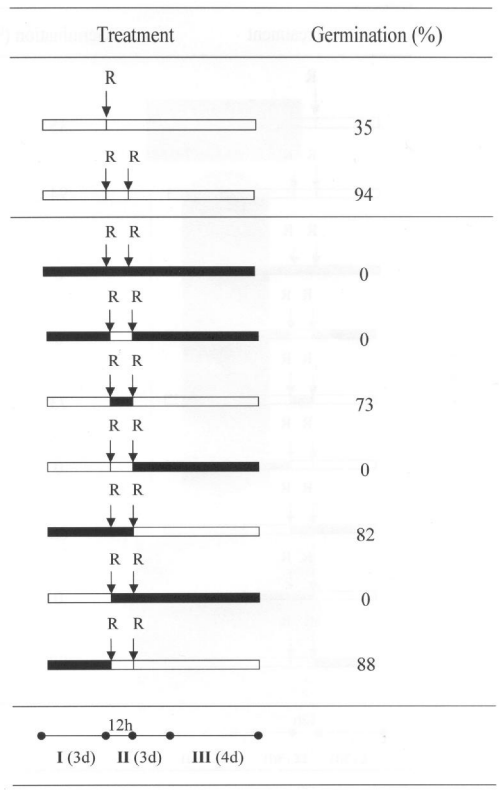


Fig. 4. Escape time from far-red light inhibitory action in water-imbibed and sodium azide-treated seeds of empress tree.
Seeds were imbibed in water for 3 days in darkness at 25°C, irradiated with 5-min red light, and returned to darkness. After that, they were either irradiated with 5-min far-red light at the indicated times (x-axis) and again returned to darkness (£); or transferred to 10^{-3} M solution of sodium azide in darkness for 72 h (period of P_{fr} activity), rinsed and irradiated with 5-min red light, and then irradiated with 5-min far-red light at the indicated time intervals (x-axis) and again returned to darkness (˜). Germination was scored 7 days after the last red light treatment.

Table 1. Effect of sodium azide in germination of *P. tomentosa* seeds with a natural long-term light requirement.
Seeds were imbibed for 3 days in darkness at 25°C in distilled water (£) or in 10^{-3} M solution of sodium azide (¢), irradiated with 5-min red light, and returned to darkness. They then were rinsed and the solutions were replaced. After a corresponding interval of 12 h, seeds were irradiated with another 5-min red light pulse, rinsed again, and transferred to distilled water or sodium azide solution. Germination was scored 7 days after the first red light treatment. I-imbibition (3 days); II-phytochrome activity (3 days); III-radicle elongation (4 days); R-5-min red light.



The pH dependence of azide inhibition

Empress tree seeds germinate in the incubation medium over a wide pH range (Turner *et al.* 1988). To evaluate the pH dependence of azide inhibition, seeds were treated with 10^{-3} M sodium azide solution adjusted to different pH. The inhibitory effect of azide was assayed at an initial pH range of 2.9 to 10.1. The degree of inhibition due to azide was found to vary as the pH varied (Fig. 5). Complete inhibition was achieved at higher pH values of the incubation medium, *i.e.* no light-induced seed germination occurred at pH of 4.6.

Effects of azide in the presence of cyanide

Potassium cyanide alone did not affect the germination of *P. tomentosa* seeds. In contrast, when seeds were treated with a mixture of azide and cyanide solution in a two-factorial experiment, germination was significantly promoted. Although cyanide did not influence light-stimulated germination of these seeds, it did revert the inhibitory effect of azide if applied simultaneously at three-fold higher concentration (Fig. 6).

Since it is known that cyanide raises the pH of the incubation medium, both the initial and final pH values at

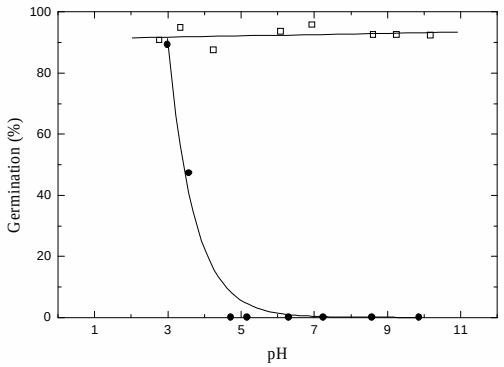


Fig.5. The pH dependance of azide inhibition.
Seeds were imbibed for 3 days in darkness at 25°C in distilled water (£) or in 10^{-3} M sodium azide solution (˜) with initial pH values in the range of 2.76-10.28. After that, they were irradiated with a 5-min red light pulse and returned to darkness. Germination was scored 7 days after the light treatment.

the highest concentrations of inhibitors were monitored. It turned out that the final pH values never fell below 6.4 (data not shown). Azide completely inhibited light-induced germination of *P. tomentosa* seeds at any pH

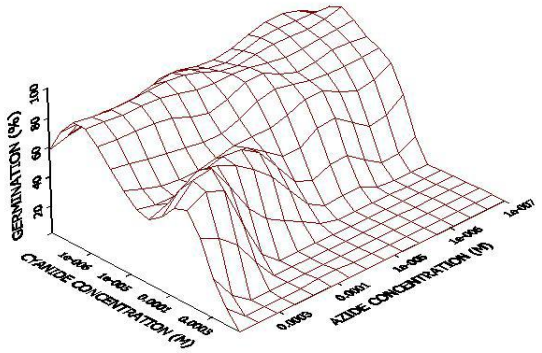


Fig.6. Effect of sodium azide and potassium cyanide on light-induced germination of *P. tomentosa* seeds.
Seeds were incubated in a mixture of different concentrations of sodium azide and potassium cyanide (10^{-2} M-10^{-6} M) for 3 days in darkness at 25°C, irradiated with 5-min red light, and returned to darkness. Germination was scored 7 days after the light treatment.

value above 4.6 (Fig. 5). Therefore, the counteracting effect of cyanide in azide-inhibited germination of seeds

of this species cannot be ascribed to cyanide-changed pH values of the incubation medium.

DISCUSSION

In light-induced seeds of *P. tomentosa*, the presence of 10^{-3} M sodium azide in the incubation medium inhibited germination. Potassium cyanide did not affect this process. In the presence of azide, an inductive 5-min red light pulse was completely ineffective. After rinsing and replacement of azide solution by distilled water, seeds still did not germinate and required an additional red light pulse for germination instead (Fig. 3). A direct azide interaction with the phytochrome molecule is questionable. Azide seemed to affect one of the (early) steps in the phytochrome transduction chain. This assumption is supported by the fact that escape from azide inhibition existed, the time needed for 50% escape being about 20 h longer than that for escape from far-red light inhibition (Fig. 2).

It was anticipated earlier that pH control of the incubation medium, when utilizing dormancy-breaking chemicals that are weak acids or bases, might improve their effectiveness and reproducibility. It has been shown in dehulled red rice seeds that the dormancy-breaking activity of azide, cyanide, and hydroxylamine are pH-dependent. In each case, activity was observed at pH values that favor formation of the uncharged form of azide (pK_a=4.7), cyanide, (pK_a=9.3), and some other compounds (Cohn and Hughes, 1986).

Azide did not substitute for light in breaking dormancy of *P. tomentosa* seeds. However, the inhibitory effect of azide in light-induced germination of seeds of this species was pH-dependent and occurred when the initial pH value of the incubation medium was 4.6 (Fig. 5). In addition, the final pH value of the azide solution differed from the initial one, varying from 4.1 to 7.3 (data not shown). Otherwise, under such experimental conditions the molecules of the inhibitor would have been overwhelmingly ionized (deprotonated). Taking into account these findings, we speculate that N_3^- represents the inhibitory form of the azide molecule in light-induced germination of empress tree seeds.

In seeds with a long-light requirement induced to germinate by two pulses of red light separated by a period of darkness, azide prevented the effect of the second pulse only (Table 1). It was previously shown that the need for two red light pulses in these seeds can be modified by addition of nitrates (Grubišić and Konjević, 1990) or different NO-releasing compounds (Giba *et al.* 1998), or by upward and downward temperature shifts (Grubišić and Konjević, 1992). All of these treatments make germination possible under suboptimal light conditions, *i.e.* after induction by one red light pulse.

It is surprising that cyanide, ineffective by itself in *P. tomentosa* seed germination, can overcome the inhibition of azide if applied simultaneously. The counteracting cyanide effect was noticed only when both inhibitors were present in the incubation medium in higher concentrations, *i.e.*, above the millimolar range. The finding that the effect of one factor (cyanide) was evident only in the presence of inhibitory concentrations of another one (azide) implies that there is an interaction between the two factors studied.

It has been suggested that the apparent resistance of germination to cyanide is an experimental artefact due to extreme cyanide volatility at the usual pH used in germination experiments (Yu *et al*. 1981). In light-induced germination of *P. tomentosa* seeds, the relief of azide inhibition by cyanide was not a result of altered pH in the incubation medium. Thus, the possibility that the "cyanide effect" might not be cyanide-specific was ruled out.

Data of the kind presented here on the effects of coincidental application of azide and cyanide have not been reported so far in studies of seed germination. However, a similar approach has been applied in experiments with isolated animal cells and tissues. For instance, the vasorelaxant effect of azide was partially reversed or prevented by an excess of free cyanide (Smith and Wilcox, 1994). In addition, the cyanide effect appeared to be competitive and reversible, although the same concentrations of cyanide alone remained without effect (Kruszyna *et al*. 1982, 1985). Thus, azide-inhibited germination of *P. tomentosa* seeds may turn out to be an appropriate tool for further studies of phytochrome-controlled germination.

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РЕВЕРЗИЈА ИНХИБИЦИЈЕ КЛИЈАЊА СЕМЕНА *PAULOWNIA TOMENTOSA* STEUD. ИЗАЗВАНЕ НАТРИЈУМ АЗИДОМ

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Испитиван је ефекат неких инхибитора дисања на клијање светлошћу индукованих семена *Paulownia tomentosa.* Потпуна инхибиција клијања, које је индуковано са 5 минута црвене светлости, могла је да се постигне већ милимоларним раствором натријум азида. Инхибиторни ефекат азида је изостајао ако се семена исперу пре активирања фитохрома осветљавањем. Натријум азид је био ефективан само ако је присутан у семенима у периоду активности P_{fr}. Време за које семена избегавају инхибиторни ефекат азида је одложено за око 24 часа у поређењу са истим за инхибиторно деловање тамно црвене светлости. Када се азид одстрани, додатни пулс црвене светлости доводи до потпуног обнављања способности за максимално клијање, а време за које семена избегавају инхибиторни ефекат црвене светлости се не разликује од истог код нетретираних семена, тј. оних који су инбибирала у води. Калијум цијанид нема никакав утицај на клијање семена индукованих светлошћу. Међутим, ако се калијум цијанид примени симултано са натријум азидом, у три пута већој концентрацији, он спречава инхибиторни ефекат натријум азида на клијање индуковано светлошћу.