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A convenient, soil-free method for the production of root nodules in soybean to study the effects of exogenous additives

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ABSTRACT

Legumes develop root nodules that harbour endosymbiotic bacteria, rhizobia. These rhizobia convert nitrogen to ammonia by biological nitrogen fixation. A thorough understanding of the biological nitrogen fixation in legumes and its regulation is key to develop sustainable agriculture. It is well known that plant hormones affect nodule formation; however, most studies are limited to model legumes due to their suitability for *in vitro*, plate-based assays. Specifically, it is almost impossible to measure the effects of exogenous hormones or other additives during nodule development in crop legumes such as soybean as they have huge root system in soil. To circumvent this issue, the present research develops suitable media and growth conditions for efficient nodule development under *in vitro*, soil free conditions in an important legume crop, soybean. Moreover, we also evaluate the effects of all major phytohormones during soybean nodulation under identical conditions. This versatile, inexpensive, scalable and simple protocol provides several advantages over previously established methods. It is extremely time-and resource-efficient, does not require special training or equipment, and produces highly reproducible results. The approach is expandable to other large legumes as well as for other exogenous additives.

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INTRODUCTION

Nitrogen is an essential element for plant growth, development and productivity. Improving the nitrogen amount available to plants results in significant increases in crop yields. Although present in huge quantities in the atmosphere (78% of earth's atmosphere), this nitrogen is not available to the plants, unless fixed by biological nitrogen fixation. This biological nitrogen occurs by the activity of specialized groups of bacteria, which exists as symbionts with the roots of leguminous plants in specialized structures called root nodules. Root nodule formation is a sophisticated process that requires strict synchronization of bacterial infection and growth as well as plant organogenesis and nodule development. The successful interactions between the host plant and the soil bacteria, *Rhizobium spp*. begins with the secretion of the bacteria-produced lipochito-oligosaccharides, known as Nodulation factors or Nod factors (NF) by root hair cells of plants. The secreted Nod factors from symbiotically compatible *Rhizobia* directly bind with and activate the Nod factor receptors (NFRs) of plants, which are LysM (Lysine motif) containing receptor like kinases [1-3]. The activation of NFRs by Nod factors induces root hair deformation, curling and entrapment of bacteria in those root hairs. The entrapped bacteria form infection threads, which enters in the root hair cells and elongates from the root hair tips to the inner cells to initiate early stages of infection. Additionally, active NFRs stimulate downstream signaling pathways through nuclear Ca^{2+} oscillations and Ca^{2+} spiking to begin nodule organogenesis from the cortical cells [4,5]. All these signaling and organogenesis events are significantly affected by the hormonal balance inside the leguminous plants, which is triggered by the Nod signals produced by bacteria [6].

Phytohormones both positively and negatively regulate nodulation and nitrogen fixation in legumes. The positive effects of plant hormones auxins and cytokinins in nodule development

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have been established for a long time. Auxins are a prerequisite during the development and differentiation of nodule primordia and the formation of the vasculature within the nodules [7-9]. Similarly, cytokinins are solely responsible for the cortical cell division, differentiation and nodule organogenesis [10-12]. In addition to auxins and cytokinins, gibberellins (gibberellic acid, GA) are also involved during regulation of nodulation likely via their cross-talk with cytokinin signaling pathways [13]. On the other hand, stress-related hormones such as jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) significantly reduce nodulation by disrupting Nod factor-induced Ca^{2+} spiking and downstream signaling pathways [14-16].

Furthermore, to control the number of nodules, legumes have evolved a systemic auto-regulation of nodulation (AON) and local hormonal inhibitory regulation, which are considered as negative feedback systems. The molecular mechanism of AON was actively investigated in different supernodulation mutants, for example *hyper nodulation and aberrant root 1 (har1), super numeric nodules 1 (sunn)*, and *nodule autoregulation receptor kinase (nark)* in *Lotus japonicus, Medicago truncatula*, and *Glycine max*, respectively [17-22]. Numerous studies suggest that auxin, JA and brassinosteroids (BRs) modulate AON signaling pathways [14,20,23,24]. Additional plant hormones such as ABA, JA, ethylene, and SA appear to act as signaling mediators in the local inhibitory regulation systems of nodulation [25-29]. Finally, all these hormonal balances coordinate a series of signal transduction events inside the root cells, which promote nodule organogenesis or mature nodule development.

Previous studies on the hormonal control of nodulation are based upon physiological approaches using a variety of leguminous species and exogenous application of phytohormones to study their effect on nodule formation. For example, exogenous application of cytokinins and auxins to pea root cortical explants induced cell proliferation required for root nodule formation

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[30]. On the other hand, exogenous ABA reduced the number of root nodules by inhibiting the cortical cell divisions during nodule organogenesis [16]. GA, an important growth regulator in higher plants, also modulate root nodule formation in legumes by exogenous application [13]. SA, a key molecule in plant disease resistance, inhibited the indeterminate nodulation of *Vicia sativa*, but not the determinate nodulation of *Lotus japonicus* after exogenous application [31]. Although each hormone had a characteristic physiological effect, it was evident that different hormones may also follow additive, synergistic, or antagonistic interactions to regulate the nodule formation.

The availability of excellent mutant populations in plants such as *Lotus japonicus* and *Medicago truncatula* led to a greater focus on model legumes and provided genetic evidence for the effect of hormones on nodule formation. These plants serve as excellent model legumes due to their modest genome sizes, short seed-to-seed generation time, higher plant transformation efficiency and the formation of a restricted number of root nodules. These traits are also useful for performing highly controlled *in vitro* assays with multiple exogenous additives, which has led to several key discoveries [13,14,28,32,33]. Conversely, it is extremely difficult to perform similar *in vitro* assays with exogenous additives in crop legumes such as soybean, due to their large stature, long life cycle, the formation of a huge number of root nodules and the requirement of soil for nodule formation. To circumvent these problems, split-root system was used in soybean to study the effects of various exogenous variable in the control of rhizobia-legume symbioses [34-37]. Most recently, a new split-root system was developed for continuous monitoring of soybean roots throughout the whole experiment after rhizobial infection [38]. Although these protocols are useful in some aspects, it is still tricky to do some of these experiments and is a bit difficult to apply to a large plant population.

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The goals of the present study were to identify suitable media and growth conditions for efficient nodule development under *in vitro* conditions in soybean, which is an important crop but not amenable to the standard *in vitro* assays used to study nodule formation in *Medicago* or *Lotus* sp. In addition, the effect of each of the major phytohormones was analyzed on nodule development under a standard set of conditions. The results presented in the following sections describe a set of optimum growth and treatment conditions for soil-free soybean nodulation and effects of phytohormones on it, which will be useful for the community at large.

RESULTS AND DISCUSSION

Optimization of soil free nodule development in soybean

Symbiotic nitrogen fixation in nodules plays a key role in the maintenance of soybean seed production. While in plants such as alfalfa, their small stature allows for growth under *in vitro* conditions on sterile media plates, the large size of soybean plants precludes such a possibility. The study of soybean nodule development in soil by using different additives is relatively hard, inaccurate and expensive. To overcome these issues, soil-free nodule production under *in vitro* condition is a suitable choice. We optimized a method where the nodules were allowed to develop in germination papers, after initial infection in soil (Fig. 1). In this method, the hairy roots were generated from trifoliate leaf containing upper part of soybean plants by applying nitrogen free media, which is an established protocol for the study of soybean nodulation [39,40]. Subsequently, hairy root containing plants were transferred to soil-rite for rhizobium infection. This step was important to avoid the bacterial infection under *in vitro* condition. Once infected, the plants were moved to the pre-wet germination paper rolls, and maintained in small containers with desired media to attain uniform root growth and nodule formation.

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Achieving uniform root growth and nodule formation in soybean after infection under *in vitro* conditions has been a challenge as it shows enormous inconsistencies in nodule numbers. The time of bacterial infection and the duration of subsequent growth of plants in soil was critical for efficient nodule development at later stages. To optimize the conditions for consistent results, we transferred plants from the soilrite to germination paper rolls at different time points after rhizobium infection and counted the nodules after four weeks of growth. Our results show that plant relocation time after rhizobium infection has a huge effect on nodule formation (Fig. 2). No nodules were formed if we transferred the plants from zero to 24 h after rhizobium infection, whereas ~2, 8 and 12 nodules were formed per plant if the plants were transferred after 48, 72 and 96 h rhizobium infection, respectively. Based on these results, all through our experiments, we have transferred the plants from soilrite to germination paper rolls 72 h after rhizobium infection as it generates a reasonable number of nodules needed for any comparative analysis.

We are cognizant of the fact that the need to grow roots in soil for up to 72 hrs post-infection excludes the possibility of assaying the effects of various additives during the early stages of infection such as on root hair curling, or infection thread formation. The method described in this research is therefore suitable for the study of additives at the later stages of nodule development only. However, as detailed in the next sections, it does result in reproducible and consistent effects of exogenous additives on nodule developments and is extremely resource- and cost-effective.

Optimization of hormone treatment time

For this study, we focused on assaying the effects of various plant hormones as exogenous additives, as these have a significant effect not only on initiation but also on organogenesis. The assays can be optimized for additional additives such as salinity, osmotic stress etc. as needed. The hormone treatments were started when the plants were transferred from soil-rite, as the

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germination paper rolls were transferred to either control or treatment media. An obvious effect on nodule development was observed for each of the plant hormones.

Effect of ABA on root nodule development

Numerous studies have highlighted the involvement of ABA during the regulation of *Rhizobium* nitrogen-fixing legume symbiotic interaction [16,41-43]. As the addition of exogenous ABA causes a marked increase in the endogenous ABA levels in plants, the application of ABA to media in which plants are growing is a suitable system to study its effect on nodulation. In *Lotus japonicus*, the number of root nodules is reduced at higher ABA concentrations, and increased in the presence of abamine (which causes lower ABA concentrations) confirming its inhibitory role during nodule development [44]. In soybean, exogenous ABA decreased nodule number in both the wild-type and a supernodulation mutant [41-43]

We evaluated the effect of ABA on soybean nodulation in a concentration-dependent manner using ABA concentrations ranging from 2 to 25 µM. An equimolar amount of ethanol were used as a control in all assays. Nodule formation showed extreme sensitivity to exogenously added ABA. Average 7 nodules were formed per plants after 4 weeks of growth under control conditions, which was reduced by 15% to 30% in presence of 2 to 15 µM ABA (Fig. 3). It was noticeable that plants treated with ABA had fewer medium and large nodules compared to the control plants. Concentrations higher than 15 µM severely affected nodule formation, with the nodule numbers reducing by more than 60% per plant in presence of 20 to 25 µM ABA. At higher concentrations, all types of nodules (large, medium and small) were significantly affected. These data confirm the extremely high sensitivity of soybean nodule formation to exogenous ABA, and by extension to other abiotic stresses, which led to increased ABA concentrations *in planta*. These results also confirm the negative role of ABA during nodule development.

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Effect of auxin (IAA) and cytokinin (BAP) on nodule development

Several studies have demonstrated the auxin and cytokinin control root nodule formation. IAA (indole-3-acetic acid), a native auxin in plants, derived from the phenylpropanoid biosynthetic pathway is a key member of the auxin family. The root nodules have a higher IAA content than uninfected root tissues, suggesting a role for IAA in nodule development [7]. The higher IAA content of infected roots promotes root cells to undergoing cell division, elongation, differentiation and vascular bundle formation to form a nodule [45-48]. Moreover, rhizobia alter the root auxin balance as a prerequisite for nodule formation [49-53]. Besides auxin content, rhizobia-legume symbiosis is also regulated by shoot-to-root auxin transport, which is an active transport process involving auxin efflux protein complexes [54]. Overall, it is well established that the changes in auxin accumulation and transport, both are essential for lateral root development, nodule primordium activation and nodule organogenesis [9,46,50].

To test the effect of exogenous auxins on soybean nodule formation, plants were treated with different IAA concentrations ranging from 10 nM to 100 µM. IAA at 10 nM caused the most obvious phenotypic differences in the nodule number, although all treatment conditions resulted in higher nodule number per plant. Approximately 4 times more nodules were formed per plant at 10 nM IAA compared to control plants with a huge increase in the number of small and medium-size nodules (Fig. 4A). Two times more nodules were formed compared to control conditions in the presence of 1 µM IAA, whereas a modest increase in nodule numbers was observed in the presence of 100 µM IAA in the media. These results indicate that even at a very high concentration, auxin still has a limited but positive effect on nodule formation. Furthermore, 10 nM exogenous auxin is an optimal concentration for increased nodule numbers in soybean.

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Extensive research on *Lotus japonicus* and *Medicago truncatula* have demonstrated that cytokinins (CKs) are key players in the regulation of rhizobium infection and nodule development [10,11,55]. The activation of the NF signaling pathway rapidly induces CK accumulation and response in the hairy root region [56-58]. The exogenous applications of cytokinins also promote cortical cell divisions and the expression of early nodulation markers in different legumes [55,56,59-61]. To study the effect of exogenous applications of cytokinins on soybean nodulation, we included different concentrations of BAP (50 nM, 100 nM, 250 nM, 1 µM and 10 µM) in the media when the plants were transferred from soil media to germination paper rolls. We detected a clear, concentration-dependent effect of BAP in soybean nodulation. Approximately 2.5 times more nodules were formed in the presence of 50 nM BAP, whereas a modest increase was observed in the presence of 100 nM BAP, compared to the control conditions (Fig. 4B). Interestingly, BAP concentrations higher than 250 nM were inhibitory and lead to the development of fewer nodules compared to control media grown plants. For example, ~ 80% less nodule developed in presence of 10 µM BAP. These data suggest that nodule formation in soybean strongly regulated by a precisely controlled cytokinin level.

Effect of GA and BR on root nodule development

Gibberellic acid (GA) is one of the vital growth regulators in higher plants. Several studies have highlighted the involvement of GA in the regulation of the *Rhizobium* nitrogen-fixing symbiotic interaction [13,62-64]. Interestingly, the effect of an exogenous application of potassium gibberellate in inhibiting nodule formation in *Phaseolus vulgaris* was reported in earlier studies [65]. On the other hand, nodules aborted in pea mutant (*na-1*) which was deficient in GA_3, but were re-established by the application of exogenous GA_3 [62]. These results suggest that exogenous GA may have different effects on *Rhizobium* infections and nodule development

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in different legumes. To assess the effect of GA_3 on soybean nodule formation, plants post-infection were treated with different GA_3 concentrations (10 nM to 1 µM) and compared with control media with no added GA_3. Plants treated with 10 nM and 100 nM GA_3 showed considerably increased nodule formation with ~40% and ~20% more nodules, respectively. Interestingly, most of the nodules were small sized in these treatments. However, similar to what was observed for cytokinins, at higher concentrations (1 µM) of GA_3, the nodule formation was severely affected and ~70% fewer nodules were formed on treated roots compared with control media (Fig. 5A). These data suggest that the reported discrepancies in the previous publications could be due to the different GA concentrations used in different assays. These observations suggest that the endogenous GA concentration in plants is tightly regulated to achieve effective nodulation.

Brassinosteroids (BRs) play pivotal roles during many aspects of plant growth and development [66,67]. BRs affect both cell proliferation and cell elongation to control shoot and root lengths and hypocotyl growth in plants [68,69]. Although the effects of brassinosteroids are well-documented on root and shoot growth, their effects on nodule development in different legumes are not as well-described. In one example, BRs affected nodule development by reducing lateral root numbers in pea as is evident from the BR synthesis mutants *lk* and *lkb* and the BR response mutant *lka* [62]. To test the effect of brassinosteroid on soybean nodule development, plants were treated with media containing different BR concentrations. In comparison to plant grown on control media, a concentration-dependent increase in nodule numbers was observed in plants grown on BR containing media. Highest nodule numbers were seen in response to 100 nM brassinolide, where ~85% more nodules were present compared to the control media roots (Fig. 5B). At lower (10 nM) and higher (1 µM) concentrations of exogenous BL, ~50 % more nodules were formed, suggesting the BRs are positive regulators of nodule formation.

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Effect of SA and JA on root nodule development

Salicylic acid (SA) strongly affects nodule formation at early stages of nodulation [70]. The exogenous application of SA resulted in both reduced and delayed nodule formation on alfalfa roots inoculated with wild-type *S. meliloti*. Moreover, exogenous SA that inhibited nodulation also strongly reduced the growth of the bacterial symbiont [15]. The effect of SA was additionally documented in different legumes. For example, the inhibition of nodule formation after exogenous SA treatment were also observed in plants like vetch (*Vicia sativa*), pea (*Pisum sativum*) and white clover (*Trifolium repens*) [31]. This study shows that the exogenous SA inhibits indeterminate but not determinate nodulation [31], which is inconsistent with two other previous reports [70,71], where they showed that exogenous application of SA to soybean, which forms determinate nodules, reduced nodulation. Another report demonstrated that SA enhances the efficiency of nitrogen fixation and assimilation in *Cicer arietinum* [72]. To examine the role of exogenous SA in soybean nodulation in our system, we used different concentrations of SA ranging from 10 µM to 1 mM in the exogenous media. All SA-treated soybean plants exhibited a significantly higher nodule number, ranging from 57% to 76% compared to the control media (Fig. 6A). The phenotypic differences were more prominent in case of higher concentration of SA treatment (100 µM to 1 mM). Our data thus suggest that exogenous SA levels positively regulate nodulation in soybean at these concentrations.

Jasmonic acid (JA) negatively regulates the plant's response to the rhizobial bacterial signal, Nod factor [28]. Primarily, JA inhibits nodule formation by suppressing calcium spiking and the frequency of calcium oscillations to modulate the Nod factor-induced gene expression [28]. In addition, even in the shoots, the autoregulation of nodulation signaling (AON) pathway is modulated by JA [24]. However, a recent study by suppression of allene oxide cyclase in *Medicago*

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truncatula, an enzyme involved in committed step in JA biosynthesis, suggested that jasmonates are not involved in the development and function of root nodules [73]. To examine if JA has any effect on soybean nodulation, we tested its effects at different concentrations (10 µM, 100 µM and 1 mM). Surprisingly, at low concentration, (10 µM), JA had a positive effect and approximately 2 times more nodules were formed compared to the control media. However, the nodule number was significantly inhibited (up to 95%) after increasing the JA concentrations from 10 to 100 µM. No nodules were formed in the presence of 1 mM JA (Fig. 6B). These data suggest that regulation of nodule formation by JA is complex and is highly dependent on the exogenous concentrations. As hormones such as JA and SA not only modulate the signaling pathways in plants but are also a core part of plant-microbe interaction [74-76], it is expected that different concentrations may have altered effects on growth and development versus survival.

CONCLUSION

Nodule development in legumes directly affects nitrogen fixation efficiency during plant growth. Here we present a method for determining the effects of ABA, auxin, BAP, GA, BR, SA and JA on soybean nodulation that is rapid, accurate, technically simple and requires minimal amounts of plant samples. This method provides several advantages over other methods as these approaches do not require time-consuming additional steps such as changing solvents and maintaining hormonal concentrations day-by-day use of large containers, which require a lot of resources and space and tedious handling. That kind of manipulations often increases potential technical errors. Moreover, the standardization of hormone concentrations and the description of resultant phenotypes will support further targeted studies, and in combination with additional genetic and genomic tools being developed in multiple labs, will greatly increase its use for the

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study of the effects of exogenous factors affecting nodulation in soybeans as well as in other larger legumes.

MATERIALS

1. Soybean seeds (*William 82*)
2. Pots (2-gallon) filled with soil rite (BM 7, 35% Bark mix, Berger, Saint-Modeste, QC, Canada)
3. Rock wool, cubic size (Hummert International, Earth City, MO, United States)
4. Petri dishes (100×15 mm) (Corning™ Falcon™, Fisher Scientific, United States)
5. Trays (2 square feet) (T.O. Plastics, Inc., Clearwater, MN, United States)
6. Pot (85×85 mm) (T.O. Plastics, Inc.)
7. Vermiculite:Perlite:Sand (3:1:1) containing pots (Therm-O-Rock East Inc, New Eagle, PA, United States)
8. Beakers (250 ml)
9. Germination paper (Anchor Paper Company, St Paul, MN, United States)
10. Light chamber under 16 h light/ 8 h dark conditions (100 µmol m^{-2} sec^{-1}) with 50% humidity and 25ºC (day/night) temperature
11. Greenhouse with 50% humidity and 31ºC/22ºC (day/night) temperature.
12. Nitrogen-free nutrient solution (1000X stock solution, pH 6.5)

Macronutrient

KSO_4	(87.135 gm/L)
KH_2PO_4	(68.045 gm/L)
$CaCl_2$, $2H_20$	(147.01 gm/L)
$MgSO_4$,7H20	(123.24 gm/L)

Fe-EDTA

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EDTA (372.24 gm/L)

$FeSO_4$, $7H_20$ (278.02 gm/L)

Micronutrient

$ZnSO_4$,$7H_2O$ (0.143 gm/L)

$CuSO_4$,$5H_2O$ (0.030 gm/L)

$MnSO_4$,H_2O (0.845 gm/L)

H_3BO_3 (1.855 gm/L)

$(NH_4)_6Mo_7O_{24}.4H_2O$ (0.099 gm/L)

$Co(NO_3)_2$ (0.003 gm/L)

$NiSO_4$ (0.026 gm/L)

13. Vincent's rich medium (per liter)

K_2HPO_4 (0.5 gm)

NaCl (0.1 gm)

$MgSO_4$, $7H_20$ (0.2 gm)

Yeast Extract (0.4 gm)

Mannitol (10.0 gm)

pH 6.8

14. Rhizobium strain (USDA136)

15. Plant hormones:

a. (+/-) Abscisic acid (ABA), (Caisson Labs, Smithfield, UT, United States)

b. Indole-3-Acetic Acid (IAA), (Caisson Labs)

c. 6-Benzylaminopurine (BAP), (Caisson Labs)

d. Epibrassinolide ≥85% (BR), (Sigma-Aldrich, Saint Louis, MO, United States)

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e. Gibberellic Acid (GA_3), (Caisson Labs)

f. Methyl jasmonate, 95% (JA), (Sigma-Aldrich)

g. Methyl salicylate *ReagentPlus*®, ≥99% (GC) (SA), (Sigma-Aldrich)

METHODS

Plant growth

Soybean seeds were grown in the greenhouse on a two-gallon pot filled with soil-rite (BM 7 35%) for 12 to 14 days ((25°C/24°C (day/night) temperature, 40–95% humidity,~300–450 µmol/m2/sec light intensity depending on the weather and time of the day) -). Twenty-five to thirty seeds were grown in each pot, with equal spacing (Fig. 1A). There should be no overlap of the seeds. The spacing is important for proper growth of the seedlings.

Hairy root formation

Hairy root were developed as per our previously established protocols [77]. Briefly, three to four sterilized rock wool cubes were placed in one petri dish and a hole was created in the middle of the rock wool by using 1 mL pipette tips. Six ml nitrogen-free nutrient solution was applied in the hole of each rock wool cube. The trifoliate leaf from the upper part of soybean plant was cut using a razor blade and put in the hole of the rock wool cube (Fig. 1B). Eight petri dishes (~30 trifoliate) could be placed in each tray.

The trays were covered with a plastic cover and kept in the light chamber with the light intensity 100 µmol m^{-2} sec^{-1}. Plants were grown for two weeks for generation of hairy roots. If needed, the nitrogen-free nutrient solution was added in the rock wool cubes. After two weeks, the rock wool cubes were carefully removed and the plants with hairy roots were transferred to the pots containing vermiculite:perlite:sand at 3:1:1 ratio. The plants were grown for one more week in the green house (Fig. 1C).

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Rhizobium treatment

Appropriate *Rhizobium* strain was cultured in Vincent's rich medium containing chloramphenicol (20 µg/ml) for 3-4 days. The culture was spun down and resuspended in equal volume of nitrogen-free nutrient solution, with the final concentration adjusted to 0.08 at OD_{600}. Ten ml of rhizobium culture was poured surrounding the roots in each pot and plants were grown for two to four days in the green house. For the study of gene silencing or overexpression or gene-editing, Agrobacterium cells (*K599*) expressing the appropriate gene constructs can be used by mixing it with a nitrogen-free nutrient solution, for the generation of transgenic soybean hairy roots.

Hormone treatment

After Rhizobium inoculation, plants were removed from the pot at specific time points (Fig. 1D), wrapped in a roll in two sheets of germination paper, pre-wet with the nitrogen-free nutrient solution. Six plants (in individual rolls) were kept in one 250 mL beaker containing 100 mL of nitrogen-free nutrient solution (Fig. 1E). Specific hormones were added to each beaker one time every week, immediately after transfer of the plants to the media. The 100 ml nitrogen-free nutrient solution level was maintained by monitoring the solution level on alternate days and adding media as required. Multiple replicates were performed for each treatment. Nodule numbers were counted after four-weeks of growth (Fig. 1 F). The nodules were divided into small (<0.5 mm in diameter), medium (0.5–2 mm in diameter), and large (>0.2 mm in diameter) categories.

Statistical analysis

All experiments were repeated two times independently and the data were averaged. Each replicate consisted of 24 plants. Statistical significance of results was calculated using Student's t-test with a *P*-value threshold of less than 0.05.

AUTHOR CONTRIBUTION

Conceptualization, Sona Pandey; Data curation, Swarup Roy Choudhury and Sarah Johnson; Formal analysis, Swarup Roy Choudhury; Funding acquisition, Sona Pandey; Investigation, Swarup Roy Choudhury; Methodology, Swarup Roy Choudhury and Sarah Johnson; Project administration, Sona Pandey; Supervision, Sona Pandey; Writing – original draft, Swarup Roy Choudhury; Writing – review & editing, Sona Pandey.

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FIGURE LEGENDS

Fig. 1. Experimental flowchart describing the details of soil-free nodule development on hairy roots of soybean. (A) Two-week-old plants grown on soil were used as the source of trifoliate leaves. **(B)** The trifoliate leaves were removed the plants and inserted in the middle of sterilized rock wool cubes for the development of hairy roots. **(C)** Plants with hairy roots were transferred to the pots with soil-rite for rhizobium infection. **(D)** Post-infected plants were removed from the pot. **(E)** Plants were wrapped with pre-wet germination paper in rolls. One plant was

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placed per roll. **(F)** Six plants were kept in each beaker containing nitrogen-free nutrient solution. **(G)** Nodule number was counted after 4 weeks of further growth.

Fig. 2. Optimization of nodule development time after infection. Rhizobia-infected soybean hairy roots were transferred to pre-wet germination paper at different time points. Nodule number was counted 4 weeks after infection. All experiments were repeated two times independently and data were averaged. Each replicate consisted of 12 plants. Asterisks denote significant difference,* $P < 0.5$, Student's t-test.

Fig. 3. Effect of exogenous ABA on nodule formation. Rhizobia-infected soybean hairy roots were treated with different concentrations of ABA when transferred to the nitrogen-free media. ABA concentration was maintained throughout the experiment (4 weeks). Nodule number was counted 4 weeks after infection. All experiments were repeated two times independently and data were averaged. Each replicate consisted of 24 plants. Asterisks denote significant difference,* $P < 0.5$, Student's t-test.

Fig. 4. Effect of exogenous auxin (IAA) and cytokinin (BAP) on nodule formation in soybean. Rhizobia-infected soybean hairy roots were treated with different concentrations of **(A)** IAA and **(B)** BAP, when transferred to the nitrogen-free media. The hormone concentrations were maintained throughout the experiment (4 weeks). Nodule number was counted 4 weeks after infection. All experiments were repeated two times independently and data were averaged. Each replicate consisted of 24 plants. Asterisks denote significant difference, * $P < 0.5$, Student's t-test.

Fig. 5. Effect of gibberellic acid (GA_3) and brassinosteroids (brassinolide, BR) on nodule formation in soybean. Rhizobia-infected soybean hairy roots were treated with different concentrations of **(A)** GA_3 and **(B)** BR, when transferred to the nitrogen-free media. The hormone concentrations were maintained throughout the experiment (4 weeks). Nodule number was

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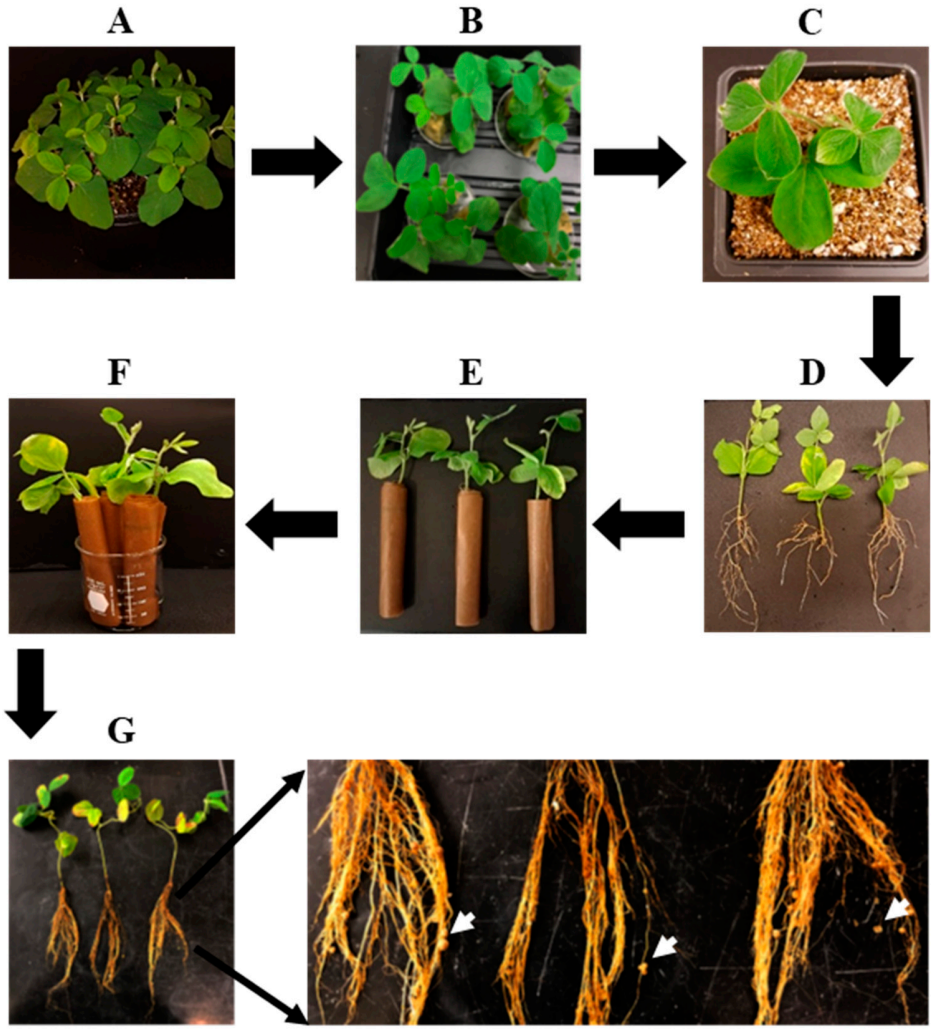

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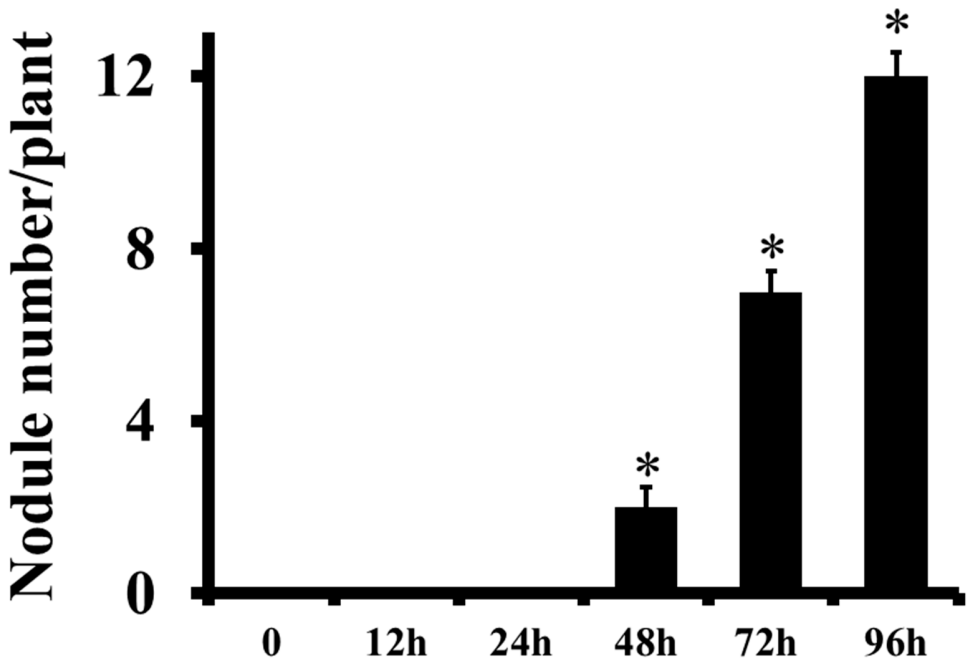

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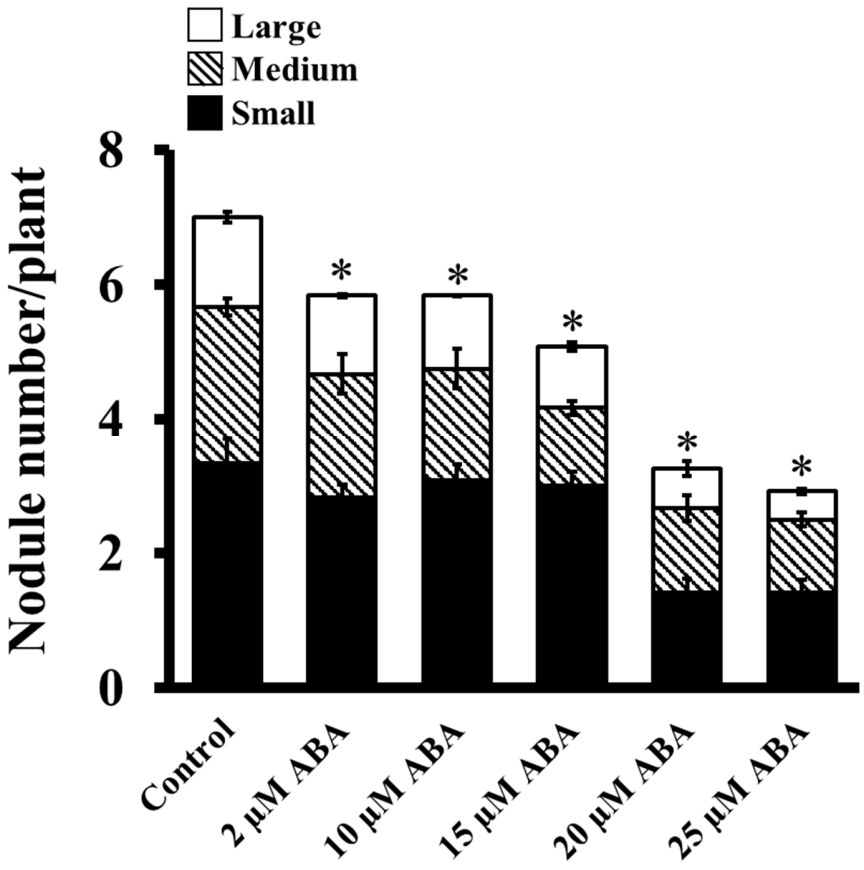

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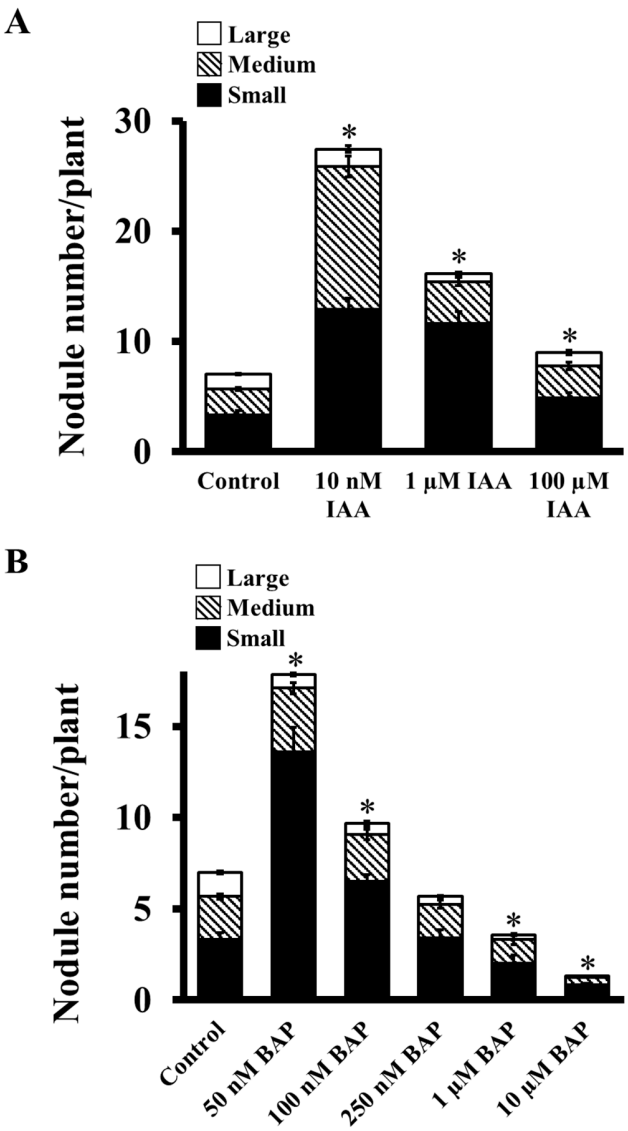

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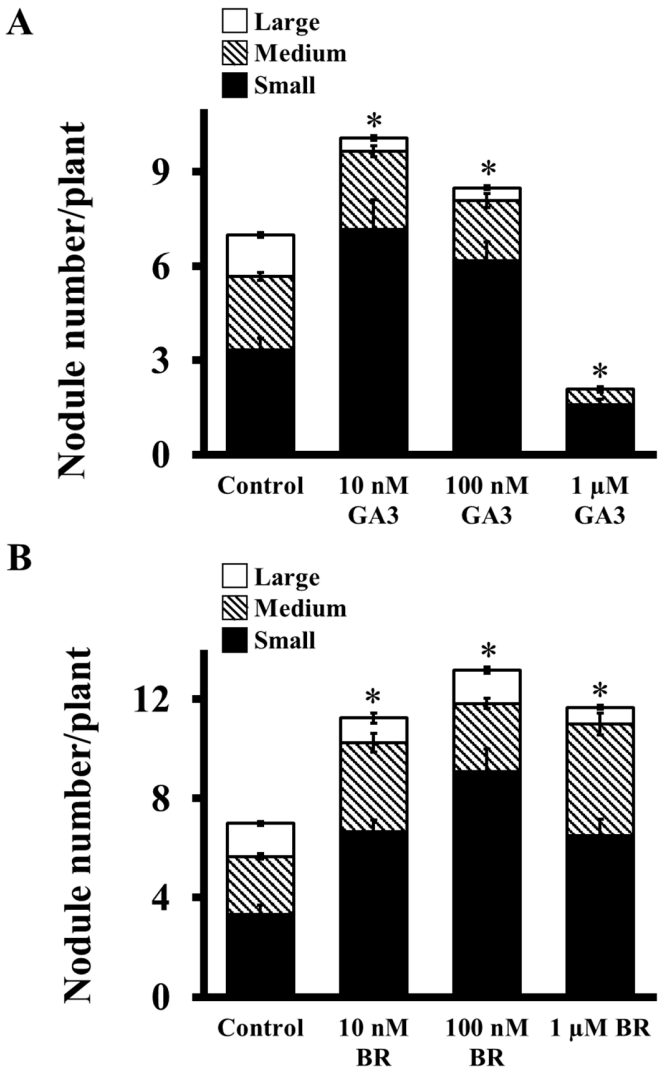

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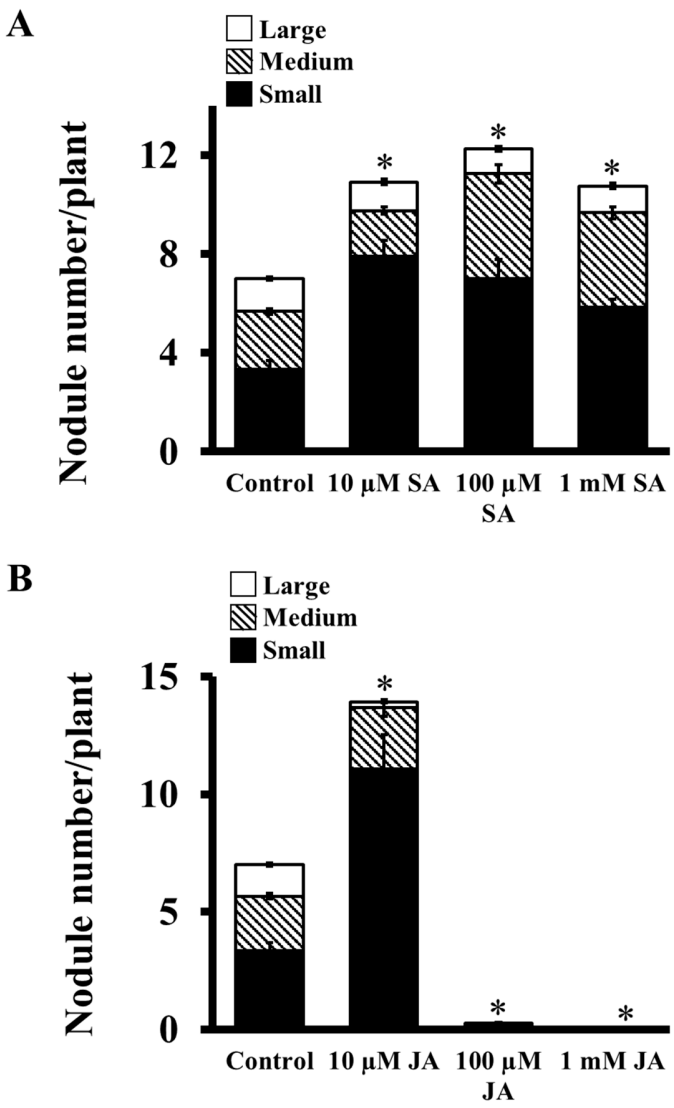

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