

Diazepam, the modulator of GABA_A receptors, alters the leaf-folding and expanding speed of *Mimosa pudica*

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Research Article

Keywords: *M. pudica*, seismonastic movement, benzodiazepines, diazepam

Posted Date: April 11th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6399180/v1>

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Additional Declarations: The authors declare no competing interests.

Abstract

Mimosa pudica L. closes leaves in response to various stimuli. This curious reflex, called seismonastic movement, is said to be advantageous for their survival from predatory insects but is a trade-off between efficient energy acquisition. While it has been revealed that action potentials and water translocation of motor cells in the pulvinus are involved in this reaction, little is known about under what conditions or substances this movement is controlled. *M. pudica* has also been known to synthesize many secondary metabolites, and the extracts are valued in some countries for their anxiolytic effects. Benzodiazepines are commonly used anxiolytics and are known as allosteric modulators of some gamma-aminobutyric acid (GABA) receptors that are involved in inhibitory neurotransmission in animals. Although the role of GABA and its receptors in plants has been gradually unraveled in recent decades, neither the role of the endogenous benzodiazepine-like compounds nor the effect of exogenous administration of benzodiazepines on the plant has been understood. In this study, we investigated the effect of exogenous benzodiazepines on touch-induced leaf behavior. We treated the *Mimosa* plant with the solution of diazepam, a major representative of benzodiazepines, via root absorption. One hour after the roots were immersed in the solution, the speed of leaf closing was slowed down, and it lasted until 6 hours. Furthermore, the leaf-expanding movement (recovery) was accelerated with the diazepam treatment. Our findings may imply that benzodiazepines affect the generation of action potentials and/or osmosis-driven water movement of motor cells by regulating anion efflux and water transport.

Introduction

Sensitive plant, *Mimosa pudica* L, quickly folds their leaves and bends the petioles in response to various stimuli such as touch, electrical, and thermal stimuli (Houwink 1935; Volkov et al. 2010). This reflex, termed as seismonastic movements, is caused by the motion of joint-like tissue called pulvinus. A Pulvinus consists of swollen tissues at the base of *M. pudica* leaves or petioles, found at three levels on the shoot: primary pulvinus, secondary pulvinus, and tertiary pulvinus (Fig. 1a and 1b). A stimulation to a leaflet is converted to electrical signals by inducing a depolarization of membrane potential in cytosolic Ca^{2+} (Hagihara et al. 2022). These action potentials (APs) are sequentially transmitted via protoxylem and phloem of the vascular bundle to the tertiary pulvini, rachillae, and petioles, triggering rapid movements by altering the turgor pressure of motor cells at each level (Houwink 1935; Sibaoka 1962; Hagihara and Toyota 2020). In the process of leaves closing, motor cells in the upper half (extensor side, Fig. 1c) shrink by the passive efflux of K^+ and Cl^- and subsequent osmosis-driven water movement through the water transporters such as aquaporins (Simons 1981; Fromm and Eschrich 1988; Fleurat-Lessard et al. 1997a, b; Morillon et al. 2001; Moshelion et al. 2002; Braam 2005; Mano and Hasebe 2021; Zeng et al. 2024). It is considered that this sudden shrinkage of extensor cells may stretch the lower half (flexor side, Fig. 1c) of motor cells and helps them to absorb water (Braam 2005; Mano and Hasebe 2021). Tamiya et al. also reported that the primary pulvini are bent by the translocation of water from the lower half of the motor cells to the upper part (Tamiya et al. 1988). On the other hand, the process of the recovery is slow (about within 30 minutes), and the proposal mechanism is different from leaf closure:

K⁺ and water are reabsorbed into the cells using the energy from H⁺-ATPase (Tamiya et al. 1988; Hagihara and Toyota 2020). This seismonastic movement of *M. pudica* is thought to play an important role in defending itself from predatory insects: scaring visitors away, shaking insects off leaves, disappearing from the sight of insects, and giving out its thorns (Pickard 1973; Eisner 1981; Braam 2005; Hagihara et al. 2022). Jensen et al. mentioned that this leaf-folding behavior is a trade-off between predation risk and energy resources: *Mimosa* keeps their leaves closed for longer time under high light conditions than under limited light conditions (Jensen et al. 2011). However, it remains unknown and mysterious what molecular factors mediates the balance of antiherbivore defenses and energy production in response to external conditions.

M. pudica is known to synthesize many compounds. For example, the toxic alkaloid mimosine has been reported to have a cell cycle inhibitory and apoptotic effect on human cells (Cannon et al. 2009). A root extract of *M. pudica* has neutralizing activity against cobra venom (Mahanta and Mukherjee 2001). In some countries, surprisingly, *M. pudica* is used to treat anxiety and depression. Mbomo et al. reported that an extract of *M. pudica* acted as a positive modulator of γ -aminobutyric acid A receptors (GABA_ARs) in mice and had a similar effect to diazepam (DZP), a well-known representative of benzodiazepines (BZs) (Ayissi Mbomo et al. 2012). GABA is a widely known amino acid that acts as an inhibitory neurotransmitter in animals, and BZs, agonists of GABA_ARs, were first developed in the 1950s (Krnjević and Schwartz 1967; Witkin and Barrett 2024). Due to their anti-anxiety effect, many BZs are currently widely used in medical situations as anxiolytics, sedatives, and general anesthetics. Although *M. pudica* synthesizes many secondary metabolites, little is known about the role of these compounds in its survival. Especially regarding the BZ-like effect of *Mimosa* extracts, specific responsible compounds and corresponding receptors have not been found. In this research, we tested the impact of DZP on the rapid movement of *Mimosa* leaves. There has been no report about the effect of BZs on the seismonastic movement of *M. pudica*, and it is very intriguing how exogenous administration of DZP works on the plants that synthesize similar compounds themselves. It may be possible that endogenous GABA and/or BZs have a role in switching between the defensive state and the active state of photosynthesis.

Material and Method

Plant material and growth conditions

Sensitive plant (*Mimosa pudica* L.) was obtained from a local garden store and maintained at 23–25°C under a 16-h light/8-h dark photoperiod in the laboratory for a few weeks. White light was provided at an intensity of 100 $\mu\text{mol}/\text{m}^2 \text{ s}$. Plants about 20 cm tall were used in the experiment.

The treatment of *Mimosa* roots with diazepam

All measurements were conducted in the laboratory at about 24°C on a stable table. Plants were taken out from the pot, and *Mimosa* roots were gently washed to remove soil with Milli-Q water (Millipore, MA, USA). After that, roots were immersed in a polycarbonate plant box (75×75×100 mm; VWR International,

PA, USA) with 250 ml of Milli-Q water (Fig. 1d). Three plants were placed in a single plant box and stood on a table overnight to avoid any vibration or stimulation. A silicon drain tube was installed in the plant box to replace the internal fluids without direct stimulation or vibration to the plants that would cause leaves to close. The next day, the liquid in the box was replaced with 250 ml of solution via the silicon drain tube: Milli-Q water containing 0.1% dimethyl sulfoxide (DMSO), 3 μ M DZP, and 30 μ M DZP. DZP was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and the stock solutions of DZP liquid were prepared by dissolving in pure DMSO. It was then diluted with distilled Milli-Q water to obtain the final concentration containing 0.1% DMSO with DZP (3 μ M, 30 μ M).

Mechanical stimulation

To mechanically stimulate the *M. pudica* leaves, a small glass bead (4 mm in diameter, 80 mg in weight) was dropped from 10 cm above a pinna (Fig. 2a). This glass bead size was sufficient to close only the laminar pulvinus but not the secondary or primary pulvinus. The first stimulus was provided immediately after the solution was replaced. Stimulation was given every 15 minutes for the first hour. Thereafter, a glass bead was dropped at 1.5 h, 2 h, and every hour until 7 hours after the start of the experiment. Three replicates were conducted by stimulating a leaflet of independent individuals in each group. The degree of leaf opening was evaluated by the ratio of the distance between the opposite edges of pinnules (d) to the maximum distance between the pinnules before being stimulated ($d_{\max}$) (Fig. 2a and 2b). The distance of pinnules in the middle of the pinna was measured with ImageJ software (ver. 1.54g, macOS Sonoma 14.4).

Imaging of leaf movements

Two cameras were set up to capture pinnule movements. To observe the process of *M. pudica* pinnae closing, images were captured at 0.1 second intervals with an iPhone 6s (iOS 12.0.1, Apple Inc., CA, USA) burst mode. The $d/d_{\max}$ ratio was measured at every 0.2 seconds from the point a bead dropped onto a pinna until 3 seconds (Fig. 3). To follow the process of the leaves opening, pictures were taken with an EOS Kiss X7 (Canon, Tokyo, Japan): every minute until 14 minutes after the start of the experiment for the first hour, every minute until 15 minutes and 20, 25 minutes for the second hour, and every minute until 15 minutes and 20, 30, 45, 60 minutes thereafter.

Statistical analysis

All numerical data were analyzed using Student's t -test using JMP[®] Pro 18.0.2 (MP Statistical Discovery LLC, NC, USA). Differences are considered as significant when $p < 0.05$.

Result

Effect of Diazepam on the folding movement of *M. pudica* leaves

The leaf touch-response after the DZP treatment of 0 min, 30 min, 1 h, 3 h, 5 h, and 7 h are shown in Fig. 4a. The value $d/d_{\max}$ indicates the degree of leaf closure (small value means leaf-closing). The $d/d_{\max}$ was calculated every 0.2 seconds up to 3 seconds after the mechanical stimulation. Figure 4b represents the data at the time points of 1, 2, and 3 seconds from Fig. 4a. There were no apparent differences among control, 3 μ M DZP, and 30 μ M DZP groups after the solution was exchanged (0 minutes) and 30 minutes after the *Mimosa* roots were immersed in the solutions. At the point of the 1 h, 3 h, and 5 h treatment, the movement of leaves was tended to be slowed in the DZP group (Fig. 4a), whereas no leaves were entirely immobilized. This inhibitory effect of DZP on the seismonastic movement was more evident in the 3 μ M DZP group than in the 30 μ M group. At 1 h of DZP treatment, 2.0 seconds after the bead stimulation, the $d/d_{\max}$ ratio in the 3 μ M DZP group was significantly higher than the control (0.08 ± 0.0 S.D. vs 0.30 ± 0.12 S.D., $p < 0.05$) as shown in Fig. 4b. At 3 h, the $d/d_{\max}$ ratio at 1.0 second after the stimulation was higher than the control (0.23 ± 0.11 S.D. vs 0.43 ± 0.10 S.D., $p < 0.05$). Similarly, at 5 h, the $d/d_{\max}$ ratio was higher in the 3 μ M DZP group than control (0.20 ± 0.09 S.D. vs 0.50 ± 0.14 S.D., $p < 0.05$) at 1.0 second. The effect of DZP on the leaf folding movement was no longer observed at the point of 7 hours.

Effect of Diazepam on the recovery of leaf expansion

Next, we observed the effect of DZP on the recovery phase (leaf expansion). First, the plants were treated with DZP for 0, 1, 3, and 5 hours. The plants analyzed for recovery correspond to those stimulated by dropping bead, as shown in Fig. 4a (the first acute change of the $d/d_{\max}$ value in Fig. 5a). The temporal changes in the $d/d_{\max}$ ratio at the recovery phase up to 15–60 minutes were photographed and calculated (Fig. 5a). Figure 5b represents that three different time points were extracted from the data shown in Fig. 5a. The time required for recovery in the DZP-treated groups was significantly shorter than the control group after the first stimulation of a glass bead, however, at the point of 1, 3, and 5 hours, no significant difference was observed between the control group and DZP-treated groups.

Discussion

Diazepam may inhibit the action potentials in *M. pudica* leaves and water shift

GABA is the major inhibitory neurotransmitter in mammals, and its receptor, the GABA_ARs, are known to be modulated by many drugs such as alcohols, barbiturates, inhaled anesthetics, and benzodiazepines (BZs) (Philip et al. 2025). BZs allosterically activate the animal GABA_ARs by binding to the extracellular BZs binding site of the GABA_ARs, causing hyperpolarization of neurons by opening the channel pore of pentameric GABA_ARs which allow chloride ion influx (Masiulis et al. 2019; Philip et al. 2025). In plants, many studies have found that GABA plays an important role in not only stress tolerance, such as low temperature, drought, and heat stress (Wallace et al. 1984; Yong et al. 2017; Li et al. 2019, 2020b), but also pathogen response, regulation of gas exchange, and pollen tube growth (Hedrich 2012; Dreyer et al. 2012; Gutermuth et al. 2013). Bread wheat (*Triticum aestivum*) aluminum-activated malate transporter1

(TaALMT1), an anion channel that enhances aluminum tolerance by secreting malate, an aluminum chelator, has been reported to be negatively regulated by GABA (Ramesh et al. 2015; Long et al. 2020). Since anion equilibrium potentials in plants are strongly positive, the inhibitory effect of GABA on the anion transportation of TaALMT1 results in hyperpolarization and desensitization of the membrane (Žárský 2015; Palmer et al. 2016). Turano et al. predicted that plants possess GABA-like receptors similar to animal GABA receptors from physiological and genetic perspectives (Kinnersley and Turano 2000). It suggests that plant GABA receptors may be modulated by BZs, as in animals.

The folding movement of *M. pudica* leaves was delayed one hour after administration of DZP in this experiment, and this time course seemed to depend on the rate at which roots absorb water. When the DZP solution reaches pulvini, the agent may act locally as an inhibitor of ALMTs or any other putative GABA receptors and cause membrane hyperpolarization. There are two possibilities regarding the delayed movement of *Mimosa* leaves: (i) the transmission of action potentials (APs) was inhibited, and/or (ii) the translocation of water in the motor cell was attenuated. For the APs, Pavlovič A et al. found diethyl ether completely diminished the APs of the trigger hair of carnivorous plant Venus flytrap (*Dionaea muscipula*) leaves (Pavlovič et al. 2020). As shown in Fig. 6, in response to external stimuli, plant APs are triggered by the Ca^{2+} influx into the cytosol, then the depolarization of the membrane promotes the Cl^- efflux via calcium-dependent chloride channels. The activated anion channels can drive the efflux of K^+ through the voltage-dependent K^+ channels by the change of electrical potentials of the cell membrane. Repolarization starts with suppressing Ca^{2+} influx and the promotion of Ca^{2+} resequestration, and this repolarization tapers off the Cl^- and K^+ efflux (Lee and Calvo 2023). Considering this mechanism of action potential generation and the effect of diazepam on ALMTs, it is imaginable that diazepam suppressed the generation and propagation of APs by the inhibition of Cl^- efflux, but this hypothesis remains to be tested. From the perspective of water movement, the modulation of ALMTs by GABA may inhibit the passive efflux of Cl ions, the major anions, in the extensor side of motor cells. Then, subsequent osmosis-driven water runoff would be slowed down. Furthermore, some reports found that exogenous GABA application elevated the transcription level of aquaporins under heat stress in white clover (*Trifolium repens*) and salt stress in creeping bentgrass (*Agrostis stolonifera* L.) (Li et al. 2020a; Qi et al. 2021). Although little is known about the exact pathway of GABA signaling in the regulation of aquaporins, pretreatment with diazepam on plants may indirectly affect transcellular water shifts by the allosteric effect on GABA receptors.

Metabolism of BZs in *M. pudica*

The inhibitory effect of diazepam on the rapid movement of *Mimosa* leaves was pronounced between 1 to 6 hours after the start of the experiment but was not obvious at 7 hours. While there have been no reports on the diazepam-metabolizing activity of *M. pudica*, it has been suggested that some plants may be able to metabolize BZs (Carter et al. 2018). Metabolic activity could be evaluated by measuring the amount of the final transformation product of diazepam such as nordiazepam or oxazepam.

Assumed role of GABA in maintaining leaf folding

During the recovery phase of folded leaves of *M. pudica* after the stimulation, the time it takes for leaves to open was shortened. As Jensen et al. mentioned, avoidance behaviors entail the risk of a reduction in light foraging (Jensen et al. 2011). Eprintsev AT et al. reported that light irradiation changes the glutamate metabolism and GABA shunt of maize (*Zea mays* L.) leaves (Eprintsev et al. 2024). Given this effect of light on GABA metabolisms, it would be fascinating to consider if *M. pudica* maintains the balance between predation risk and energetic reward by photo perception and subsequent GABA signaling. While it is difficult to interpret why the difference in the speed of leaf opening was no longer apparent after the second stimulation, one possible reason is due to the lack of energy to keep the leaves folded. Furthermore, it could be caused by the plasticity of *M. pudica* leaves in response to repeated mechanical or electrical stimuli, which was reported in 1972 by Applewhite PB (Applewhite 1972).

Potential significance of natural BZs in plants

The natural BZs in plants were first found in wheat grains and potato tubers, and it has been reported that the content of BZs in wheat and potatoes increases during germination (Wildmann et al. 1988; Wildmann 1988). Kavvadias et al. proved plants can synthesize endogenous BZs without exposure to microorganisms and environmental contamination (Kavvadias et al. 2000). Although BZs could be assumed to have some effect on the timing of seed germination, little is known about the role of BZs in mature plants. One possible answer is that endogenous BZs may modulate and regulate the GABA receptors' activity and water transportation, contributing to heat or drought tolerance. Since it has been implied that GABA regulates the central circadian clock of the human brain, it would be very interesting if plant natural BZs are involved in the nyctinastic movement of the *M. pudica* leaves by oscillating intracellular GABA concentration and/or activity on their receptors.

Conclusions

The seismonastic movement of *M. pudica* was delayed by the administration of diazepam, and the recovery period of the leaflet was accelerated after the first stimulation. Our findings on the effect of diazepam on *M. pudica* leaves might help us to have a profound understanding of the plant GABA dynamics, the biochemical properties of plant GABA receptors, and the metabolisms of BZs in plants. Not only in the decision-making process of striking a balance between predation risk and energetic reward, but endogenous BZs might have a role in regulating the periodical movement of *M. pudica* leaves, which is controlled by a biological clock.

Declarations

Conflict of interest

The authors declare no competing interests.

Acknowledgment

The authors thank the Akiyama Life Science Foundation for the partial financial support.

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Figures

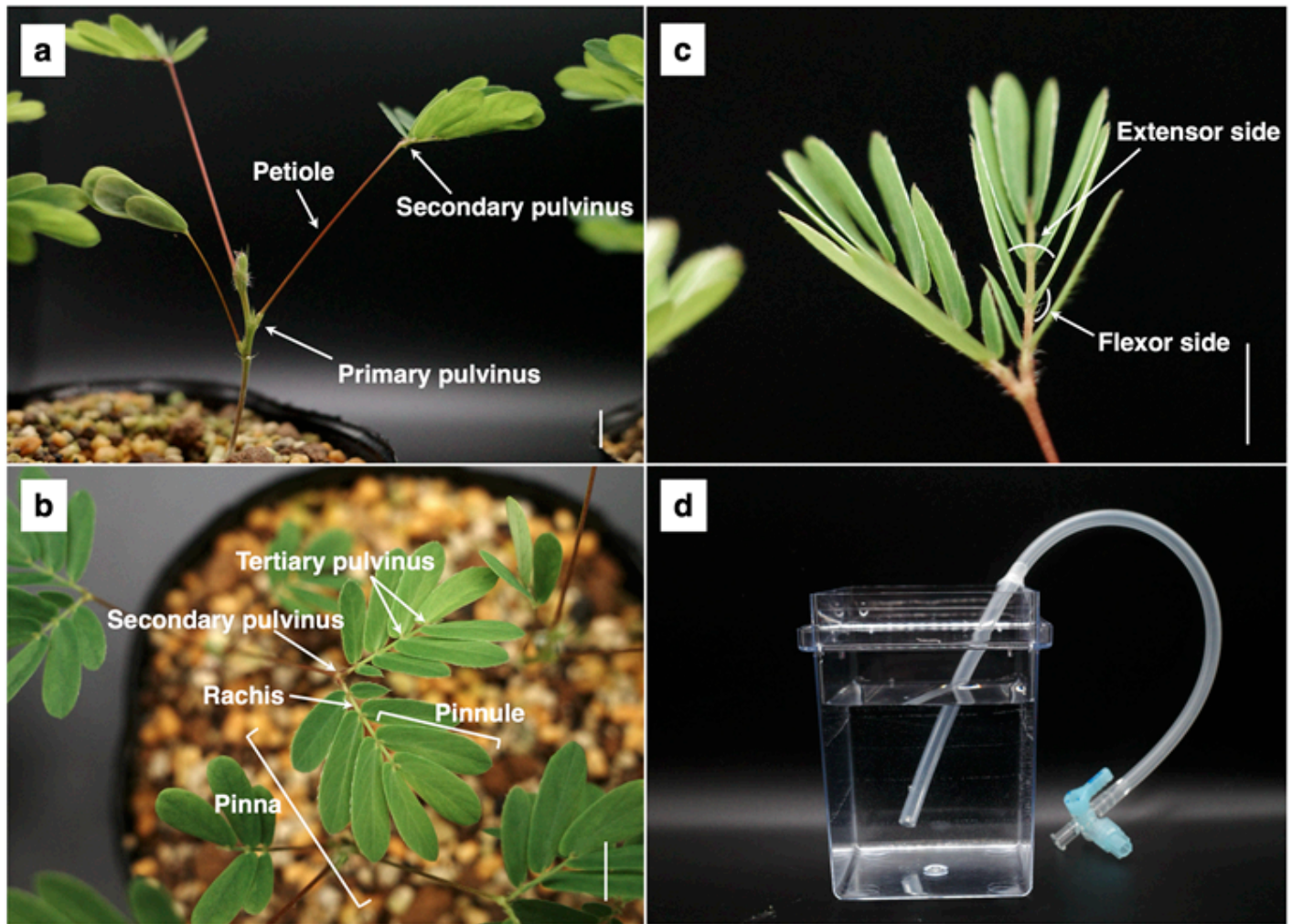


Figure 1

The structure of *M. pudica* and experimental set-up. (a) The arrows indicate primary pulvinus, secondary pulvinus, and petiole. The pulvinus is a joint-like tissue that allows leaves to fold. (b) The arrows indicate secondary pulvinus, tertiary pulvinus, rachis (stalk within the blade), pinnule (secondary leaflet), and pinna (primary leaflet). (c) Extensor side and Flexor side of pinnules. Motor cells of the tertiary pulvinus change size in response to stimuli to move the pinnule. (d) Experimental set-up: *M. pudica* roots were soaked in a plant box with a drain tube. Scale bars, 5 mm.

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Figure 2

Quantification of the folding state of pinnula. d : distance between the edges of pinnules in the middle of pinna (b), $d_{\max}$: maximal distance of the edges of pinnules in the middle of pinna before stimulation (a). A glass bead was dropped from a height of 10 cm to stimulate leaflets.

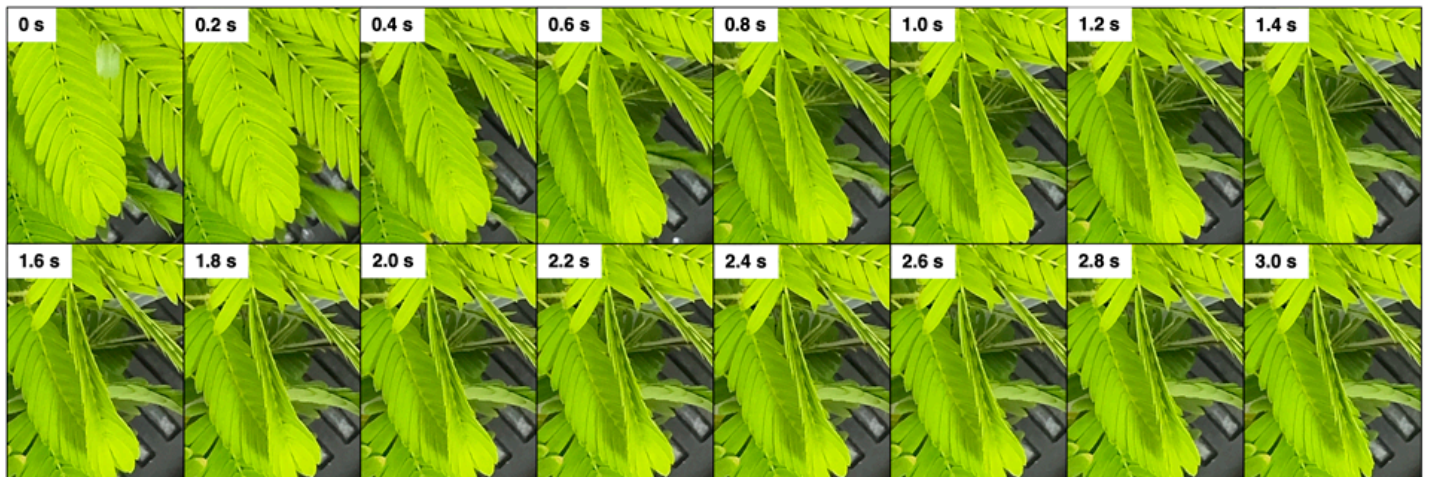


Figure 3

Examples of a serial shot of pinna after stimulation. Photos were taken every 0.2 seconds for three seconds after the stimulation.

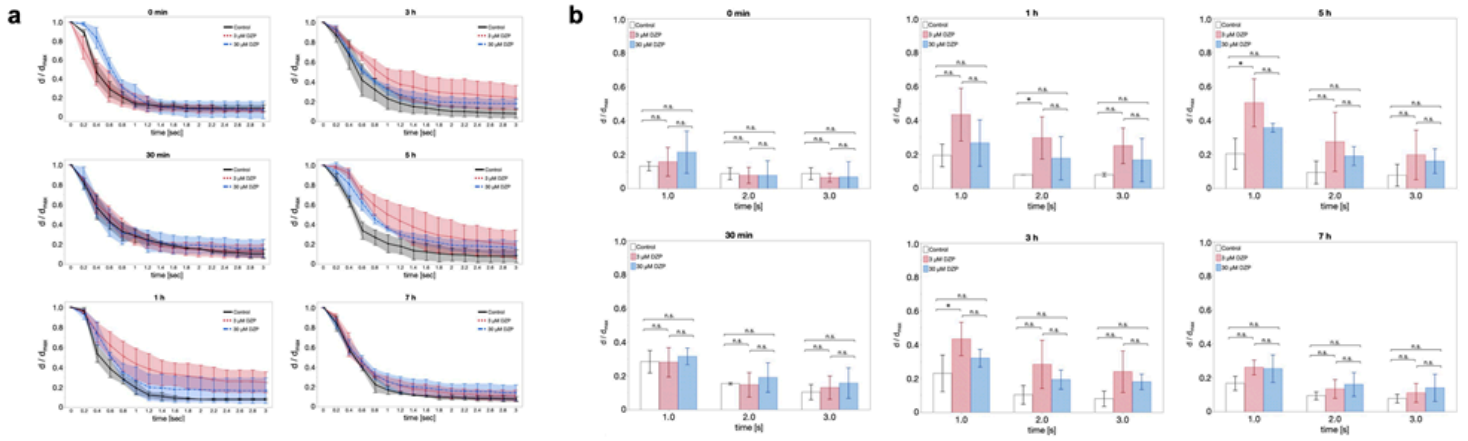


Figure 4

Effect of Diazepam on the folding movement of *M. pudica* leaves. (a) Time course of the d/d_{max} after mechanical stimulation at 0 min, 30 min, 1 h, 3 h, 5 h, and 7 h from the beginning of the experiment. Plants are divided into three groups: control (0.1% DMSO containing MilliQ water, black), 3 μ M DZP (0.1% DMSO, red), and 30 μ M DZP (0.1% DMSO, blue). Three individuals were put in a single plant box. A single leaflet of three different individuals was stimulated in each group ($n = 3$). Error bars represent the standard deviation. (b) Representation values of the d/d_{max} at 1.0 sec, 2.0 sec, and 3.0 sec. Error bars represent the standard deviation. * $p < 0.05$.

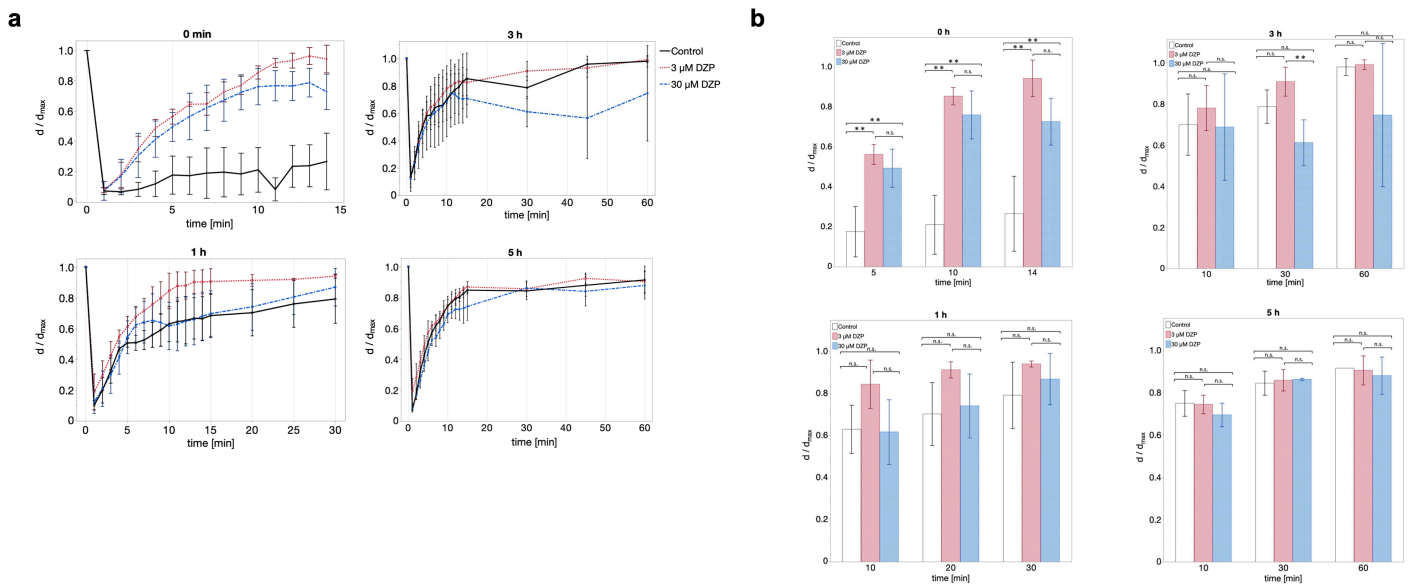


Figure 5

Effect of Diazepam on the recovery of leaf expansion. (a) Time course of the d/d_{max} at the recovery phase. (b) Representation values of the d/d_{max} . Error bars represent the standard deviation. * $p < 0.05$, ** $p < 0.01$.

Extensor motor cell

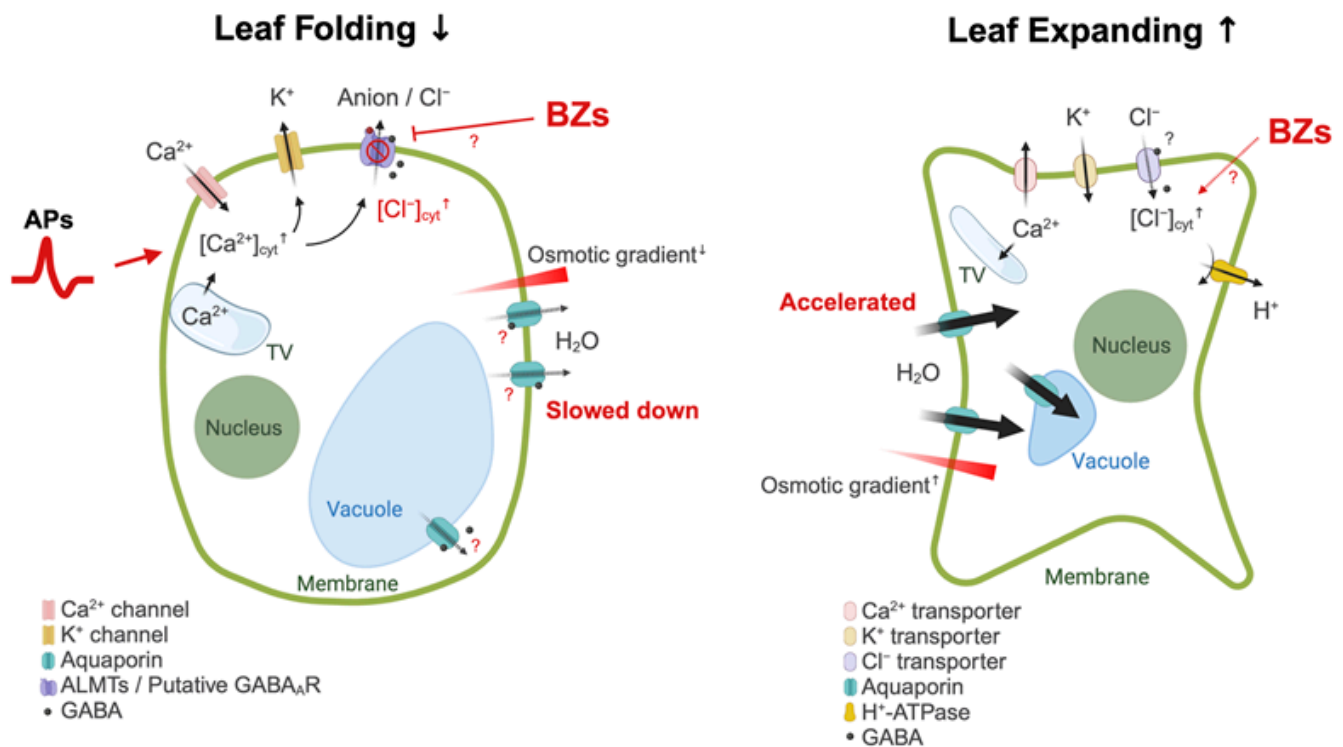


Figure 6

Possible effects of BZs on the extensor motor cells. For the folding movement, transmitted APs increase cytosolic Ca²⁺ levels ([Ca²⁺]_{cyt}) by an influx of Ca²⁺ via plasma membrane and an efflux of Ca²⁺ from the tannin vacuole. This increase in cytosolic Ca²⁺ levels activates voltage-dependent K⁺ and Cl⁻ channels, resulting in K⁺ and Cl⁻ efflux. Subsequently, water moves out of the cell via aquaporins according to an osmotic gradient. GABA inhibits the anion channels such as ALMTs or putative GABA receptors, thereby limiting Cl⁻ efflux. BZs may potentiate the GABA effect by binding to GABA receptors. Water translocation may be slowed down due to the insufficient osmotic gradient. For the expanding movement, water reabsorption may be enhanced by higher intracellular anion concentrations. ALMTs; aluminum-activated malate transporters, APs; action potentials, BZs; benzodiazepines, GABA; gamma-Aminobutyric acid, TV; tannin vacuole.